Enhanced bone formation by rapidly formed bony wall over the bone defect using dual growth factors

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Enhanced bone formation by rapidly formed bony wall over the bone defect using dual growth factors

Directed by Professor Young-Beom Park

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ABSTRACT

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Purpose: As a method of enhancing GBR, this study induces bone formation in the early stage of healing by applying BMP-2 to the collagen membrane, isolates the inside of the defect from external stimuli to create a structurally stable bone regeneration environment during the healing period, and applies bone graft material and FGF-2 inside the isolated bone defect to increase bone

regeneration. It also investigated the most effective GF concentration for bone formation in the designed GBR model to enhance bone formation.

Methods: This study used 24 New Zealand white rabbits. Four bone defects with a diameter of 8mm were formed in the calvaria of each individual to perform GBR. When applying GBR, collagen membrane and BCP were applied to the control group, and collagen membrane + BMP-2 (0.5, 1.0 mg/ml) and BCP + FGF-2 (0.5, 1.0 mg/ml) were applied to the four experimental groups, respectively. After 2, 4, and 8 weeks of healing, the rabbits were sacrificed for histological and histomorphological analysis.

Results: In the histological analysis, continuous forms of new bones were observed in the upper part in the experimental groups, and no continuous forms were observed in the control group. New bones derived from existing bones were observed in both the control and experimental groups. In the histomorphological analysis, Group 3 (BMP-2 0.5 mg/ml, FGF-2 1.0 mg/ml) showed statistically

significantly higher new bone formation (30.65 ± 11.39%, $p<0.05$). Also, there was no statistically significant difference in new bone formation according to the healing period at 2 and 4 weeks, but it was statistically significantly higher at 8 weeks (2=4<8 weeks, $p<0.05$).

Conclusion: In this study, it was confirmed that the new dual scaffold matrix method using a collagen membrane applied with BMP-2 and BCP applied with FGF-2 was effective for bone regeneration in GBR. The concentration combination of BMP-2 0.5mg/ml and FGF-2 1.0mg/ml showed a higher osteogenic ability compared to the experimental groups with other concentrations. In addition, the dual scaffold complex is quantitatively and qualitatively advantageous for bone regeneration and bone maintenance over time.

Key words: Growth factors, Animal experiments, wound healing, Guided bone regeneration

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I. INTRODUCTION

Implants have been spotlighted as one of the primary treatment options for edentulous patients whose condition is related to periodontitis, trauma, tumors, or congenital deformity. However, osseointegration of implants is difficult in many cases due to the size and location of bone

defects, so various clinical studies have been conducted to overcome this problem [1,2]. Guided bone regeneration (GBR) is the most common treatment method for reconstructing the alveolar bone or regenerating bone defects around implants [3-6]. The principle of GBR is to protect the slow-growing internal bone regeneration tissue from the fast-growing epithelium and connective tissue using a barrier membrane, and bone grafts can also be used inside the defect to aid bone regeneration [3,4,7,8]. Membranes play a crucial role in GBR, which started with non-resorbable membranes and developed into resorbable membranes [3,9]. Non-resorbable membranes have excellent biocompatibility and space maintenance but have an increased risk of exposure, and require an additional surgery to remove the membrane [10]. Resorbable membranes are widely used in GBR clinical practice due to their advantages such as low cost, lower rate of exposure, and no need for additional surgery to remove the membrane [11]. Several studies have reported no significant differences in the clinical results between non-resorbable and resorbable membranes [12-14]. However, resorbable membranes show lower mechanical strength, making them less efficient in space-making than non-resorbable polytetrafluoroethylene (PTFE) and titanium mesh. Therefore, in GBR, bone substitutes are combined with degradable membranes

to maintain the membrane shape and enable space-making [15]. When resorbable collagen membranes are used for GBR, bone-related proteins such as alkaline phosphatase (ALP), osteopontin, and osteocalcin are observed in the lower part of the membrane (16), and growth factors involved in bone formation, such as bone morphogenic proteins-2(BMP-2), transforming growth factor-β(TGF-β), fibroblast growth factor-2(FGF-2), and vascular endothelial growth factor (VEGF) are gradually expressed in the collagen membrane compartment [16]. Therefore, membranes play an active role and fulfill a passive barrier function in GBR [3,16].

Several studies have been conducted that attempted to induce fast and effective bone regeneration by applying growth factors (GF) with collagen membranes in GBR [17-22]. Collagen membrane is a core biomaterial capable of continuously releasing GF for a certain period and providing ideal GF release kinetics [23]. Caballé-Serrano et al. reported that the combined use of GF and collagen membrane accelerated the effect of GBR [24]. BMP-2 and FGF-2 are the most widely used in clinical practice among the various GFs [25-27]. Of the rhBMP (recombinant BMP) family, only rhBMP-2 and rhBMP-7 are currently approved for clinical use [25]. In dentistry, rhBMP-2 is used to treat cleft lip and palate defects [28,29], generate bone around implants [30],

and in periodontal tissue reconstruction [31]. rhFGF-2(recombinant FGF-2) is used to reconstruct alveolar bone defects [32,33], reconstruct periodontal soft tissue [34], and in pulp capping [35]. The chondro-osteogenesis and osteoinduction effects of BMP-2 are the results of inducing the migration and proliferation of osteoprogenitor cells, osteoblasts, chondrocytes, and endothelial cells [27]. FGF-2 is a protein that strongly increases the proliferation of fibroblasts, and was first discovered in pituitary extracts in 1974 [36]. It is a heparin-binding cytokine that promotes angiogenesis and osteogenesis [34]. In clinical practice, FGF-2 is used to regenerate periodontal tissue [26,33,34,37], induce the proliferation of periodontal ligament cells [38], reconstruct bone defects around implants [39], and support pulp capping [40]. On the other hand, FGF-2 is known to down-regulate the expression of type-I collagen mRNA [38]. This inhibitory effect is reversible, and is apparently correlated with the temporary inhibition of cytodifferentiation of periodontal ligament cells into hard-tissue forming cells, such as osteoblasts and cementoblasts [26]. In other words, while not directly involved in osteogenesis, the proliferation of immature mesenchymal cells is sufficiently achieved at the early stage of osteogenesis, resulting in a favorable environment for continuous bone

formation [41].

Studies have been conducted to enhance osteogenic effects by combining the use of these two GFs in GBR [31,42-45] in addition to research on bone regeneration using the interaction between BMP-2 and FGF-2 [45-47]. Studies have also been performed to induce new bone formation by expressing the osteoinduction effect of BMP-2 and the angiogenesis effect of FGF-2 together. In a study by Khorsand et al. comparing the amount of bone formation in a rabbit long-bone model defect model [45], the group using a co-delivery of BMP-2 and FGF-2 showed a significant improvement in bone regeneration compared to the group using BMP-2 or FGF-2 alone. Hanada, K. et al. found through in vivo and in vitro research that the combined treatment of basic FGF (bFGF and BMP-2 was an effective cell-growth factor therapy for healing wounds in osteoporosis and fracture treatment [48]. Nosho et al. reported that BMP-2 could be suitable for application in extramedullary bone regeneration, whereas FGF-2 could be suitable for medullary bone regeneration [49]. A study on the sequential application of bFGF and BMP-2 also reported that these two GFs increased periodontal stem cell proliferation to improve periodontal tissue regeneration [50]. A study on healing calvarial defects in old mice showed that combining low

doses of BMP-2 and FGF-2 increased the total volume and histological bone formation compared to using BMP-2 alone [44]. However, most studies used the same scaffold to apply FGF-2 and BMP-2 [42,44,49-52]. Yin et al. [53] encapsulated BMP-2 in chitosan-collagen (CS/COL) scaffolds by improving the double emulsion/solvent evaporation technique, and attached FGF-2 to the surface to differentiate the release time, but still applied the same scaffold. FGF-2 is an upstream regulator of BMP-2 expression in calvarial osteoblasts [54], and BMP-2 and FGF-2 have synergistic effects in healing fractures [55], so the combined use of BMP-2 and FGF-2 can shorten the healing period and induce an increase in bone regeneration.

Through our previous study, we have found that applying BMP-2 with bone graft material resulted in poor spatial stability due to the increase of adipose tissue in the later phase of healing, and applying FGF-2 to the collagen membrane showed no difference in new bone formation compared to the control group [41]. Applying FGF-2 to the collagen membrane and BMP-2 to the bone graft material also showed no difference in new bone formation. Based on these results, a design of applying BMP-2, a significant element to improve bone regeneration, to the membrane, while applying bone graft material and FGF-2 inside the defect was considered

to enhance the bone regeneration effect of GBR.

As a BMP-2 carrier, the collagen membrane continues to release BMP-2 during the healing period, increases ALP and osteocalcin, which evaluates bone regeneration activity [56], and also exhibits a rapid release effect in the early stage of healing [57], making it a good combination for early healing containment. As a bone graft material, Biphasic Calcium Phosphate (BCP) has macro-sized pores and excellent interconnectivity with collagen membranes for bone formation [58,59], and applying FGF-2 to BCP led to excellent space maintenance during the healing period [60] and increased ALP activity [61].

The FDA (U.S. Food and Drug Administration) approved 1.5mg/ml concentration of BMP-2 for human use through nonhuman primate efficacy testing [62]. In clinical use, the concentration of BMP-2 is limited to 1.5mg/ml or less [63], and the concentration of FGF-2 is 0.2~0.8mg/ml [64]. A study on calvarial defects in a rabbit model used different concentrations of BMP-2 and FGF-2 (0.5~1.0mg/ml) to assess new bone formation [65]. Studies reported that the concentration of FGF-2 in rat skulls affects the stimulation of osteoblast differentiation of BMP-2 (67, 68). However, its

exact mechanism is unknown, and there is a lack of prior studies on the concentration of BMP-2 and FGF-2 in combined applications in large animals and clinical trials.

As a method to enhance GBR, this study induces bone formation in the early stage of healing by applying BMP-2 to the collagen membrane, isolates the inside of the defect from external stimuli to create a structurally stable bone regeneration environment during the healing period, and applies bone graft material and FGF-2 inside the isolated bone defect to increase bone regeneration. It also investigated the most effective GF concentration for bone formation in the designed GBR model to enhance bone formation.

II.MATERIALS AND METHODS

A. Animals and materials

a. Experimental animals

24 New Zealand white rabbits weighing 3~3.5kg aged 16-20 weeks were prepared. After bringing in the experimental animals, they were quarantined and acclimatized for one week and fed a standard laboratory diet. The breeding, management, and surgical procedures followed the animal testing standards of the Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea (Approval no. 2016-0062).

B. Materials

a. BMP-2

rhBMP-2 (E.coli-derived, GENOSS, Suwon, Korea) was used for BMP-2. 0.5mg/ml and 1.0mg/ml concentration BMP solutions were prepared through dilution with saline. The same amount of BMP dilutions (50㎕) was applied to each group, and each contained 25㎍ and 50㎍ of rh-BMP2, respectively. Only 50㎕ of physiological saline was used for the control group.

b. FGF-2

rhFGF-2 (E.coli-derived, GENOSS, Suwon, Korea) was used for FGF-2. 0.5mg/ml and 1.0mg/ml concentration FGF-2 solutions were prepared through dilution with saline. Collagen membranes were soaked in 50㎕ of FGF-2 dilutions of each concentration, and each contained 25㎍ and 50㎍ of rh-FGF2, respectively. Only 50㎕ of physiological saline was used for the control group.

c. Biphasic calcium phosphate

Osteon II (Genoss, Suwon, Korea) was used for Biphasic calcium phosphate (BCP). The ratio of Hydroxyapatite(HA) and β-Tricalcium

phosphate(β-TCP) was 30:70, and had a porous structure. Pore size was 250 ㎛, porosity 70%, and each particle size was 0.5 ~ 1.0mm. The appropriate dose for 8mm diameter bone defects in rabbit calvaria was 70 mg, and the same dose was used for each bone defect.

d. Collagen membrane

In terms of the collagen membrane, highly pure type I collagen (Dentium, Seoul, Korea) derived from the bovine tendon was used. It was 300㎛ thick and lasted for more than 6 months in the human body.

C. Methods

a. Control / Experimental groups

BCP (Osteon II) and collagen membrane were used on the control group without applying BMP-2 and FGF-2.

Four groups were set by combining BCP with two concentrations of FGF (0.5mg/ml, 1.0mg/ml) and collagen membrane with two concentrations of

BMP-2 (0.5mg/ml, 1.0mg/ml) (Table 1).

Table 1. Control / Experimental group

Group	BCP + BMP-2 + Membrane + FGF-2
Control group	BCP + Membrane
Experimental Group 1 (G1)	Collagen membrane + BMP-2 (1.0mg/ml) + BCP + FGF-2 (1.0mg/ml)
Experimental Group 2 (G2)	Collagen membrane+BMP-2(1.0mg/ml) +BCP+FGF-2 (0.5mg/ml)
Experimental Group 3 (G3)	Collagen membrane+BMP-2(0.5mg/ml) +BCP+FGF-2 (1.0mg/ml)
Experimental Group 4 (G4)	Collagen membrane+BMP-2(0.5mg/ml) +BCP+FGF-2 (0.5mg/ml)

Four bone defects were created in the calvaria (temporal bone) of each of the 24 rabbits (Figure 1). Four concentration groups were arranged in each of the four bone defects of each individual to reduce individual specificity. However, due to the difficulties in fixing 4 collagen membranes of each concentration to prevent interference with each other during the surgical procedure, only two BMP-2 concentration groups were set for each individual in each of the 2 bone defects on the left and right (Figure 2). The healing period was set at 2 weeks, 4 weeks, and 8 weeks. There were 8 animals in each period.

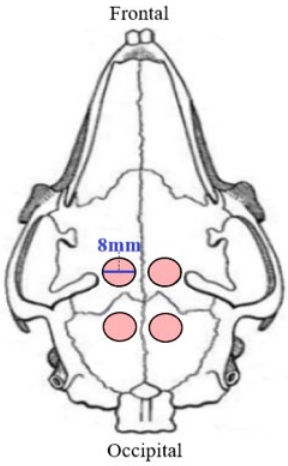

Figure 1. Schematic diagram of surgery on parietal bone of rabbit

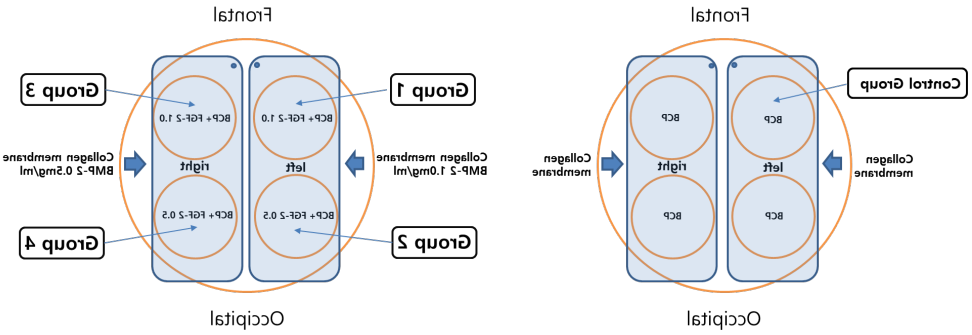

Figure 2. Schematic diagram of surgery of control/Experimental groups.

b. Surgical procedures

The New Zealand white rabbits were anesthetized by subcutaneous injection of 1.5mg/kg Zolazopam (Zoletil, Virbac Korea Co., Seoul, Korea) and intramuscular injection of 5mg/kg Xylazine HCl (Rompun, Bayer Korea Co., Seoul, Korea). After 10 minutes of general anesthetic injection, the skull skin was sterilized with Povidone-iodine, and local anesthesia was performed with 2% Lidocaine (lidocaine HCl, Huons, Seongnam, Korea) containing 1:80,000 epinephrine. The skin and periosteum were incised from the anterior part of the frontal bone through the parietal bone to the anterior part of the occipital bone for about 2.0 - 2.5cm, and the periosteum was elevated with a full-thickness flap.

As shown in Figure 1, four bone defects were created on the exposed calvarial surface, on each side and on the front and back. Circular bone defects with a depth of 1~2mm were formed up to the inferior cortical bone without damaging the underlying dura mater using a Trephine bur (Mr. Curette Tech, Seongnam, Korea) with an outer diameter of 8mm. The bone fragments of the formed defect were gently elevated using a curette. The dura mater was not damaged, and there was no bleeding. The distance

between each defect was at least 3mm.

BMP-2 dilutions at each concentration, collagen membrane with a size of 10×20 ㎟, and physiological saline were prepared in advance, and the collagen membrane was soaked in 50㎕ BMP-2 dilutions for 30 minutes to absorb BMP-2 into the collagen membrane. The control group was soaked in 50㎕ of physiological saline.

FGF-2 and 70 mg of BCP for each concentration were put into a tube and soaked for 30 minutes before transplantation. After implanting the prepared BCP in each bone defect, the collagen membrane was covered and fixed using micropins (Figure 3). The periosteum was first sutured with an absorbable suture (4-0 Vicryl, Ethicon, Somerville, NJ, USA), and then the skin was closed (Figure 4).

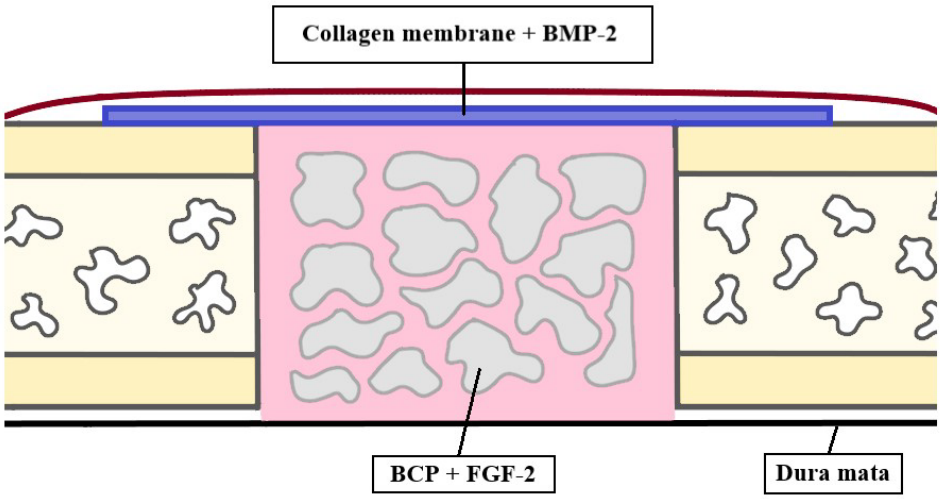

Figure 3. Schematic diagram of the GBR section of the defective part of the experimental group

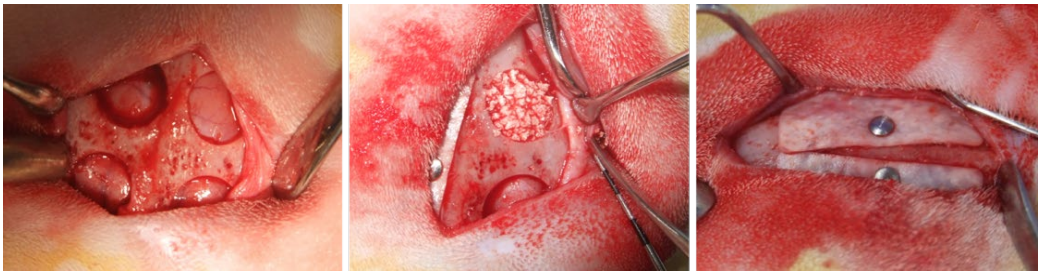

Figure 4. Photo of GBR performed on rabbit skull using collagen membrane and BCP, BMP-2, FGF-2

c. Sacrifice

The rabbits were sacrificed after 2, 4, and 8 weeks of healing. After inducing deep anesthesia, the skin was incised to separate the periosteum. Maintaining a sufficient distance from the healed bone defect, a specimen was taken by removing the marginal bone using a handpiece. The front side of the specimens was marked to identify the direction.

D. Histological and Histomorphometric Analysis

After washing the specimens fixed in 10% formalin for 6 weeks, demineralization was performed in 2.5% NaOCl and 17% EDTA solution for 18 days. Dehydration and paraffin penetration processes were performed using ethanol and xylene. After embedding, the specimens were sliced to a thickness of 3~4㎛. They were cut along the sagittal plane, passing through the center of the bone defect in the front and rear direction. Hematoxylin-Eosin (HE) stain and Russell-Movat pentachrome stain (American MasterTech) were performed to prepare tissue slides.

The specimens were imaged at 12.5 times and 40 times magnification using

an optical microscope (Leica DM 2500, Leica Microsystems, Wetzlar, Germany), and histological measurements were performed using H-E stain tissue slides. New bone area and remaining bone graft material area were measured using Image-pro plus (Media Cybernetics, Silver Spring, Maryland, USA). The areas of new bone and remaining bone graft material were calculated as percentages with respect to the total area.

Russell-Movat Pentachrome Stain tissue slides, a tissue-specific staining method according to tissue development, were used to observe bone development and analyze the differentiation of new bone. (Bone: dark yellow, New osteoid: red, Cartilage: green)

E. Immunohistochemical analysis

Anti-Osteocalcin antibody (OCG3) immunohistochemical staining was conducted to examine the degree of vascularity and density in osteoblasts. The tissue sections were used by diluting the Anti-Osteocalcin antibody (abcam; ab 13420; 1/150) and Anti-Mouse HRP (abcam; ab205719; 1/150).

F. Statistical analysis

Of the 58 specimens, 56 were used for analysis after excluding two outliers. Normality was confirmed using the Shapiro-Wilk test. The data were presented by mean and standard deviation.

Two-way ANOVA was conducted to check whether there was a difference in bone formation rate according to the BMP-2 and FGF-2 concentration combination and healing period, and a post-hoc test was performed to confirm the difference between groups.

SPSS version 25.0 (IBM Corp., Armonk, NY, USA) was used to make the statistical calculations, and the significance level was set at 5%.

All experiments were approved by Yonsei University College of Dentistry, Intramural Animal Use and Care Committee and they were performed in accordance with the guidelines of this committee.

III. RESULTS

A. Anatomical findings

Four bone defects were created in each of the 24 New Zealand white rabbit calvaria, and the animals were sacrificed after 2, 4, and 8 weeks to collect tissue samples (Figure 5). We failed to make two tissue specimens (1 in 2 weeks group 3, 1 in 4 weeks group 4), so 56 out of 58 were used for statistical analysis.

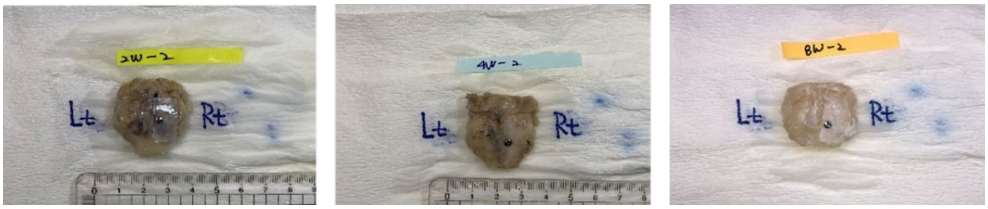

Figure 5. Experimental specimens at 2weeks, 4weeks, 8weeks

B. Histological findings (H-E stain)

a. Analysis of histological specimens after a two-week healing period (H-E stain)

Figure 6 below shows images of H-E stained tissue specimens of the control and experimental groups after a two-week healing period. At 2 weeks, the formation of new bone around the existing bone and bone graft material begins, in both the control and experimental group. Most of the new bone was in the form of woven bone, but in experimental groups 2 and 3, the mature bone in the upper side seemed to be thinly derived from the existing bone. At 2 weeks, most graft sites were filled with residual bone graft and connective tissue, with many inflammatory cells.

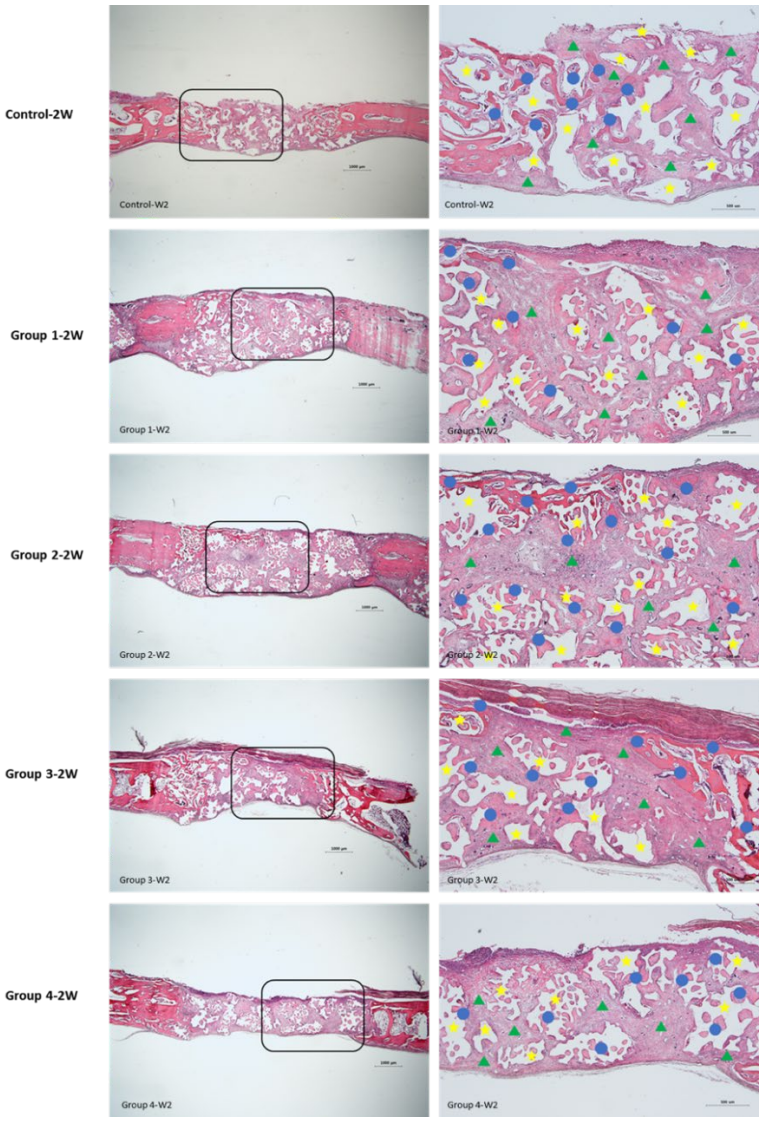

Figure 6. H&E stain of two-week healing period.
Histological specimens for each group after a two-week healing period (Left raw original magnification X12.5, Right raw original magnification X40). Blue circles, yellow stars, and green triangles indicate new bone, remaining bone graft materials, and connective tissues, respectively.

b. Analysis of histological specimens after a four-week healing period (H-E stain)

Figure 7 below shows images of H-E stained tissue specimens of the control and experimental groups after a four-week healing period. In the control group, new bone was observed around the existing bone and bone graft material, similar to the two-week group. Upper new bone originating from the existing bone was observed, loose connective tissue was observed around the remaining bone graft material, and connective tissue with dense inflamed tissue was observed around the existing bone. In the experimental group, mature new bone extending from the existing bone was observed at the top, and the thickness of the upper new bone and its maturity was also increased compared to the two-week group. Connective tissue with dense inflamed tissue was also observed in the experimental group.

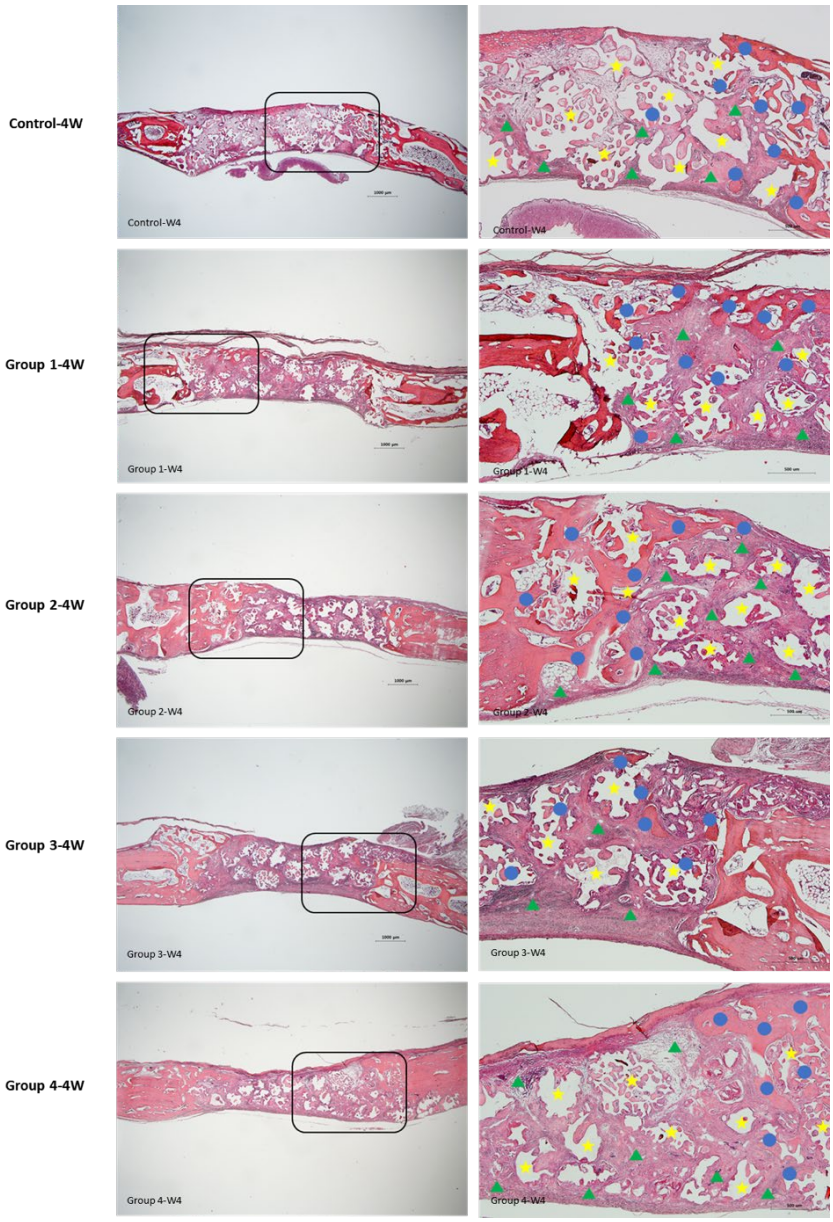

Figure 7. H&E stain of four-week healing period.

Histological specimens for each group after a four-week healing period. (Left raw original magnification X12.5, Right raw original magnification X40). Blue circles, yellow stars, and green triangles indicate new bone, remaining bone graft materials, and connective tissues, respectively.

c. Analysis of histological specimens after an eight-week healing period (H-E stain)

Figure 8 below shows images of H-E stained tissue specimens of the control and experimental groups after an eight-week healing period. There were differences between the control and experimental groups. All experimental groups had mature new bone maintaining connectivity with the upper part, which was not observed in the control group. In the control group, new bone was observed around the remaining bone graft material and near the existing bone. The experimental group showed a significant decrease in remaining bone graft material, and adipose tissue was observed inside when the new bone covered or wrapped around the bone graft material. Also, no new bone was observed in the dura mata in all experimental groups, which only had connective tissue with many inflammatory cells.

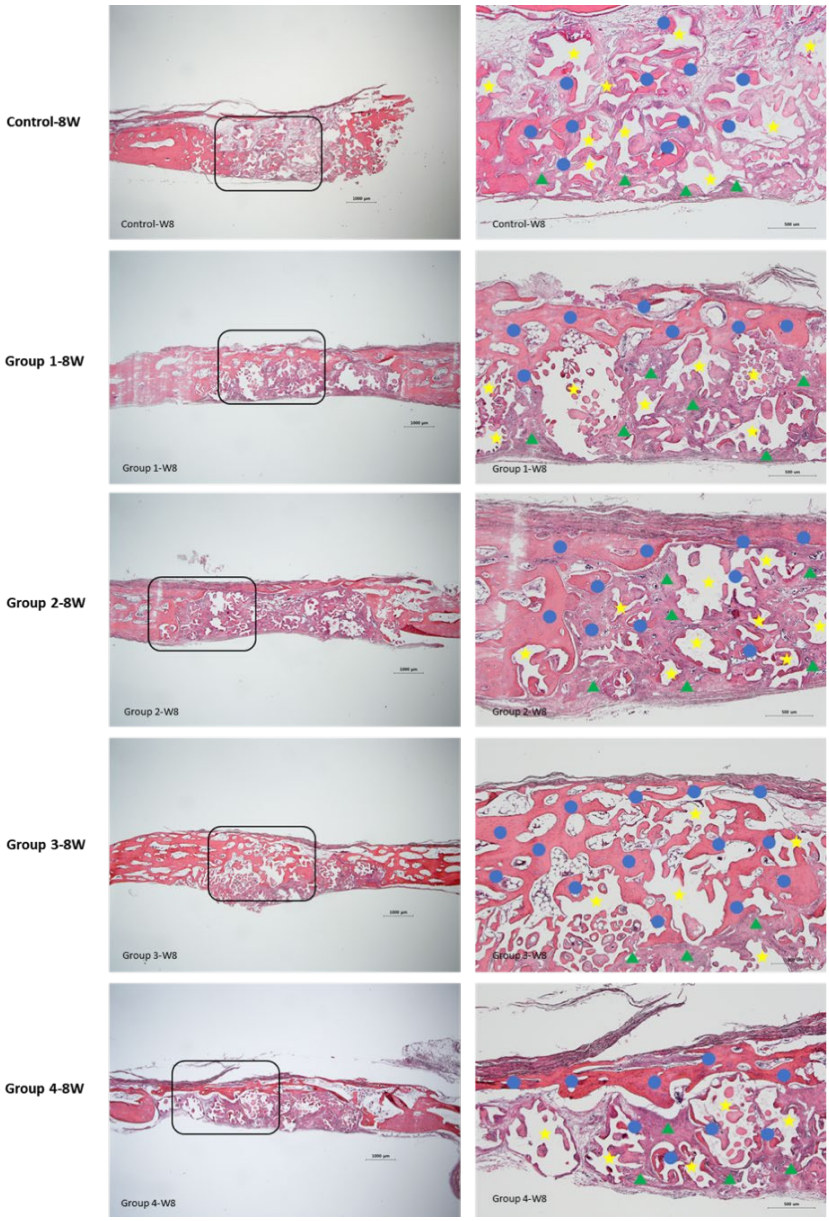

Figure 8. H&E stain of eight-week healing period.

Histological specimens for each group after an eight-week healing period (Left raw original magnification X12.5, Right raw original magnification X40). Blue circles, yellow stars, and green triangles indicate new bone, remaining bone graft materials, and connective tissues, respectively.

C. Histomorphometric analysis

New bone area and residual bone graft material area were measured. The areas of new bone and remaining bone graft material with respect to the total area were calculated as percentages.

D.New bone formation

In the two-way ANOVA to determine whether there was a difference in bone formation rates according to BMP-2 and FGF-2 concentration combinations and the healing period, the p-values were 0.04 and <0.001, respectively, which were less than 0.05, so there were differences in bone formation rates according to BMP-2 and FGF concentration combinations and the healing period, respectively, with a significance level of 5% (Table 2, Figure 9).

In terms of BMP-2 and FGF-2 concentration combinations, the average bone formation rate was 22.14%, 22.42%, 22.51%, 23.87%, and 30.65%, for Group 4, Control, Group 2, Group 1, and Group 3, respectively. In other words, the BMP-2 0.5mg/ml and FGF-2 0.5mg/ml group had the lowest bone formation rate, and the BMP-2 0.5mg/ml and FGF-2 1.0mg/ml groups had the

highest. In the post-hoc analysis using the LSD method, the relationship between each group was Control = Group 1 = Group 2 = Group 4 < Group 3.

In terms of the healing period, the average bone formation rate was 19.15%, 20.8%, and 32.20%, in the order of 2, 4, and 8 weeks. That is, the longer the healing period, the higher the rate of bone formation. In the post-hoc analysis using the LSD method, the relationship between each healing period was 2 weeks = 4 weeks < 8 weeks.

Table 2. New bone formation analysis.

Variables	N	New bone (%)	p-value	Post-hoc analysis with LSD method
Group				
Control	12	22.42 ± 9.06	0.040	Control = G1 = G2 = G4 < G3
BMP-2=1, FGF-2=1 (G1)	11	23.87 ± 11.23		
BMP-2=1, FGF-2=0.5 (G2)	12	22.51 ± 8.48		
BMP-2=0.5, FGF-2=1 (G3)	10	30.65 ± 11.39		
BMP-2=0.5, FGF-2=0.5 (G4)	11	22.14 ± 7.35		
Healing time				
2 weeks	18	19.15 ± 7.31	<0.001	2weeks = 4weeks < 8weeks
4 weeks	19	20.80 ± 6.46		
8 weeks	19	32.20 ± 9.56		

Values were presented by mean ± SD and tested by Two-way ANOVA.

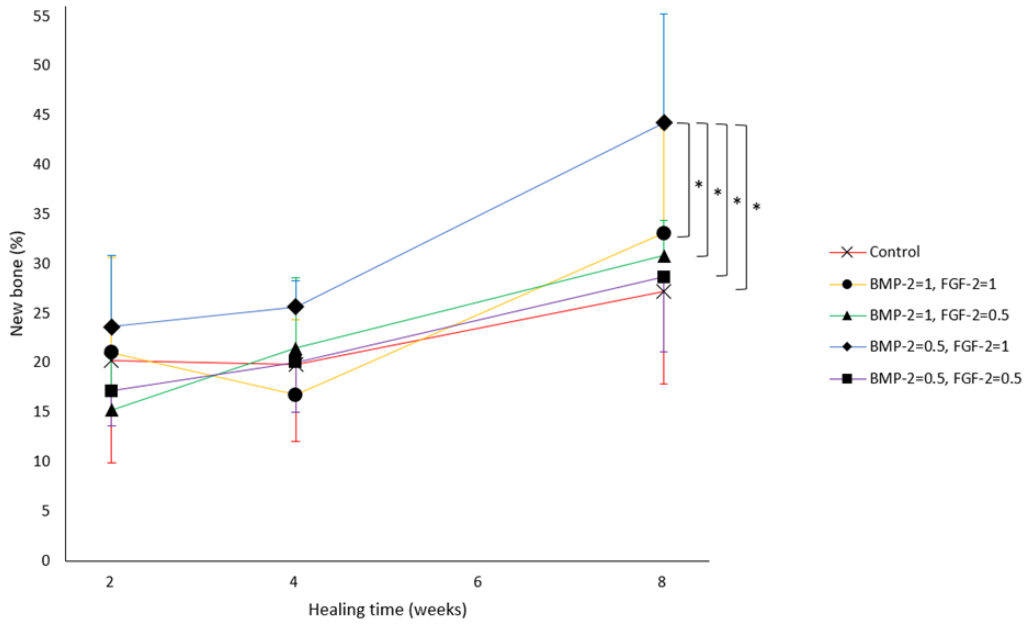

Figure 9. New bone formation between the groups during 2-4-8weeks healing times

a. Histological components

Excluding the new bone and bone graft, the rest of the defect was fibrous tissues. Fig. 10 shows a graph of the manner in which the three compositions change in each group according to the healing period. In all groups, the amount of new bone increased and the amount of bone graft decreased as the healing period elapsed (Figure 10).

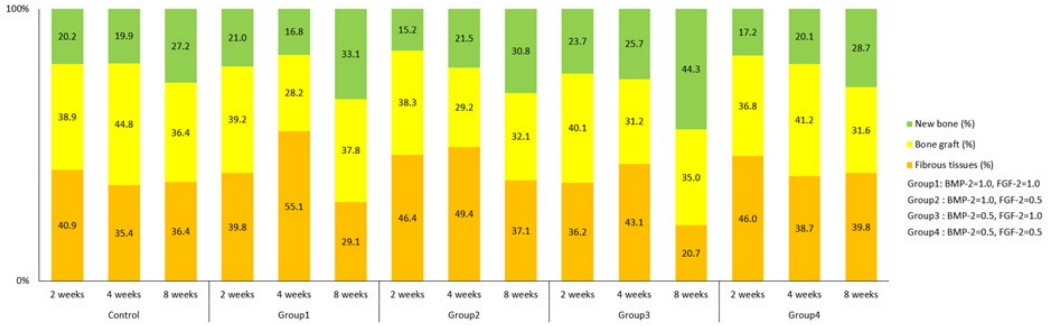

Figure 10. Cumulative graph of histologic components at groups.

E. Histological findings (Russell-Movat Pentachrome stain)

a. Analysis of histological specimens after a two-week healing period (R-M Pentachrome stain)

Figure 11 shows images of the Pentachrome-stained tissue specimens of the control and experimental groups after a two-week healing period. In the control group, new bone was observed near the existing bone, and collagen fibers were observed around the bone graft material. In the experimental group, mature new bone was observed around the existing bone, and new bone was observed in the upper part and inside the bone graft material. These new bones were not yet mature, and many collagen fibers were observed in the upper part and around the bone graft material.

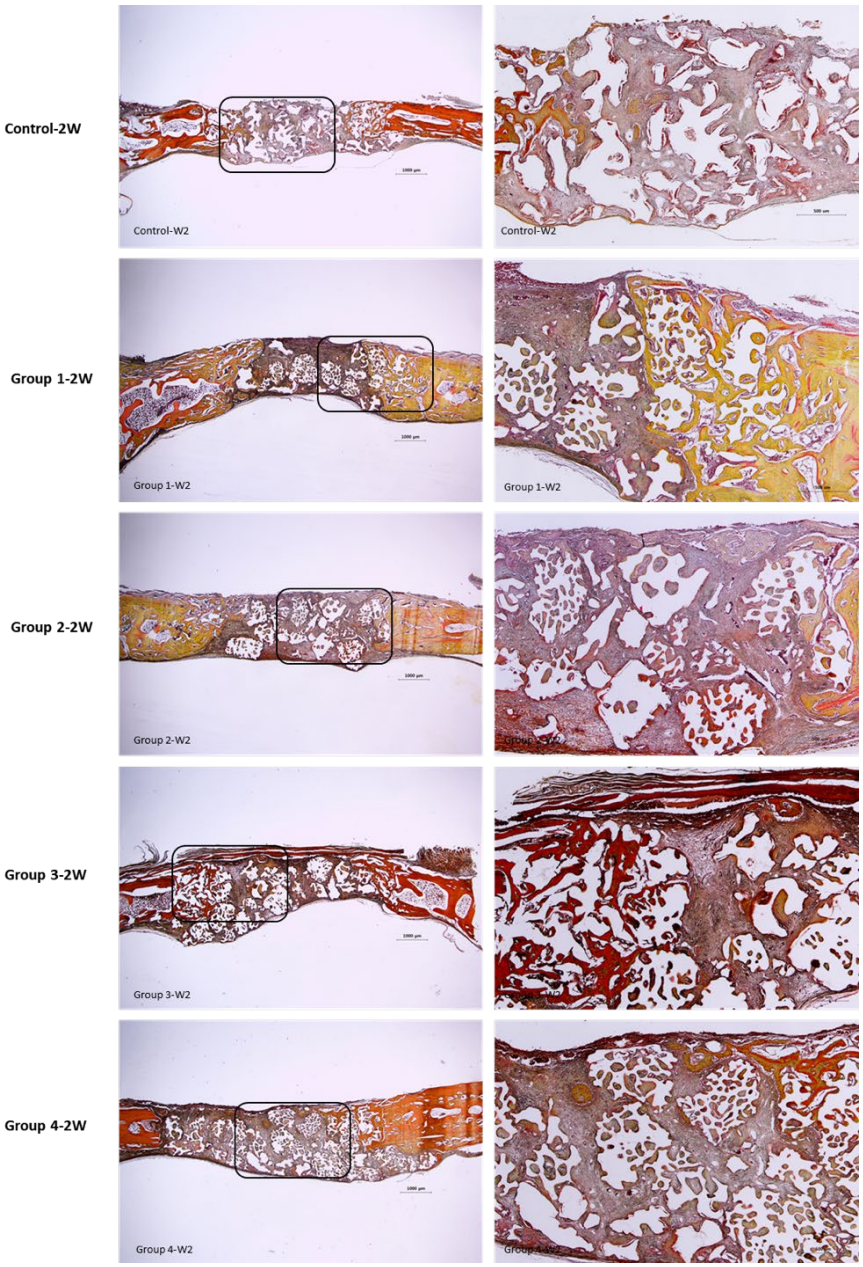

Figure 11. Pentachrome stain of two-week healing period.
Histological specimens for each group after a two-week healing period (Left raw original magnification X12.5, Right raw original magnification X40).

b. Analysis of histological specimens after a four-week healing period (R-M Pentachrome stain)

Figure 12 below shows images of the Pentachrome-stained tissue specimens of the control and experimental groups after a four-week healing period. In the control group, mature new bone was observed around the existing bone, and immature new bone was observed only inside the bone graft in the defect. In the experimental group, mature new bone was observed around the existing bone, similar to the control group, but these bones were observed alone in the upper part or derived from the existing bone. These were not fully connected, but were in the form of a mature bone. New immature bone was also observed inside the bone graft material, as in the control group.

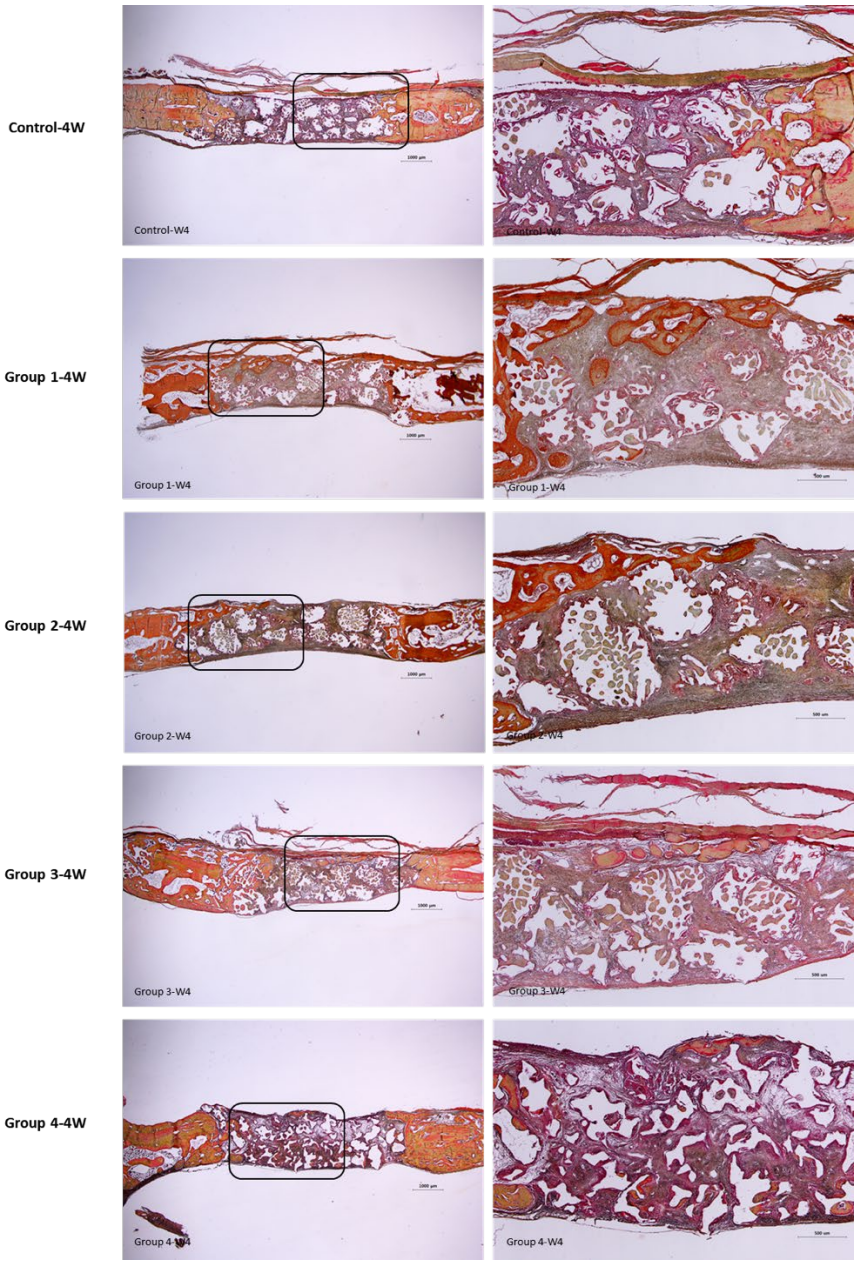

Figure 12. Pentachrome stain of four-week healing period.
Histological specimens for each group after a four-week healing period (Left raw original magnification X12.5, Right raw original magnification X40).

c. Analysis of histological specimens after an eight-week healing period (R-M Pentachrome stain)

Figure 13 shows images of the pentachrome-stained tissue specimens of the control and experimental groups after an eight-week healing period. In the control group, mature new bone was observed around the existing bone, but inside the defect, new bone and collagen fibers were observed only around the bone graft material. The experimental group had continuous mature new bone that completely sealed the upper part of the defect, while in the existing bone, it became thinner toward the center of the defect. No new bone was observed in the lower part of the defect in both the experimental and control groups.

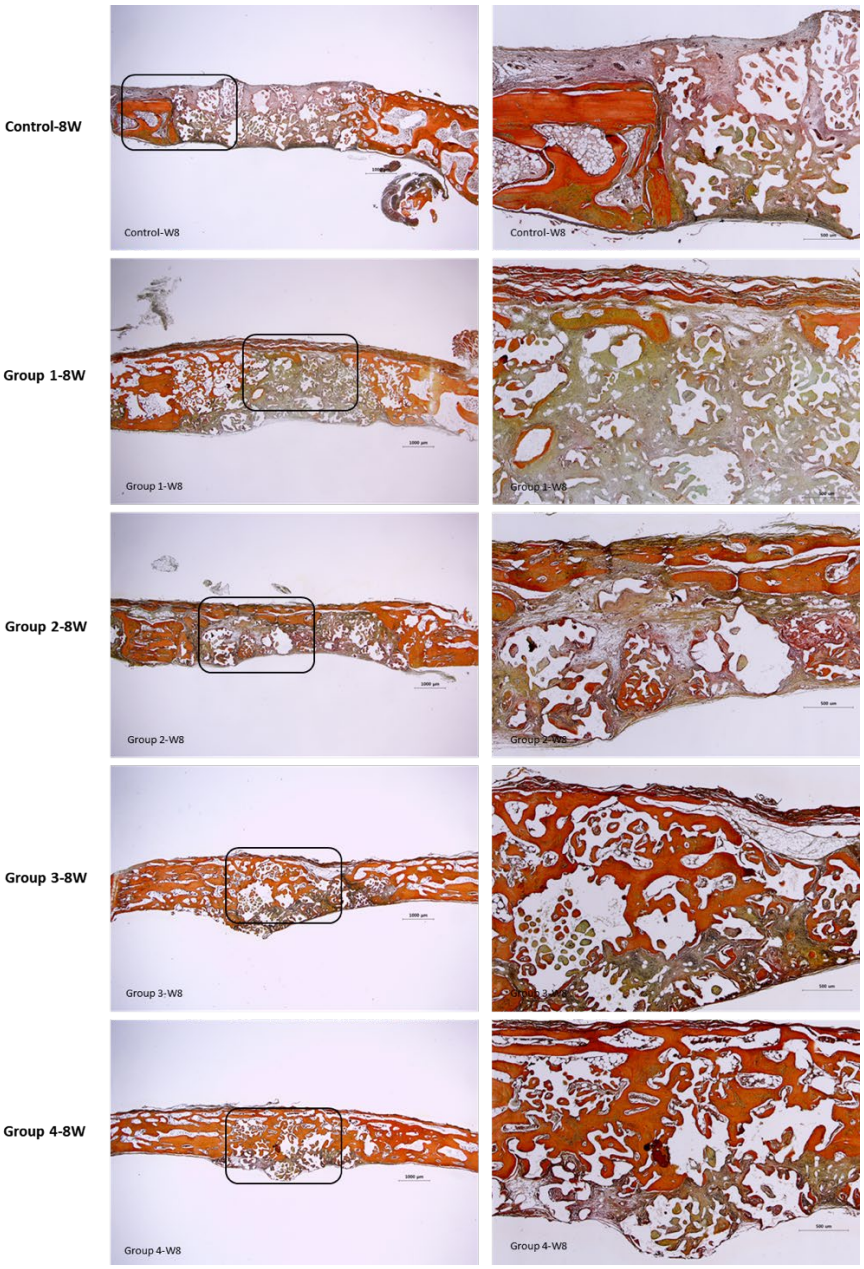

Figure 13. Pentachrome stain of eight-week healing period.
Histological specimens for each group after an eight-week healing period (Left raw original magnification X12.5, Right raw original magnification X40).

F. Immunohistochemical findings (Anti-Osteocalcin antibody stain)

a. Analysis of histological specimens after a two-week healing period (Anti-Osteocalcin antibody stain)

Figure 14 below shows images of anti-osteocalcin antibody (OCG3) stained tissue specimens of the control and experimental groups after a two-week healing period. Osteocalcin was observed around the bone graft material in both the control and experimental groups. However, the expression of osteocalcin was higher in the bone graft material around the existing bone than in the center of the defect.

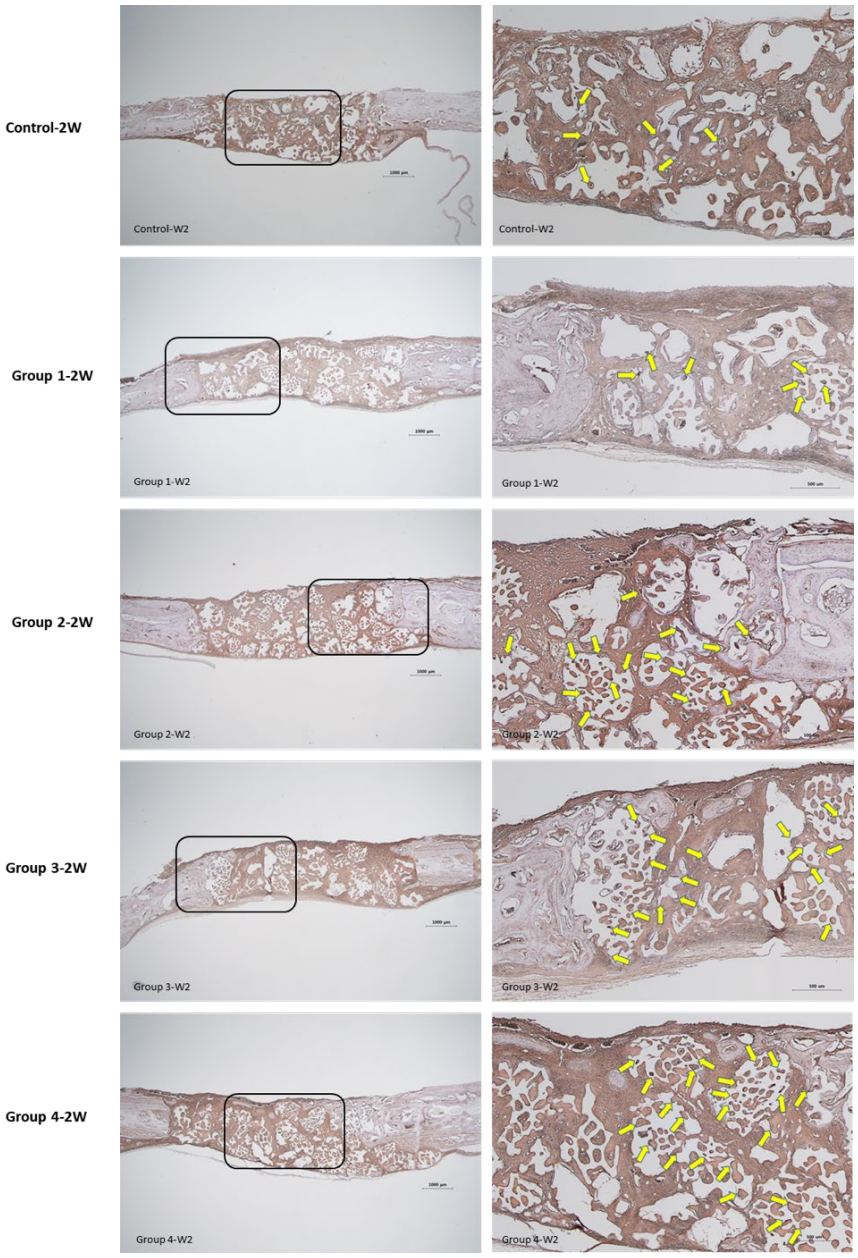

Figure 14. Osteocalcin expression of two-week healing period.
Histological specimens for each group after a two-week healing period (Left raw original magnification X12.5, Right raw original magnification X40). Yellow arrows indicate osteocalcin.

b. Analysis of histological specimens after a four-week healing period (Anti-Osteocalcin antibody stain)

Figure 15 below shows images of anti-osteocalcin antibody (OCG3) stained tissue specimens of the control and experimental groups after a four-week healing period. The control group showed osteocalcin expression sites similar to those of the two-week group. These were mainly observed around the bone graft material, and there was nothing significant in the defect. The 4-week experimental group had different osteocalcin expression sites from the 2-week group. These were mainly observed toward the collagen membrane, and when the upper part was sealed, as shown in Group 2 in Fig. 16, they were near the bone graft material at the center of the defect rather than directly below the new bone. In the experimental group, osteocalcin was not observed in the dura mata of the defect.

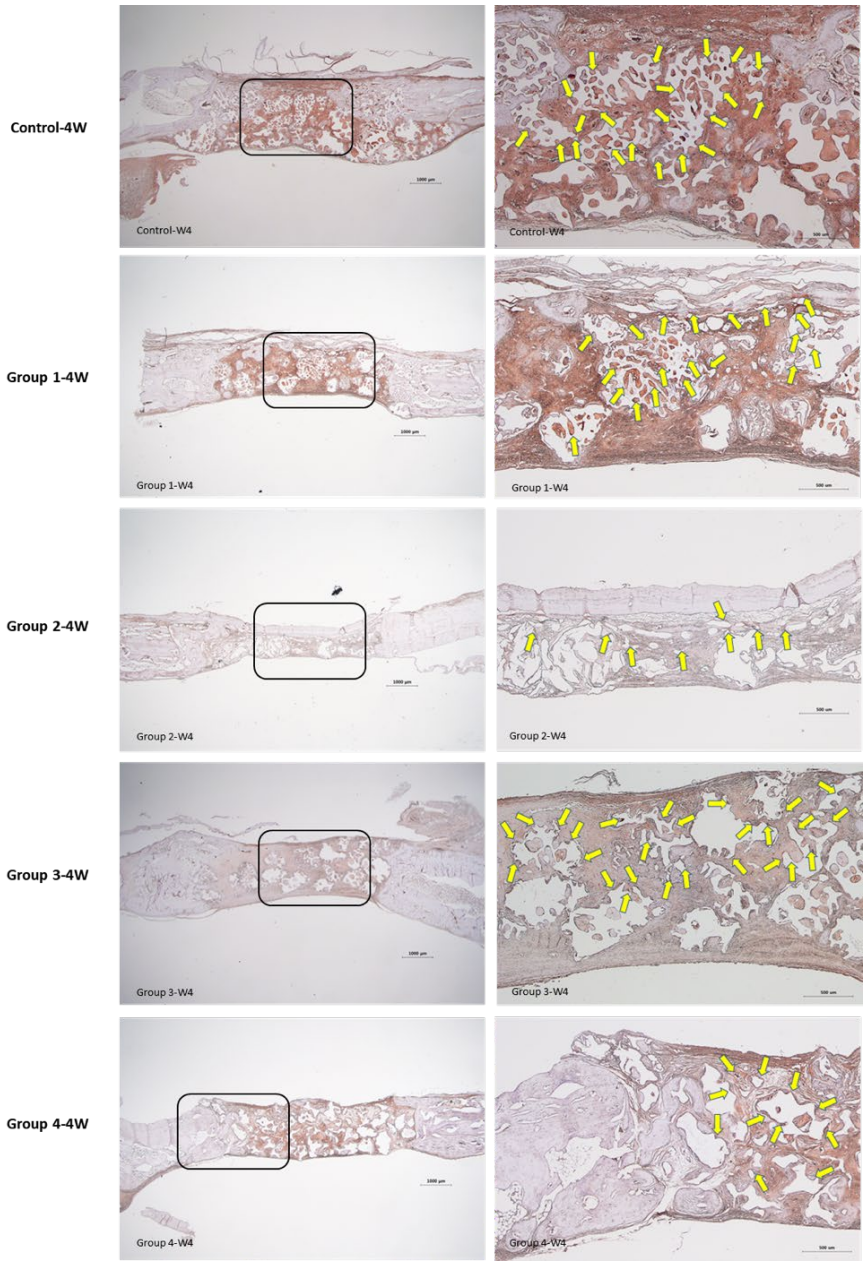

Figure 15. Osteocalcin expression of four-week healing period.
Histological specimens for each group after a four-week healing period (Left raw original magnification X12.5, Right raw original magnification X40). Yellow arrows indicate osteocalcin.

c. Analysis of histological specimens after an eight-week healing period (Anti-Osteocalcin antibody stain)

Figure 16 below shows images of anti-osteocalcin antibody (OCG3) stained tissue specimens of the control and experimental groups after an eight-week healing period. In the control group, similar to the 2 and 4-week groups, osteocalcin was observed near the bone graft material around the existing bone, but there was nothing significant in the defect. In the 8-week experimental group, osteocalcin expression was decreased compared to the 2 and 4-week groups, and when the new bone of the collagen membrane filled the defect, osteocalcin was observed near the internal bone graft material rather than below the new bone. Also, osteocalcin was not observed between or near the new bone in the upper part, nor in the lower part of the dura mata of the defect, as in the 4-week group.

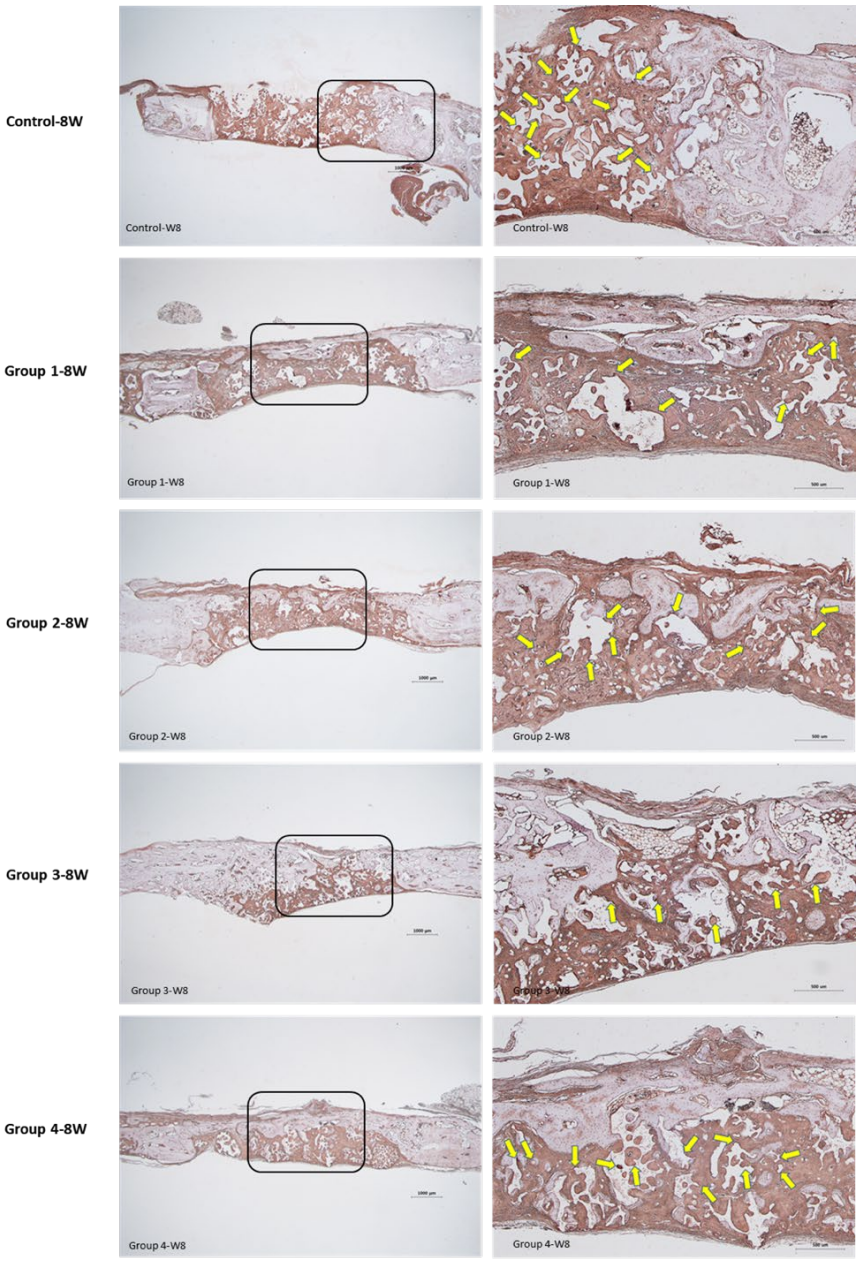

Figure 16. Osteocalcin expression of eight-week healing period.
Histological specimens for each group after an eight-week healing period (Anti-Osteocalcin antibody stain. Left raw original magnification X12.5, Right raw original magnification X40). Yellow arrows indicate osteocalcin.

IV.DISCUSSION

The aim of this study was to investigate whether guided bone regeneration (GBR) using a collagen membrane applying BMP-2 and biphasic calcium phosphate (BCP) applying FGF-2 was effective for bone regeneration in critical size defects (CSD) in rabbit calvaria. Schmitz et al. defined CSD as "the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal" [66]. However, in preclinical studies, due to limitations of the evaluation period and the species traits of experimental animals, CSD in animal studies refers to the size of a defect that will not heal during the study period [67]. In a systematic review of CSDs in rabbit calvaria, Delgado-Ruiz et al. reported that circular defects with a size of 6-8mm did not completely occlude spontaneously, even after 12 weeks [68]. Kramer et al. also stated that 8 mm bone defects in rabbit calvaria did not completely occlude after 24 weeks [69]. Also, when four 8 mm CSDs were created in the rabbit skull, four defects were acceptable for one animal, and the model was suitable for comparing biomaterials while avoiding

various individual deviations [70]. Cranial osteogenesis occurs through an intramembranous pathway, such as the jaw, so calvaria is an appropriate animal model for alveolar bone healing [71].

This study was conducted after creating CSDs with a diameter of 8mm in the rabbit calvaria, and the experimental group showed a statistically higher level of new bone formation than the control group ($p<0.05$) (Table 2, Figure 9). Group 3 (BMP-2 0.5mg/ml, FGF-2 1.0mg/ml) was found to have formed more new bone in the histomorphological analysis than the other groups, which became significantly higher as the healing period elapsed (LSD analysis 2 weeks = 4 weeks <8 weeks). Also, in terms of pentachrome staining, new mature bone was observed only around the existing bone in the control group even after 2-4-8 weeks of healing. However, the experimental group showed similar patterns to the control group at 2 weeks, but after 4 weeks, thin mature new bone was observed in the collagen membrane, and at 8 weeks, continuous mature new bone was observed in the collagen membrane, and the thickness increased from 4 weeks. However, in the anti-osteocalcin stained specimens, osteocalcin (OCN) was not observed

below the mature new bone generated at the upper part, but a small amount was observed around the internal bone graft material, so new bone formation will not increase rapidly in the experimental group after 8 weeks of healing. New bone was also observed near the bone graft material in the control group, which did not use GFs. Yellow collagen fibers were observed between the bone graft materials, and OCN was observed around the bone graft material, indicating that new bone formation will continue even after 8 weeks of healing. OCN, a non-collagenous protein, is a major component of the bone extracellular matrix and is secreted from osteoblasts [72,73]. It is often used as a biochemical marker of osteogenesis by reflecting the number and activity of osteoblasts [74,75]. The presence of osteocalcin indicates that osteoblasts are in the maturation stage and will continue to form new bone [76]. In the results of this study, the experimental group showed faster bone regeneration than the control group, indicating that GFs play a role in GBR.

BMP-2 is a potent osteogenic agent that accelerates osteoblast differentiation to secrete a new bone matrix, including early collagen

and mineral precursors [77], thereby inducing rapid bone regeneration [30]. To confirm the quick bone regeneration ability of BMP-2, Chung et al. used collagen membranes as a BMP-2 carrier, and reported that alkaline phosphatase (ALP) activity increased after 2 weeks and OCN increased immediately after surgery (4 days) [56]. The early formed new bone in the upper part can play a role in protecting internal bone regeneration. Several previous studies also reported that rapid upper bone formation can protect internal bone regeneration during the healing period by preventing the penetration of external connective tissue [22,30,78]. In the experimental group, continuous new bone was observed in the collagen membrane, and the 8-week group showed complete defect occlusion, confirming the role of protecting internal bone regeneration.

FGF-2 also affects new bone formation. Klagsbrun et al. [79] reported that FGF-2 has a high affinity for heparin and prevents heparin degeneration and proteolysis by binding to heparin-like molecules in the basement membrane and extracellular matrix. Takayama et al. [38] reported that FGF-2 played a significant role in cell differentiation and homeostasis of

gingival epithelial cells in the early stages of wound healing. With these mechanisms, the main action of FGF-2 in wound healing is angiogenesis through revascularization [26]. In addition to angiogenesis, FGF-2 plays a significant role in bone regeneration by inducing marrow-derived mesenchymal cells and promoting osteoblast differentiation [26]. This action or effect can be confirmed by ALP activity and OCN increase [80]. Through an in vitro study, Frank et al. reported that FGF-2 was related to the expression of BMP-2 [81], while Naganawa et al. showed that null mice without the FGF-2 gene do not express BMP-2 [82]. Mouse studies by Nakamura et al., Tanaka et al., Kuhn et al., and Sabbieti et al. found that the concentration of FGF-2 may modulate or interfere with the action of BMP-2 [83-86]. Among the functions of FGF-2, its interaction with BMP-2 is not yet completely determined, but previous studies have confirmed that FGF-2 can affect the bone regeneration function of BMP-2.

However, combined applications of growth factors may result in diverse complications during bone regeneration. Clinical complications associated with the use of BMP-2 include tumorigenesis, ectopic bone formation,

osteolysis, urinary system disorders, osteolysis, bone cyst, and inflammation [87]. The complications associated with FGF-2 include tumorigenesis [88] and diabetes-related vascular diseases [88,89]. There is also a clinical report in which high concentrations of BMP-2 and FGF-2 were found in ossified sites of adenocarcinoma [90]. When applied with bone graft materials, an increase in fat cells and cystic change also lead to a decrease in the quality of the grafted bone [91]. Studies have been conducted on approaches to reducing the side effects of BMP-2, including research on using other growth factors, such as using different carrier systems [92], using vascular endothelial growth factor (VEGF) [93], and using bFGF conjugates [94]. It was also reported that using multiple GFs with low concentrations can reduce the risk of side effects compared to using a single GF with a high concentration [44]. Lee et al. reported that the use of BMP-2 inside the defect resulted in excessive adipose tissue after healing, which affected the stability of the graft site or new bone quality [91]. When applying BMP-2 and FGF-2, it is necessary to set each concentration to reduce side effects.

This study used two different BMP-2 and FGF-2 concentrations, in which 0.5mg and 1.0mg of each was diluted in 1.0ml of physiological saline. Based on a concentration of 1.0mg/ml, 0.5～1.0μg/mm^3 of BMP-2 and FGF-2 were used [41]. The experiments involved creating multiple defects in the rabbit calvaria, so the paracrine effect with adjacent defects should have been considered. but the effect on the sites to which a low concentration of growth factors was applied is limited [95,96], so it was not considered in the results. However, the same concentration of FGF-2 was applied to the same side of the left and right defects to obtain maximum fixation of the collagen membrane and reduce left and right interference.

This study measured the amount of new bone according to the use of different BMP-2 and FGF-2 concentrations, and Group 3 (BMP-2 0.5mg/ml, FGF-2 1.0mg/ml) showed a statistically significant difference in new bone formation compared to other groups ($p<0.05$) (Table 2, Figure 9). This suggests that the combined use of a relatively high concentration of FGF-2 (1.0 mg/ml) and a relatively low concentration of BMP-2 (0.5 mg/ml) is

effective for bone regeneration. These results are consistent with the findings of a previous study [65] on rabbit calvarial defects, which showed that BMP-2 0.5mg/ml resulted in more bone formation than 1.0mg/ml. Lee et al. [41] reported no statistically significant difference in new bone formation between FGF-2 0.5mg/ml and 1.0mg/ml, but the 1.0mg/ml group showed 6-7% more bone growth on average than the control group. Based on the experimental results, it is considered an effective and useful method for bone regeneration in bone defects by reducing the probability of side effects through using high BMP-2 concentrations and reducing the total use of growth factors.

In terms of comparing new bone formation by healing period, there was no statistical difference between the 2-week group and the 4-week group. However, the 8-week group showed a statistically significant difference ($p<0.01$) compared to the 2-week and 4-week groups (Table 2, Figure 9). According to Misch [97], the bone remodeling cycles of rabbits and humans are 6 and 17 weeks, respectively. This study divided the healing period into 2, 4, and 8 weeks to check the early phase in the bone healing

process and the late phase after the bone remodeling cycle. The groups show differences after the mid-phase in wound healing rather than the early stage due to BMP-2 expression. Charles et al. [44] reported that BMP-2 maintained persistence during the osteogenic period for 8 weeks in mouse calvarial models. On the other hand, in the in vitro test, 40% of FGF-2 was released in the early phase, and 60% maintained persistence. In addition, through analyzing tissue composition inside the defect according to the healing period, it was confirmed that new bone increased while bone graft material tended to decrease (Figure 10). BCP causes osteoinduction by a mechanism that induces the differentiation of mesenchymal stem cells through the surface topography of graft materials [98]. β-TCP has a higher chemical dissolution than slowly absorbed HA, and the resorbed BCP is replaced by newly formed bone [99,100]. Cellular degradation of BCP is due to the extracellular process characteristic of osteoclastic resorption [101]. The osteogenesis of osteoblasts and bone resorption of osteoclasts are related to each other during the bone remodeling process [102], and osteoclasts also play a significant role in bone regeneration [103]. HA is degraded by cellular action such as osteoclasts, and β-TCP has a faster chemical dissolution rate than

cellular action [104,105]. As the bone graft progresses and the healing period elapses, the bone graft material gradually decreases due to cellular action and chemical dissolution; the goal of bone regeneration in GBR is to replace this space with new bone. Considering the histological analysis results in which the 8-week group showed more new bone formation, and the histological analysis results of observing continuous mature bone in the collagen membrane, the GBR method adding GFs brings space maintenance and continuous bone regeneration compared to the control group, even after the healing period.

The purpose of this study was to perform GBR using BMP-2 + collagen membrane and FGF-2 + BCP on rabbit CSDs to form new upper bone in the early stage to isolate bone defects, thereby securing space for bone regeneration and improving bone regeneration ability. During the 8-week healing period, the histological findings showed that forming mature new bone in the collagen membrane increased total new bone. Also, the concentration combination of BMP-2 (0.5mg/ml) and FGF-2 (1.0mg/ml) was statistically significantly higher in new bone formation ($p<0.05$). However,

some limitations could not be excluded due to the lack of experimental subjects and the specificity of inter-individual healing caused by the limits of animal testing. It will be necessary to find effective internal scaffolds for bone regeneration in defects by deriving experimental designs that show the total volume increase or decrease as an absolute value during the healing period. Also, while we confirmed the possibility of using a low dose of BMP-2, further research should be conducted in order to reduce the overall concentration. Additional studies on the combined use of GFs other than FGF-2, which is known to interact with BMP-2, for selecting GFs inside defects will lead to the discovery of effective methods for bone regeneration.

V. CONCLUSION

In this study, it was confirmed that the new dual scaffold matrix method using a collagen membrane applied with BMP-2 and BCP applied with FGF-2 was effective for bone regeneration in GBR. The concentration combination of BMP-2 0.5mg/ml and FGF-2 1.0mg/ml showed a higher osteogenic ability compared to the experimental groups with other concentrations. In addition, the dual scaffold complex is quantitatively and qualitatively advantageous for bone regeneration and bone maintenance over time.

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ABSTRACT (In Korean)

두 가지 성장인자를 이용하여 신속 상부골 형성을 통한 골형성능 증진에 관한 연구

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박 재 한

본 연구의 목적은 GBR을 증진시키는 방법으로 collagen membrane에 BMP-2를 적용하여 치유 초기 골형성을 유도하여, 결손부 내부를 외부 자극으로부터 격리함으로써 치유 기간 동안 구조적으로 안정적인 골재생 환경을 조성하고, 이렇게 격리된 골결손부 내부에서 골이식재와 FGF-2를 적용하여 골재생을 증가시키는 방법을 제시하고자 하였다. 또한 이렇게 디자인한 골형성 증진 GBR 모델에서 골형성에 제일 효과적인 GF의 농도를 찾아보고자 하였다.

New zealand white rabbit 24마리를 본 연구에 사용하였다. 개체별 calvaria에 직경 8mm의 4개의 골결손부를 형성하고, GBR을 시행하였다. GBR 적용 시에 대조군에는 collagen membrane과 BCP를 적용하였고, 4개의 실험군에는 collagen membrane + BMP-2 (0.5, 1.0 mg/ml), BCP + FGF-2 (0.5, 1.0

mg/ml)를 각각 적용하였다. 2, 4, 8주 치유기간을 가진 후 희생하여, 조직학적, 조직형태학적 분석을 시행하였다

조직학적 분석에서 실험군에서 상부에 연속적인 형태의 신생골이 관찰되었으며, 대조군에서는 연속적 형태는 관찰되지 않았다. 기존골에서 파생되어지는 신생골은 대조군과 실험군에서 모두 관찰된다. 조직형태학적 분석에서 신생골형성은 Group 3(BMP-2 0.5 mg/ml, FGF-2 1.0 mg/ml)에서 통계적으로 유의차 있게 높게 나왔다(30.65 ± 11.39%, $p<0.05$). 또한 치유기간별 신생골형성은 2주와 4주는 통계적으로 차이가 없었으나, 8주에서는 통계적으로 유의차 있게 높게 나왔다(2=4<8 weeks, $p<0.05$).

GBR을 이용한 골결손부 골재생에 collagen membrane과 BMP-2, BCP와 FGF-2를 사용한 방법은 신생골 형성에 있어서 효과적이다. 신생골 형성에 효과적인 성장인자의 농도는 BMP-2 0.5 mg/ml, FGF-2 1.0mg/ml 이다. 신생골 형성은 치유기간이 길수록 증가한다

핵심되는 말: 성장인자, 동물실험, 창상치유, 골재생유도