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Synergistic Effect of BMP2 with Bone Grafts Covered by FGF2 Soaked Collagen Membrane on New Bone Formation in Mongrel Dogs

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Synergistic Effect of BMP2 with Bone Grafts Covered by FGF2 Soaked Collagen Membrane on New Bone Formation in Mongrel Dogs

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June 2016

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ABSTRACT

Synergistic Effect of BMP-2 with Bone Grafts Covered by FGF-2 Soaked Collagen Membrane on New Bone Formation in Mongrel Dogs

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Purpose: The purpose of this study is to evaluate, through histomorphometric analysis, synergistic effect of sequential use of both BMP2 and FGF2 on new bone formation by using BMP2 with Biphasic Calcium Phosphate (BCP) covered by FGF2 soaked collagen membrane in the maxillary lateral augmentation defect model of Mongrel dog. **Methods**: Six male mongrel dogs were used in this study. All maxillary premolars (P1, P2, P3) were extracted bilaterally and 8 weeks after extraction, box-shaped lateral defects with dimension of 5 mm x 5 mm x 5 mm^3 were surgically created. Then, the defects were filled with BCP containing BMP2

and covered by either collagen membranes soaked with saline (BMP2 group) or collagen membranes soaked with FGF2 (BMP2+FGF2 group). 4 and 12 weeks following treatment, dogs were sacrificed and histological and histomorphometric analyses were performed. **Results**: Histological analysis revealed that a greater amount of newly-formed immature bone was observed in BMP2+FGF2 group than that of BMP2 group. In histomorphometric analysis, at 4 weeks post-treatment, BMP2 group showed greater amount of new bone formation (23.04±4.67%), connective tissue volume (24.06±7.73%), and osteoclast number (76.86±36.76 unit), than those of BMP2+FGF2 group; (21.03±8.80%), (23.36±6.27%), and (76.17±22.61 unit), respectively. Furthermore, BMP-2 group showed a smaller amount of the graft material remained (15.21±7.44%), and blood vessel count (95.33±22.46 unit), than those of BMP2+FGF2 group; (19.60±6.82%) and (106.33±29.17 unit), respectively. In contrast, at 12 weeks post-treatment, BMP2+FGF2 group showed a greater amount of new bone formation (35.55±8.35%), connective tissue volume (12.48±3.32%), blood vessel count (64.83±8.80 unit) and number of osteoclast (123.17±49.93 unit) than those of BMP2 group; (30.12±8.25%), (17.02±8.15%), (47.33±15.59 unit), and (103.00±39.14 unit), respectively. However, BMP2+FGF2 group showed a smaller amount of the graft material remained (3.11±2.80%) than BMP2 group (8.76±4.16%). **Conclusions**: Within the limitation of this study, while their short-term effect is limited, the delivery of

both BMP2 and FGF2 may have long-term synergistic effect on new bone formation and therefore, may supply a promising treatment modality for repairing large bone defects.

Key words: Guided bone regeneration (GBR), Biphasic Calcium Phosphate (BCP), Bone morphogenetic proteins 2 (BMP2), Fibroblast growth factor 2(FGF2).

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I. Introduction

In order to improve quality of life in edentulous patients, treatment using the conventional dental implants which had been used successfully for many years to restore function and esthetics in edentulous patients has been steadily increasing. In 1965, Branemark studied the contact between titanium and bone and called it osseointegration, which was the start of theoretical and historical beginning of implants (Becker and Becker, 1989; Brånemark et al., 1985). However, when

placing implants, there were many anatomically vital structures such as nasal sinus, maxillary sinus and inferior alveolar nerve that did not allow adequate space for implant placements in both maxilla and mandible. Also, bone quality was not favorable in maxilla compared to mandible and resulted in relatively higher implant failure rate. Higher implant failure rate found in maxilla, especially in the posterior region may be due to reasons such as pneumatization of sinus, low bone density, and unfavorable bone quality and quantity (Galindo et al., 2005). In order to overcome these limitations of implant placements, Guided Bone Regeneration (GBR), which was originated from guided tissue regeneration (GTR) in 1980's (Dahlin et al., 1989), was introduced. However, the amount of new tissue generation using this method was still limited.

With the recent improvements in tissue engineering, studies on clinical application of growth factors, bone forming proteins and many biologically controlled agents to promote new bone formation are being studied extensively. In particular, there are many studies to induce new bone formation using Bone Morphogenetic Protein-2(BMP2) (Amini et al., 2012). BMP2 is a strong bone inducer that can promote bone formation by inducing chondroblast and osteoblast differentiation from undifferentiated mesenchymal stem cells (Gerstenfeld et al., 2003; Sykaras and Opperman, 2003; Tickle, 2002). Bone regenerating ability of BMP2 had been verified in various animal studies with rats (Lutolf et al., 2003), rabbit (Chang et al., 2004), and mongrel dogs (Murakami et al., 2003), and due to

its proven superiority, it is also being widely used in clinical settings. Nevertheless, BMP2 is not completely free from limitations. BMP2 has short half- life due to ‘early busting’ effect. Moreover, it has reported complications such as ectopic bone formation, edema and severe inflammatory response when used in high dose (Winn et al., 2000). Consequently, there have been discussions on additional factors necessary to overcome the aforementioned limitations while maximizing the effects of BMP2 (Baltzer et al., 2000; Franceschi et al., 2004).

Among many factors that have been discussed, Fibrous Growth Factor-2 (FGF2) had been reported to play important role in natural bone formation and bone healing. Even when FGF2 was used alone, it was reported that FGF2 had ability to induce new bone formation by promoting angiogenesis (Kim et al., 2010; Nomi et al., 2006; Oh et al., 2012). In fact, during embryonic period, FGF2 stimulates formation of lateral plate of mesoderm and form ectodermal layer, which will later become apical ectodermal ridge, and is also involved in crystallization of mesenchymal cells that will form zone of polarizing activity (Martin, 1998). FGF2s secreted from apical ectodermal ridge promote mesenchymal cell proliferation from progress zone of the limb bud while BMP2s determine the location of mesenchymal condensation that will form the skeleton. Throughout this series of progress, the sequential and spatial expression of FGF2 and BMP2 is known to regulate skeletal and bone formation (Bitgood and McMahon, 1995). Also, during the fusion of fracture site, macrophages and

multinuclear neutrophils from the early inflammatory phase move to the fracture site and remove microorganisms and necrotized tissues while increasing expressions of PDGF, IGFs, TGF-β, and FGF2 form hematoma. With time, progenitor cells are known to be differentiated into chondroblasts and osteoblasts as PDGF, IGFs, TGF-β, and FGF2 expressions increase along with proliferation of fibroblasts from the granulation tissue (Gerstenfeld et al., 2003; Khan et al., 2000).

Based on these aforementioned results, it can be anticipated that the interaction between BMP2 and FGF2 plays vital role in producing adequate amount of bone formation during natural bone formation and bone healing process. The enhanced bone regeneration of combinational use of growth factors has been verified in recent studies, which reported that bone forming ability of BMP2 was promoted when recombinant protein of FGF2 and BMP2 was delivered at the same time using Type I collagen carrier (Kakudo et al., 2006; Nakamura et al., 2005).

Therefore, in this study, based on the knowledge of interaction of growth factors, the effect of sequential and spatial application of BMP2 and FGF2 using BCP and collagen membrane as a scaffold on improving new bone formation will be analyzed histologically and histomorphometrically.

II. MATERIALS AND METHODS

1. In vitro release kinetics of BMP2 and FGF2

To evaluate release kinetics of BMP2 and FGF2, 0.07 g of BCP soaked with 50 μL of BMP2 (Bone Morphogenetic Protein-2, Genoss, Korea), 10 x 20 mm^2 collagen membrane soaked with 50 μL of FGF2 and their combination were prepared and measured with BMP2, FGF2 enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK) as per the manufacturer's instruction. To collect the supernatant, each samples were immersed in 100 μL and incubated at 37°C with constantly reintroducing fresh Phosphate-buffered saline (PBS) solution to maintain the same volume. 100 μL of each samples were collected after 1, 3, 5, 7 and 12 h, and 1, 3, 5 and 7 days. Absorbance was red with an ELISA reader (Molecular Devices, Workingham, UK) at a wavelength of 450 nm and the cumulative release of BMP2 and FGF2 were calculated and plotted on a graph.

2. Animals

Six male mongrel dogs, 1-year-old weighting approximately 25-30 kg each, were used in this study. The animals were fed with a standard laboratory diet in a purpose-designed room for experimental animals. All animal care and treatment protocols followed the routines approved by the Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea (Approval no. 2013-0109). The surgical procedures of extraction and implant placement were performed under general anesthesia in accordance with previous study (Jung et al., 2007). The six dogs were randomly assigned to two observation period groups, with three dogs each in the observation period groups of 4 weeks and 12 weeks after surgery.

3. Tooth Extraction

One week before the tooth extraction, Oral prophylaxis comprising of calculus removal and chlorhexidine swabs was performed. Surgical procedures were performed under general anesthesia. Briefly, the animals were intravenously injected with atropine (0.05 mg/kg; Kwangmyung Pharmaceutical Ind. Co., Seoul, Korea), and then intramuscularly injected with a combination of 2 mg/kg xylazine Rompun; Bayer Korea Co., Seoul, Korea) and 10 mg/kg ketamine

(Ketalar; Yuhan Co., Seoul, Korea). After that, 2% enflurane was administered to the animals by endotracheal intubation. The maxillary premolars P1, P2 and P3 in the dogs were extracted bilaterally. After premolar extraction, resorbable sutures (VICRYL 4.0; Ethicon, Norderstedt, Germany) were performed for wound closure. The dogs were fed with a soft diet.

4. Surgical procedure

Eight weeks after tooth extraction, implantation and regenerative surgery was performed. In brief, following a mid-crestal incision of 3 cm in length was created, one side of the full-thickness mucoperiosteal flaps was carefully elevated at the maxillary first molar (P1) and third molar (P3). After that, a box-shaped defect (5 mm in the mesio-distal direction, 5 mm in the apical–coronal direction, and 5 mm in the bucco-oral direction) was created at the lateral aspect of each maxillary molar. The defects were then filled with a biphasic calcium phosphate (BCP) synthetic bone graft materials (OSTEON II™, Genoss, Co., Ltd Suwon, Korea). Prior to the bone grafting, the BCP bone graft materials were soaked in 50 μL of 1mg/ml rhBMP2 (Genoss, Co., Ltd Suwon Seoul, Korea) solution for 10 min. We then used collagen membranes (Genoss, Co., Ltd Suwon Seoul, Korea) with dimension of 10 x 20 mm^2 to cover the BCP bone graft materials.

The collagen membranes were either soaked in 50 μL of saline solution or in 50 μL of 1mg/ml FGF2 solution for 30 min, allowing the FGF2 to penetrate into the membrane. The flaps were closed with resorbable sutures (VICRYL 4.0; Ethicon, Norderstedt, Germany). The sutures were removed after 7-10 days after surgery. After the surgical procedures, the animal received analgesic to reduce postoperative pain. The animals were sacrificed 4 and 12 weeks after regenerative surgery with intravenous sodium pentobarbital under deep anesthesia. Bony defects were harvested for histological and histomorphometric analysis.

5. Histological preparation

The harvested specimens with intact bony defects were fixed in 10% formaldehyde for 6 weeks. Following fixation, fixed specimens were decalcified by immersing in 2.5% sodium hypochlorite/5M EDTA, which was changed daily for 2 weeks. After decalcification, specimens were dehydrated in a graded ethanol series and xylenea and paraffin-embedded. Blocks were sliced into sections with 4-5 μm thickness and adhered to poly-L-lysine-coated glass microscope slides and they were stained with Hematoxylin & Eosin

6. Histological and histomorphometric Analysis

The following measurement as illustrated in Fig.1 were performed at X100 magnification

using Image analysis program (Image-Pro Plus, Media cybernetics, Silver Spring, Maryland, USA)

- □New bone volume (%): The areas of new bone in the defect.
  (Newly formed bone volume/total defect volume x 100)
- □Residual particle, (%): The areas of residual bone graft.
  (Residual bone graft volume /total defect volume x 100)
- □Connective tissue volume, (%): The areas of fibers in the defect.
  (Newly formed connective tissue volume/total defect volume x 100)
- □The number of new blood vessels (unit): The number of blood vessels in the defect.
- □The number of osteoclast (unit): The number of osteoclast in the defect.

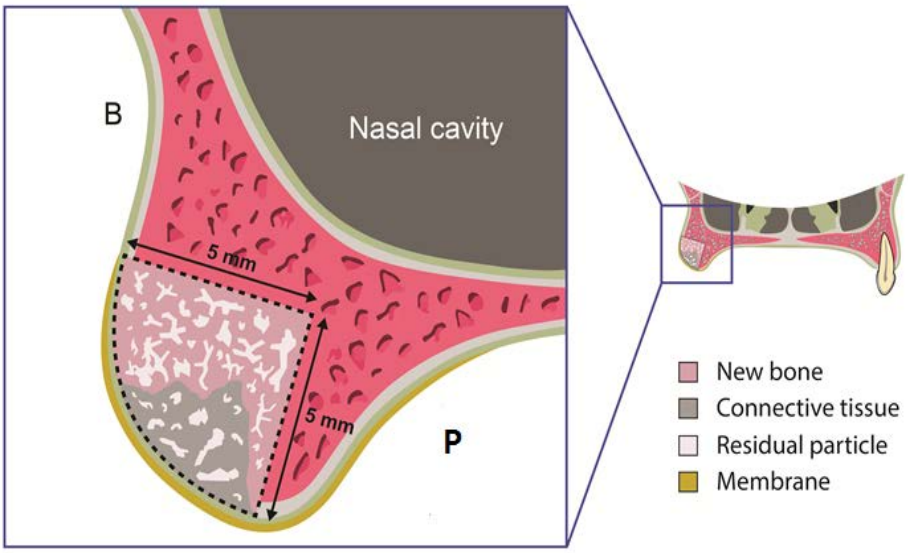


Fig1. Schematic diagrams depicting the parameters used in histomorphometric analysis B: buccal aspect P: Palatal aspect

7. Immunohistochemical analysis

Immunohistochemistry was performed to detect the expression of macrophages, osteoclasts and endothelial cell using Anti-CD68 (1:100, abcam, ab22506, Cambridge) antibody, Anti-ACP5 (1:100, Aviva Systems Biology, ARP41471_P050, San Diego, USA) and Anti-CD34 (1:100, abcam, ab81289, Cambridge), respectively.

Immunohistochemical analysis of CD68, ACP5 (TRAP) and CD34 was performed using the avidin–biotin DAB system. Endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in PBS for 30 min. The sections were blocked with PBS containing 5% BSA at room temperature for 1 h and reacted with the primary polyclonal antibody (1:100, Aviva system biology). Sections were washed with PBS, and reacted with the secondary antibody biotinylated-conjugate (1:200, Vectastain ABC kit, Vector laboratories) at room temperature for 1 h. The bound antibodies were visualized using the avidin-biotin complex reagent, followed by 3, 3-diaminobenzidine (Vector Laboratories, Burlinggsme, CA, USA). The sections were washed, dehydrated, and mounted using mount solution (Fisher Scientific, NJ, USA).

8. Statistical analysis

Statistical tests presented as mean and standard deviation (mean ± SD) were performed by the IBM SPSS 23.0 (IBM Corp., Armonk, NY, USA). Statistical significance was analyzed by One-Way ANOVA test and pot hoc Duncan's multiple range tests was used to compare the statistically significant difference of each groups, with the significance level of 5%.

III. Results

1. In vitro release kinetics of BMP2 and FGF2

It was noticed that both BMP2 and FGF2 were released from 1 h and more than half of BMP2 and FGF2 were released within 24 h. The release kinetics of BMP2 and FGF2 were shown to be completely different, indicating that release rate of FGF2 was higher than that of BMP2 at a given time. In a sample with both BMP2 and FGF2, more sustained release pattern was identified from 24 h compared to when used alone (Fig.2).

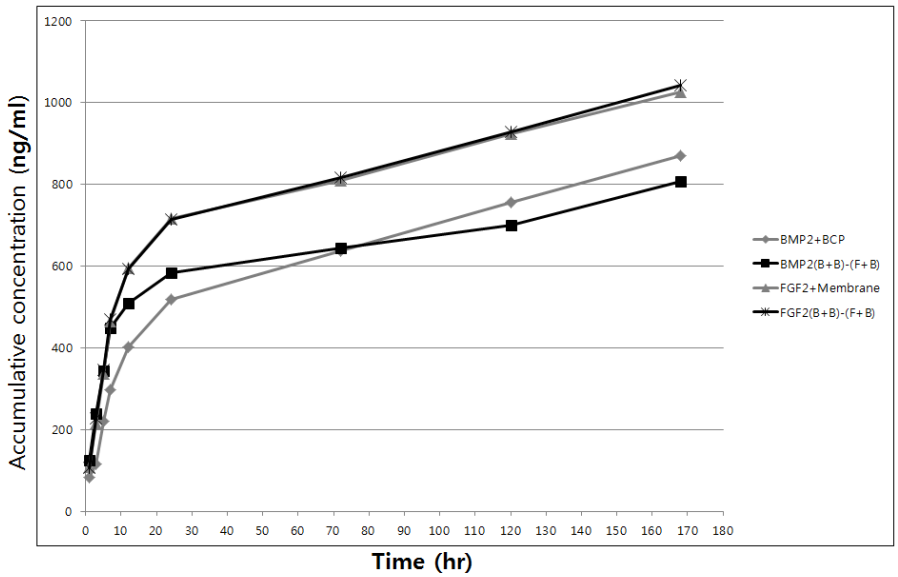


Fig2. Cumulative concentration of BMP2 and FGF2 measured for 7 days with different scaffolds.

2. Clinical observation

The inflammation of gingival margin in the animal was alleviated after 3 days of dressing and antibiotics injection, and every individual in all groups had no excessive expansion or pyogenic, maintaining healthy condition without wound dehiscence or bone graft exposure.

3. Histological findings

BMP2 Group

In the 4 weeks finding, new bone interface of defect surgically created with basic bone was observed, which confirmed the pattern of new bone starting to differentiate from marginal surface of damaged bone irregularly from around absorbed bone graft, and most of new bone patterns were trabeculated and callus bone pattern. At the bone defect, inflammatory cells were identified due to capillary proliferation a few scattered blood vessels were observed. Large amounts of loose fibrous connective tissues were identified in the bone defect. In the 12 weeks finding, it was hard to find the boundary of the defect and it had a pattern of more mature bone structure and island-shaped new bone around

absorbed bone grafts. A decreased amount of loose connective tissue was observed, and inflammation cell infiltration was not observed. Instead, continuous capillary proliferation was observed. (Fig. 3, Fig. 4, Fig. 5)

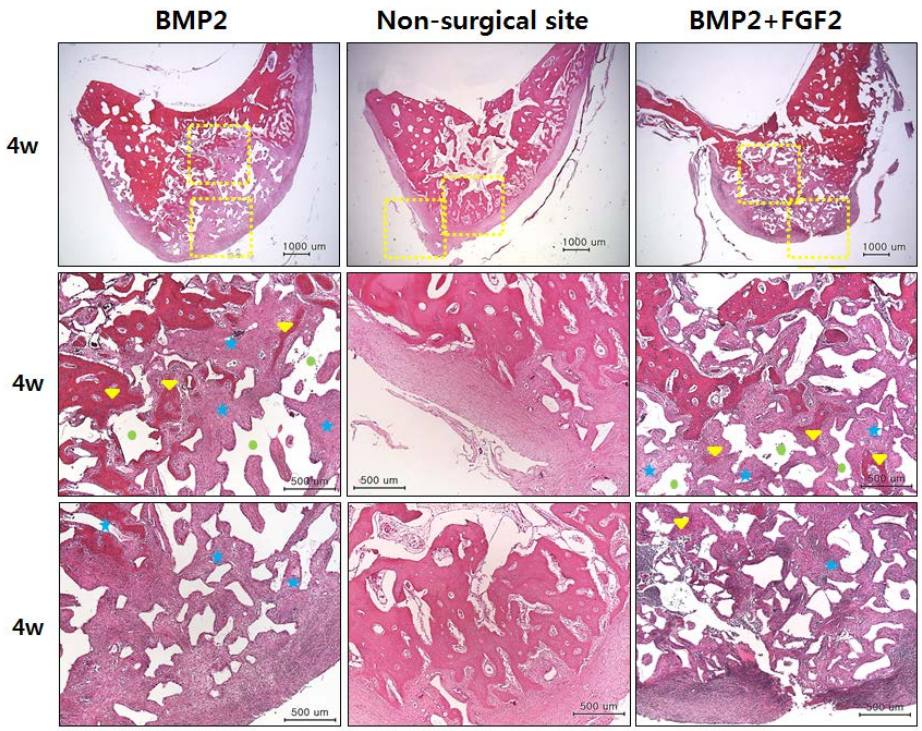


Fig3. **Light-microscopic views of BMP2 group and BMP2+FGF2 group sacrificed at 4 weeks post-surgery (H&E stain, upper raw original magnification x12.5, middle raw original magnification x50 and bottom raw original magnification x50).** Yellow triangle, blue star and green circle indicate new bone, connective tissues and remaining bone graft.

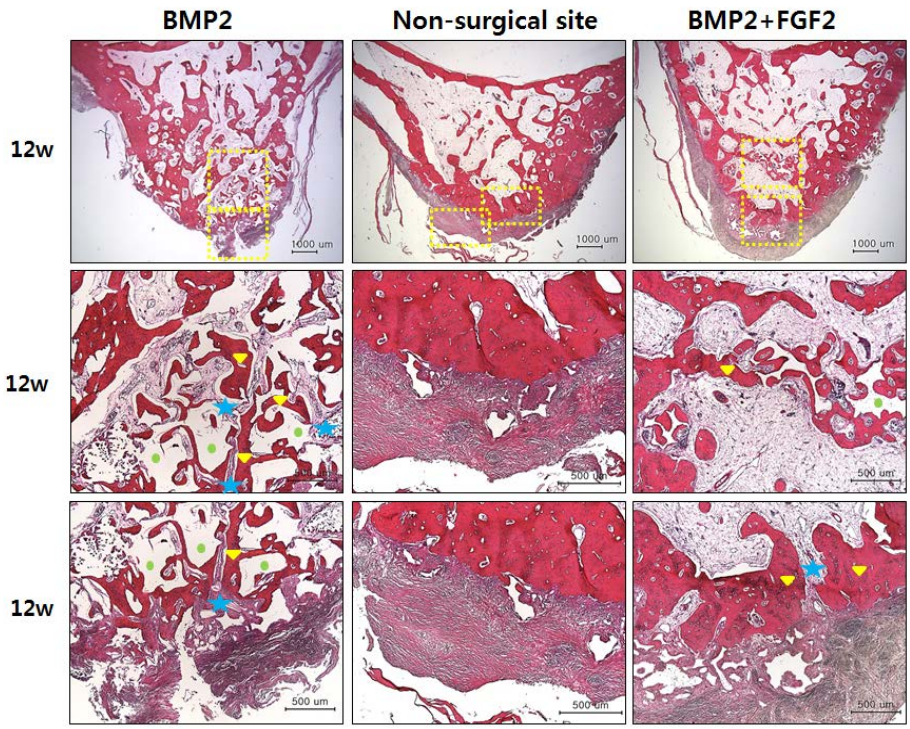


Fig4. Light-microscopic views of BMP2 group and BMP2+FGF2 group sacrificed at 12 weeks post-surgery (H&E stain, upper raw original magnification x12.5, middle raw original magnification x50 and bottom raw original magnification x50). More mature bone (yellow triangle) was identified with decreased amount of connective tissue (blue star) and remaining bone graft (green circle).

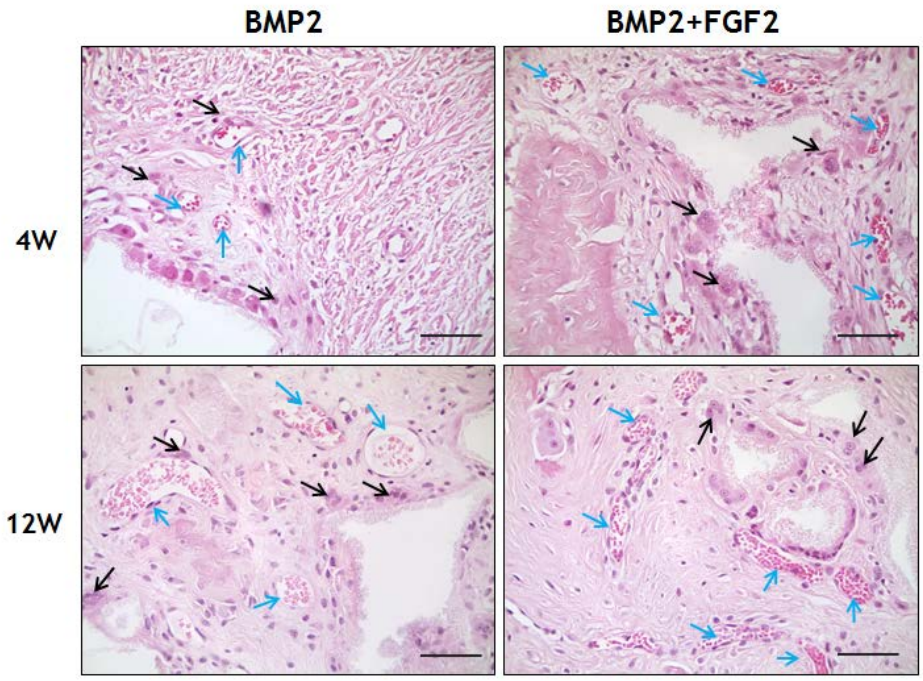
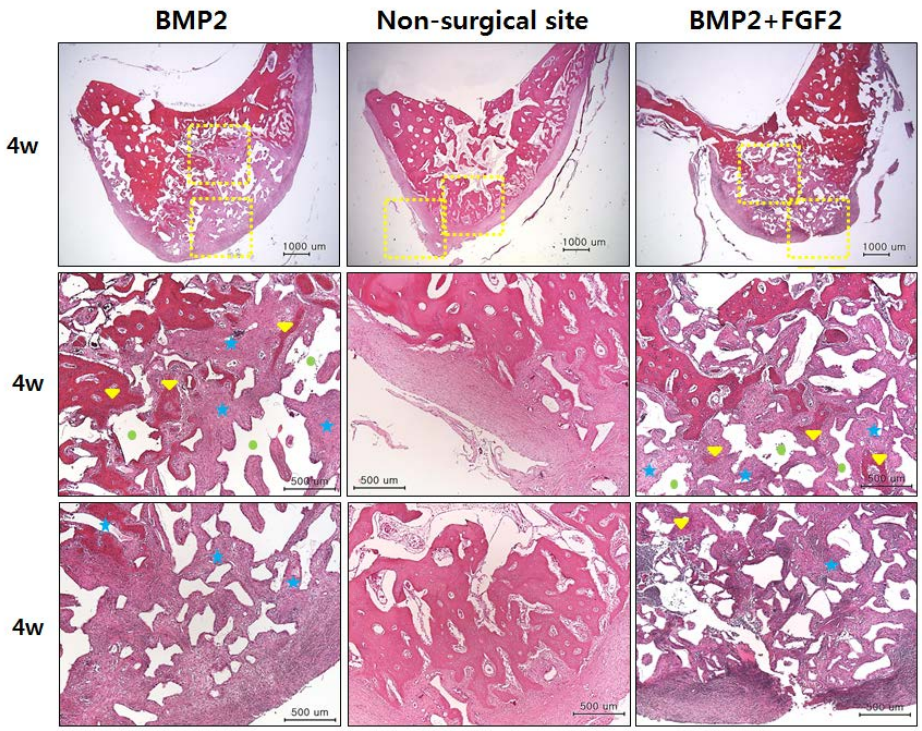


Fig5. Light-microscopic views of BMP2 group and BMP2+FGF2 group sacrificed at 4 weeks and 12 weeks post-surgery (H&E stain, original magnification x20). Significantly increased number of new blood vessels (blue arrow) were identified next to surrounding macrophages (black arrow) in BMP2+FGF2 group.

BMP2+FGF2 Group

In the 4 week finding, the boundary of existing bone and new bone of defects surgically created was distinct, and it was possible to observe little immature bones created within bone graft surroundings and connective tissues at the center of defect and the surface of damaged bone, and also healing process was observed to be progressed more actively in existing bones at the surface of the defect. In the marginal parts of the defect, dense connective tissue, capillary proliferation around this area, as well as inflammatory cells were infiltrated. Most of the defect was filled with connective tissue without bone structure. In the 12 week finding, it was hard to find the boundary of new bone and residual bone of the defect, and mature new bone was formed around absorbed bone graft. Also, connective tissues decreased dramatically in the center and marginal zone of the defect compared to that of the 4 week finding. No osteoblast and osteoclast were observed in the surrounding bone. Dense connective tissues decreased a lot and no inflammatory cells were infiltrated in the defect, and the number of blood vessels was decreased compared to that of 4 week finding. (Fig. 3, Fig. 4, Fig. 5)



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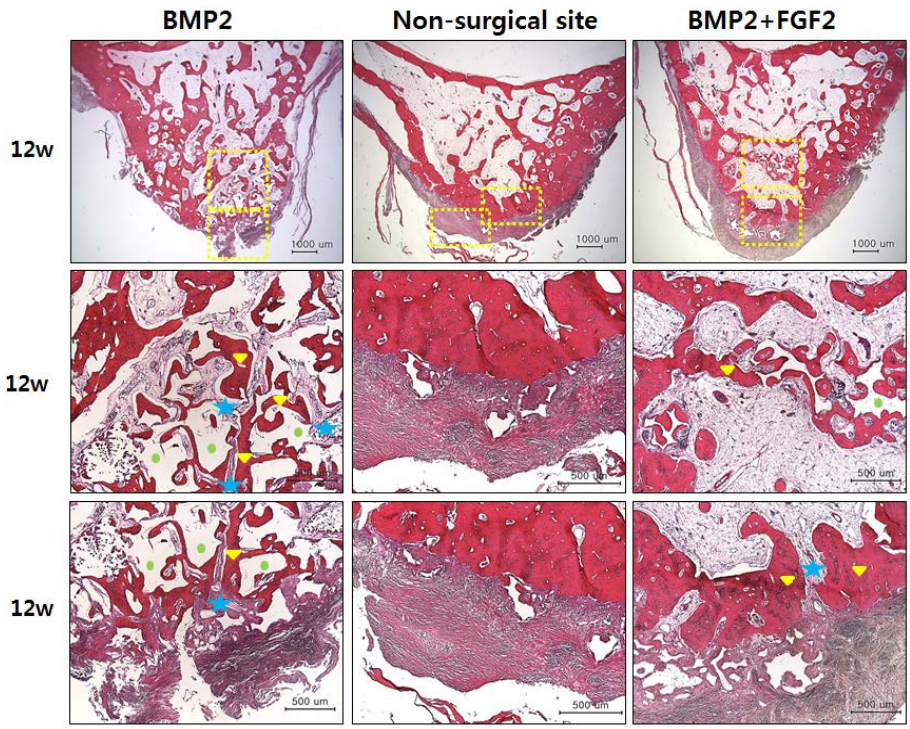

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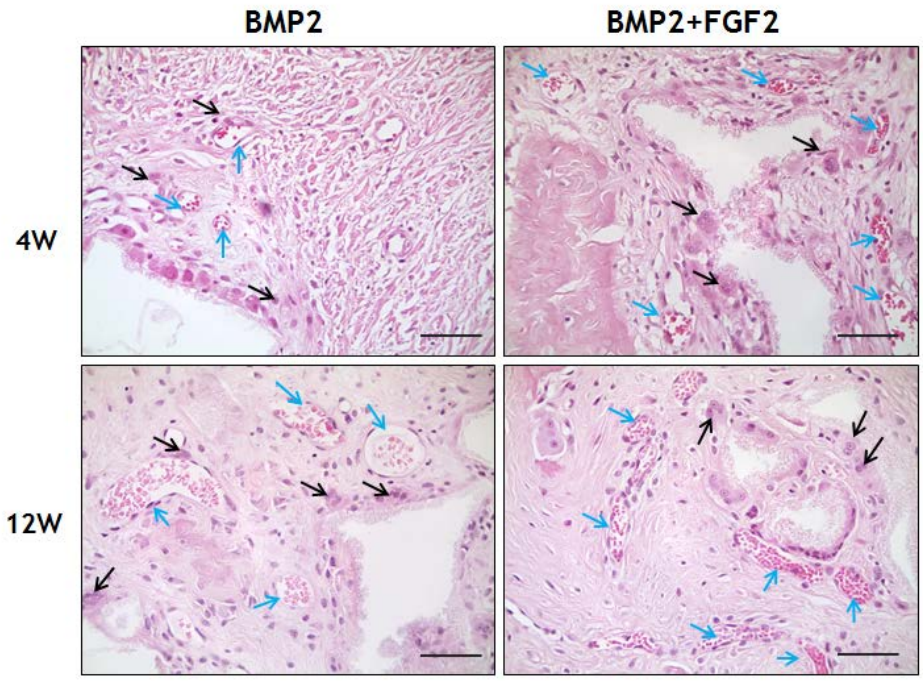

Fig5. Light-microscopic views of BMP2 group and BMP2+FGF2 group sacrificed at 4 weeks and 12 weeks post-surgery (H&E stain, original magnification x20). Significantly increased number of new blood vessels (blue arrow) were identified next to surrounding macrophages (black arrow) in BMP2+FGF2 group.

4. Histomorphometric analysis

The results of histomorphometric analysis were shown in Table 1. There was no statistically significant difference between BMP2 group and BMP2+FGF2 group in new bone formation(%) measured 4 weeks and 12 weeks after regenerative surgery (p=0.636 and p=0.283, respectively). However, the residual bone graft measured 12 weeks after regenerative surgery was significantly less in BMP2+FGF2 group.(p=0.020). Also, while connective tissue volume(%) and osteoclast count(unit) displayed no significant different between BMP2 group and BMP2+FGF2 group regardless of the time of sacrifice, number of blood vessel count(unit) measured 12 weeks after surgery was significantly increased in BMP2+FGF2 group compared to that of BMP2 group.(p=0.038). When comparing within each BMP2 group and BMP2+FGF2 group, there was tendency of increase in new bone formation(%) and osteoclast count(unit) as well as decrease in residual partible(%), connective tissue volume(%) and blood vessel count(unit) as the time increases. This time-dependent difference was found to be statistically significant in BMP-2+FGF+2 group (Table, Fig. 6, Fig. 7).

Table . Measurements in histomorphometric analysis

	BMP2			BMP2+FGF2			BMP2 vs BMP2+FGF2 (4w, 12w)
	4w	*p-value*	12w	4w	*p*-value	12w	*p*-value
New bone %	23.04±4.67	0.097	30.12±8.25	21.03±8.80	0.015 *	35.55±8.35	0.636 0.283
Residual particle (%)	15.21±7.44	0.093	8.76±4.16	19.60±6.82	0.0002*	3.11±2.80	0.312 0.020 *
Connective tissue volume(%)	24.06±7.73	0.156	17.02±8.15	23.36±6.27	0.003 *	12.48±3.32	0.867 0.234
Blood vessel count (unit)	95.33±22.46	0.002 *	47.33±15.59	106.33±29.17	0.008 *	64.83±8.80	0.481 0.038 *
Osteoclast count (unit)	76.86±36.76	0.260	103.00±39.14	76.17±22.61	0.062	123.17±49.93	0.971 0.454

Results are given as mean ± SD **** Significant difference (p < 0.05)***

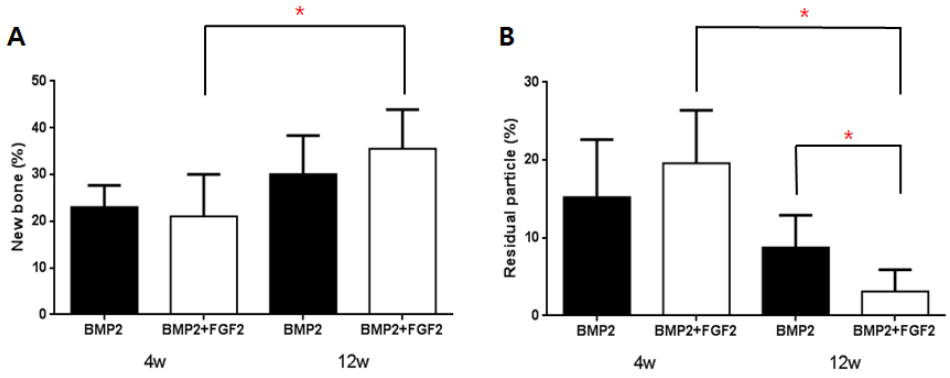

Fig6. Histomorphometric comparison of BMP2 group and BMP2+FGF2 group in new bone formation (%) and residual particle (%)

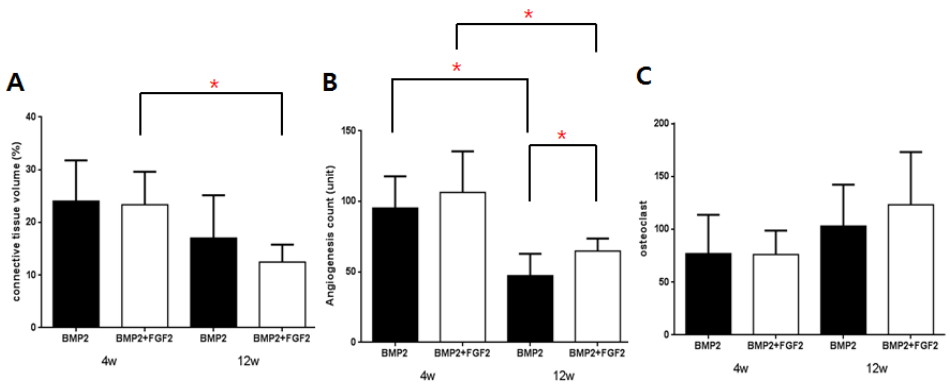

Fig7. Histomorphometric comparison of BMP2 group and BMP2+FGF2 group in connective tissue volume (%), angiogenesis count (unit) and number of osteoclast (unit)

5. Immunohistochemical findings

From immunohistochemistrical analysis, it was observed that macrophages were relatively more perivascular in both BMP2 and BMP2+FGF2 groups at 4 week. However, macrophages were denser in BMP2+FGF2 group compared to BMP2 group. After 12 week, the amount of macrophages decreased in both groups compared to 4 week but more macrophages were observed in BMP2+FGF2 group than in BMP2 group. (Fig. 8)

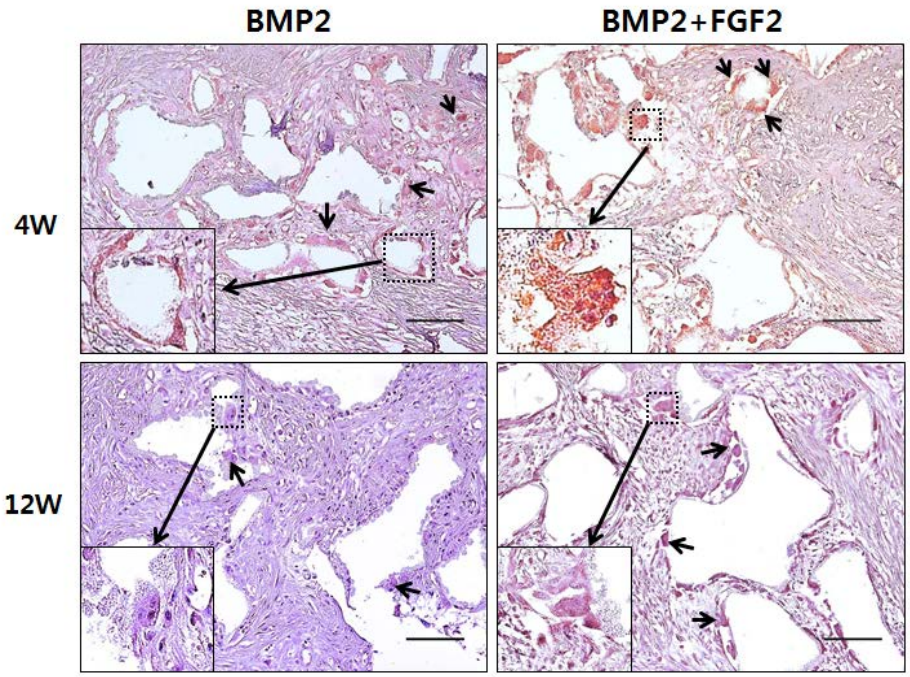

Fig8. Immunohistochemical analysis of CD68 in BMP2 group and

BMP2+FGF2 group at 4 weeks and 12 weeks post-surgery. Significantly higher staining level were identified in BMP2+FGF2 group.

Increased proliferation of osteoclasts around the new bone was observed in BMP2+FGF2 group compared to BMP2 group at 4week via immunohistochemical staining. The amount of osteoclasts at 12 week was greater than at 4 week, and the osteoclasts were more densely attached to the new bone in BMP2+FGF2 than in BMP2 gtoup (Fig. 9).

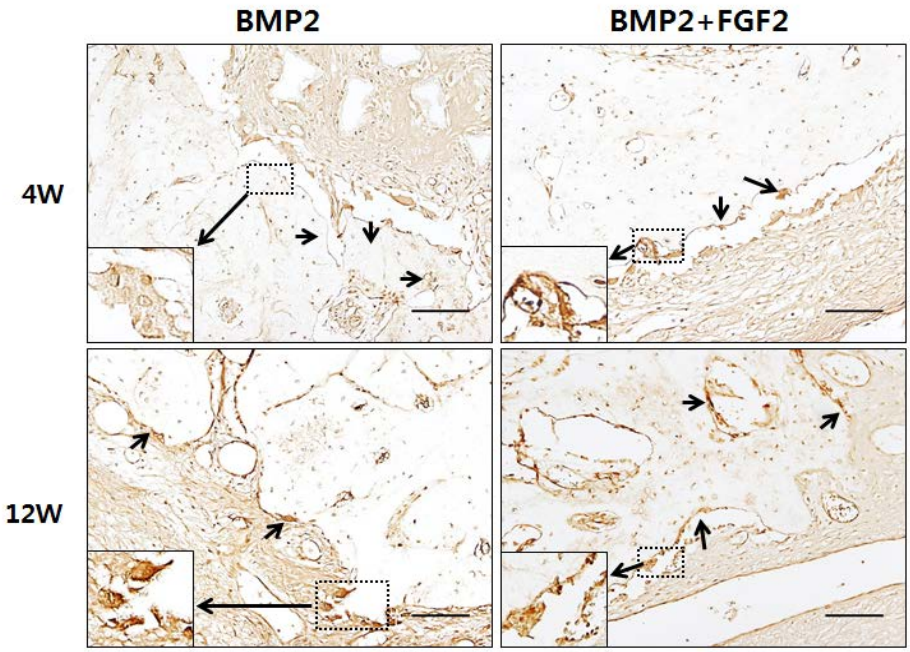

Fig9. Immunohistochemical analysis of ACP5 (TRAP) in BMP2 group and BMP2+FGF2 group at 4 weeks and 12 weeks post-surgery. Significantly higher staining level were identified in BMP2+FGF2 group.

A greater number of blood vessels were identified in both BMP2+FGF2 group and BMP2 group a 4 week. On the contrary, reduced number of blood vessels were observed in both BMP2+FGF2 group and BMP2 group at 12 week compared to that of 4 week. However, more mature blood vessels were identified in BMP2+FGF2 group. (Fig. 10)

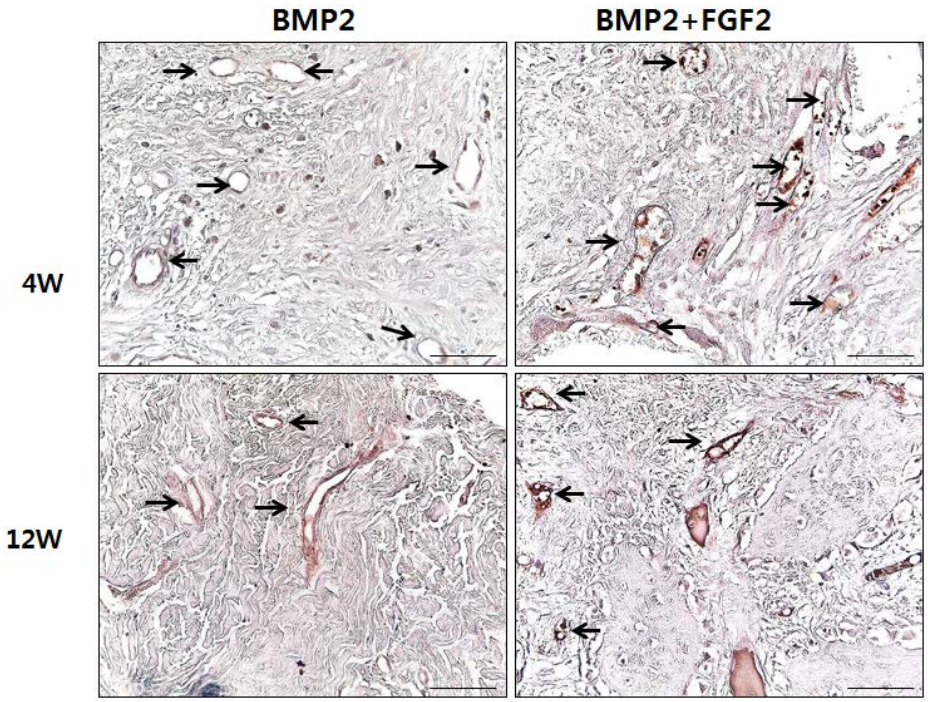

Fig10. Immunohistochemical analysis of CD34 in BMP2 group and BMP2+FGF2 group at 4 weeks and 12 weeks post-surgery. Significantly higher staining level were identified in BMP2+FGF2 group.

IV. DISCUSSION

In the current study, a maxilla of mongrel dog was chosen as the study site since the mongrel dogs are anatomically and histologically very similar to humans. Also, it has been reported that there is a high correlation between surgical procedures for maxilla in dogs and humans and therefore, suggested that experimental findings from mongrel dog model could be applied clinically to humans (Aerssens et al., 1998; Lee et al., 2007; Wetzel et al., 1995). It is widely accepted that the rate of bone formation in maxilla is the half of a rate of bone formation in mandible (Wetzel et al., 1995) and in this study, 5 x 5 x 5 mm^3 defect was created in the posterior region of maxilla to simulate poor bone quantity and quality with slow rate of bone formation and low self-healing capacity. Clinically, human's bone turnover rate is four times slower than in dogs. First four month of healing process is considered early healing period in humans where immature bone is formed in the defect area and additional eight months are required for callous bone to reduce in size and for bone to mature (Misch, 1999). Accounting for the difference between humans and dogs, the healing time of 4 weeks and 12 weeks was set up for this study.

Currently, barrier membranes made with various bioresorbable materials are being used in both clinical and experimental setting (Brunel et al., 1998) and

collagen membrane is commonly used due to its biocompatibility and ease at clinical use (Tawil et al., 2001). Previous studies reported that microfilaments in collagen membrane act as a scaffold during early vessel proliferation (Blumenthal, 1993). In this study, based on the known characteristic of collagen membrane from previous studies, FGF2, a growth factor known to promote angiogenesis was added on the membrane of experimental group and BCP was soaked with BMP2 and was inserted in the defect to increase alveolar bone conservation as well as new bone formation. The benefit of using this combination of growth factors, BMP2+FGF2, can be anticipated that they may be able to promote both bone formation and blood vessel formation, which was already verified in vitro previous studies (Nagayasu-Tanaka et al., 2015). However, not many in vivo studies has been reported regarding the effect of sequential and spatial application of different growth factors on new bone formation.

From histological and histomorphometric analysis, there were more new bone formation found in BMP2 group than BMP2+FGF2 group at 4 weeks whereas more bone formation was identified in BMP2+FGF2 group at 12 weeks. ($p>0.05$) These difference between two group at different time of sacrifice may be attributed to the fact that BMP2 plays an important role in early bone formation (Choi et al., 2013) and its ability to enhance osteogenic differentiation, thereby increasing bone formation as indicated by previous in vitro and in vivo studies

(Na et al., 2007). In addition to BMP2, FGF2 has been also reported to promote differentiation and proliferation of osteoblasts and osteoblast adhesion by regulating their function (Lozano et al., 2012). Enhanced bone healing upon local application of FGF2 on bone defect has also been reported (Radomsky et al., 1998). Likewise, the spatial and sequential expression of BMPs and FGFs is thought to modulate the degree of bone formation (Bitgood and McMahon, 1995). FGF2 is known to increase BMP2 expression in bone marrow stromal cells (Frank et al., 2002) and through cooperation of FGF2 and BMP2 increased bone formation was observed in the study by Kudota et al (Kubota et al., 2002). Interestingly, in a study where FGF2 gene was removed in mice, BMP2 expression, which was thought to play an important role in bone formation was suppressed (Naganawa et al., 2008). Generally, FGF2 is known to stimulate proliferation of osteoblasts (Lieberman et al., 2002) and BMP2 is known to promote differentiation of osteoblasts and bone mineralization in the early stage of bone formation (Choi et al., 2013). The results of this study is in accordance with the findings of previous studies on effects of growth factors on bone formation. BMP2 group showed better bone formation at the early stage of bone formation. However, as the time progressed, greater bone formation was found in BMP2+FGF2 group. Better bone formation found in BMP2 only group at 4 weeks confirms the initial bursting effect of BMP2 as reported in previous studies. Also, better bone formation was observed in BMP2+FGF2 group as time

progressed, which may be attributed to the prolonged BMP2 expression by FGF2 stimulation as explained above.

In normal bone healing process, undifferentiated connective tissue is formed in the defect area through fibrous proliferation, and newly formed blood vessels, fibroblasts, and undifferentiated mesenchymal cells are included in this tissue. The undifferentiated connective tissue is later matured into osteoid and becomes woven bone through bone remodeling process (Albrektsson et al., 2003). Young et al. reported that mesenchymal stem cells could be obtained from connective tissue of muscles and subcutaneous tissue of adults and elderly. These mesenchymal stem cells could be differentiated into chondroblasts, adipocytes, and muscles cells when appropriate inducing agents including growth factors such as FGF and TGF β are added (Young et al., 1995; Young et al., 2001). When growing connective tissue between bone grafts was examined, the area of fibrous connective tissue decreased more in BMP2+FGF2 group than in BMP2 group regardless of the time of sacrifice. In particular, in BMP2 +FGF2 group, the decrease in the area from 4 weeks to 12 weeks was statistically significant. ($p<0.05$) The area of connective tissue had decreased and the area where it was previously filled with connective tissue had been filled with newly formed bone. This finding is in consistence with the results of the study by Arnold et al. It can also be used as the evidence to verify the report that FGF2 promoted formation of blood vessels and fibrous connective tissue by promoting differentiation of

fibrous connective tissue. Previous studies report that FGF2 enhances bone modeling process by promoting formation of undifferentiated mesenchymal cells. Densely formed fibrous tissue was observed in sutured area and this may be due to proliferation of fibroblasts by FGF2 expression, contributing to better space maintaining ability.

The histological examination of newly formed blood vessels conducted at 4 weeks showed better proliferation of blood vessels in BMP2+FGF2 than in BMP2 group. At 12 weeks, blood vessel proliferation was observed around densely formed fibrous tissue and new bone, whereas this finding was not as active as at 4 weeks. More number of blood vessels were formed in BMP2+FGF2 group than BMP-2 group and the difference was statistically significant at 12 weeks, which was in accordance with the result from imunohistochemical analysis, showing the prominent, mature blood vessels identified in BMP2-FGF2 group at 12 weeks. Normal bone healing process begins with inflammatory phase by forming blood clot formation and inflammatory phase (Opal, 2000). Therefore, inflammatory cells including monocytes and macrophages are essential in this process, inducing blood vessel formation by themselves (Filbin, 2006). All growth factors and cytokines that are known to regulate angiogenesis could be produced by macrophages (Koch et al., 1986) and in turn these blood vessel-forming factors could promote the activity of inflammatory cells (Danese et al., 2007). Interestingly, this reciprocating mechanism is seen in both blood

vessel formation and inflammation (Williams et al., 1999). Among many blood vessel-forming factors, FGF2 is known as a strong blood vessel-forming factor in healing process (Kardami et al., 2007). Immunohistochemical staining was performed complete to confirm the blood vessel formation by FGF2 and their correlation with macrophages. The result from immunohistochemical analysis revealed more macrophages around new blood vessels in BMP2+FGF2 group compared to BMP2 group at both 4 weeks and 12 weeks. This finding is identical to the findings of Yang et al and confirmed. The suspected interrelationship between inflammatory response and new blood vessel formation (Yang et al., 2013). This angiogenic effect of FGF2 had been reported in many in vivo and in vitro studies and it is known to act as a promoter in wound healing process.

In order to evaluate the distribution pattern of osteoclasts, TRAP staining was conducted. Less osteoblasts and osteoclasts were seen at 4 weeks in both groups and a row of large multinucleated cells that are thought to be osteoclasts were observed around the initial bone and the graft. In BMP2+FGF2 group, at 12 weeks, in the area that is separated from the initial bone and where there was great resorption of the graft material, multiple blood vessels and new bone were seen surrounded by connective tissue. The graft material in this area was surrounded by osteoclasts, showing bone remodeling was in progress. Osteoclasts are originated from hematopoetic stem cell of mononuclear macrophages and they participate mainly in bone resorption (Teitelbaum, 2000).

Bone homeostasis is maintained by bone forming osteoblasts and bone resorbing osteoclasts. Osteoblasts and osteoclasts interact closely with each other and proliferation of osteoclasts is strictly regulated by osteoblasts (Suda et al., 1999; Takahashi et al., 1988). It was reported that FGF2 was involved in the activation and differentiation of osteoclast and Nakagawa et al. reported that FGF2 promotes the level of RANKL which induces differentiation of osteoclast and this results was in consistent with following results from out study; When the area of initial graft material was assessed at 4 weeks, there was very minimal difference between BMP2 group and BMP2+FGF2 group, however at 12 weeks, significantly more resorption of initial graft was examined in BMP2 +FGF2 group. The area of remaining initial graft at 12 weeks was 8.76±4.16% in BMP2 group and 3.11±2.80% in BMP2+FGF2 group and the difference was statistically significant. While densely arranged osteoclasts were examined around the graft material, osteoid and osteoblasts were examined around newly formed bone. More active new bone formation is expected in BMP2 +FGF2 group than in BMP2 only group based on greater amount of bone formation observed histomorphometrically in BMP2+FGF2 group.

From in vitro release study, the release kinetics of BMP2 and FGF2 were shown to be completely different, indicating that release rate of FGF2 was higher than that of BMP2 at a given time. This results are in accordance with result from our histomorphometrical study and may attributed to the higher bone formation

resulted from proliferation of surrounding mesenchymal stem cells and more pronounced angiogenesis. Also, in a sample with both BMP2 and FGF2, more sustained release pattern was identified from 24 hours compared to when used alone. Therefore, it can be anticipated that this sustained release pattern may contribute to the synergistic effect of BMP2 and FGF2, resulting in greater new bone formation even in long-term.

Taking all the measurements and findings obtained from current study into consideration, it can be concluded that the effect of combinational use of BMP2 and FGF2 on angiogenesis, inflammatory response, fibrous connective tissue and new bone formation and their interaction resulted in enhanced bone formation in the long term and confirmed once again in large animal model. Since there has not been in vivo study, showing synergistic effects of BMP2 and FGF2 introduced in this study.

In the current study, FGF2 was used interchangeably from left to right. However, exact determination of the defect area was not possible. Due to this reason, there may have been error in bone formation based on their anatomical defect, and further study examining the effect of FGF2 on confined space is thought to be necessary. Nevertheless, the results showed enhanced bone formation and reduced fibrous tissue formation when there was timely and spatial application of both BMP2 and FGF2 than when BMP2 was only applied. In addition, FGF2 showed the potential to overcome the limitations of BMP2, the

use of BMP2 with FGF2 especially in the area where bone formation is difficult, such as maxilla may allow better bone formation than using BMP2 alone.

V. Conclusion

In this study, we proved that a synergistic effect in bone and dense fibrous tissue formation was confirmed by using BMP2 and FGF2 then BMP2 alone. And it will be a treatment for the maxillary model, that use of a mixture of growth factors such as BMP2 + FGF2.

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국문요약

잡견에서 FGF-2/BMP-2 와 콜라겐 차폐막/합성골 이식재의 혼합 적용 시 신생골 형성에 미치는 상승효과

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목적: 본 연구의 목적은 잡견 상악 측방결손 모델에 FGF2/BMP2 와 콜라겐 차폐막/합성골 이식재를 혼합 적용시 신생골 형성에 미치는 상승효과를 조직학적, 조직계측학적 분석을 통해 평가하는 것이다. **방법:** 6 마리의 잡견을 실험동물로 사용하여 잡견상악 좌우 P1, P2, P3 을 발치하고 8 주의 치유기간을 거치고 난 후 상자모양의 결손부 (5 mm x 5 mm x 5 mm^3)를 상악 양쪽 P1, P3 위치에 형성하였다. 각기 결손부위에 1mg/ml BMP2+BCP 를 이식 후 생리식염수를 적신 콜라겐 멤브레인을 피개한

BMP2 그룹과 결손부위에 1mg/ml BMP2+BCP 를 이식 후 1mg/ml FGF2 를 적신 콜라겐 멤브레인을 피개한 BMP2+FGF2 그룹으로 설정하였다. 이식술 시행 4 주와 12 주 후 실험동물을 희생하고 조직슬라이드를 제작하여 조직학적, 조직계측학적 분석을 수행하였다. **결과:** 조직학적 분석 결과 BMP2+FGF2 군에서는 BMP2 그룹보다 미성숙 골들이 더 많이 형성된 것이 관찰되었다.

조직계측학적 분석 결과 4 주 후 BMP2 그룹에서는 신생골 형성이 23.04±4.67%, 결합조직의 면적은 24.06±7.73%, 파골세포의 수는 76.86± 36.76 개 인 것으로 계측 되었고, BMP2+FGF2 그룹에서는 신생골 형성이 21.03±8.80%, 결합조직의 면적은 23.36±6.27%, 파골세포의 수는 76.17±22.61 개로 BMP2 그룹보다 조금 적은 양이 계측 되었다. 그리고 BMP2 그룹에서 잔존골이식재의 면적은 15.21±7.44%, 혈관의 수는 95.33±22.4 개, BMP2+FGF2 그룹에서 잔존골이식재의 면적은 19.60±6.82%, 혈관의 수는 106.33±29.17 개로 보다 적은 수치가 계측되었다. 이와 반대로 12 주 후 BMP2 + FGF2 그룹에서는 오히려 신생골의 면적은 35.55±8.35%, 결합조직의 면적은 12.48±3.32%, 혈관의 수는 64.83±8.80 개, 파골세포의 수는 123.17±49.93 개 인 것으로 계측 되었고, BMP2 그룹에서는 신생골의 면적은 30.12±8.25%, 결합조직의 면적은 17.02±8.15%, 혈관의 수는

47.33±15.59 개, 파골세포의 수는 103.00±39.14 개 인 것으로 계측 되었다. 또한 BMP2 + FGF2 그룹에서는 잔존골이식재의 면적이 3.11±2.80%로 BMP2 그룹의 8.76±4.16%보다 통계적으로 유의차 있게 줄어든 수치가 계측되었고, 반대로 골형성은 늘어난 결과를 보여주었다. **결론:** 본 연구의 한계 내에서 BMP2 와 FGF2 의 순차적 사용은 장기적으로 신생골 형성에 상승효과가 있다는 것을 확인할 수 있었다. 따라서, 임상적으로 골 형성이 불리한 환경에서 골형성을 극대화 할 수 있는 하나의 치료방법으로 제시 될 수 있을 것이라 사료된다.

핵심되는말: 골유도재생술, 합성골이식재, 골형성 단백질2, 섬유 아세포 성장인자2.