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ORIGINAL ARTICLE

TERM

Comparative Study of BMP-2 Alone and Combined with VEGF Carried by Hydrogel for Maxillary Alveolar Bone Regeneration

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The effect of vascular endothelial growth factor (VEGF) combined with bone morphogenetic protein-2 (BMP-2) for bone regeneration is still controversial as to whether or not VEGF has a synergistic or additive effect. This study attempted to evaluate the synergistic effect of VEGF and BMP-2 compared to BMP-2 alone for maxillary alveolar bone regeneration using collagen sponge/hydrogel complex sheets in a canine model. After mixing BMP-2 and VEGF with a hyaluronic acid-based hydrogel (HAH), the collagen sponge/hydrogel complex was transplanted into maxillary alveolar bone defects (n=14) after the extraction of canine upper first molars on both sides. Bone regeneration was evaluated in three groups (control group without growth factors, experimental groups I and II with BMP-2 alone and BMP-2 and VEGF, respectively) using micro-computed tomography and histological staining. The total amount of new bone formations and bone mineral density were significantly higher in the group with BMP-2 only and the group with BMP-2 combined with VEGF than it in the control group. The area with positive staining of von Willebrand factor bone defect was significantly greater in the group with BMP-2 only and with dual growth factors than the control. BMP-2 released from the HAH promoted new bone formation. However, the combination of BMP-2 and VEGF did not show a synergistic or additive effect on bone regeneration at canine maxillary alveolar bone defects.

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Key Words: Maxillary alveolar bone defect; Bone regeneration; Bone morphogenetic protein-2; Vascular endothelial growth factor; Synergistic effect

INTRODUCTION

Alveolar regeneration of the jaw bone is essential for implant-prosthetic oral rehabilitation, and adequate bone quality and quantity are required to ensure stability upon implant insertion. Several bone-grafting materials such as autogenous bone, allogeneic and xenogenic bone, and alloplastic bone substitutes have been used in mandibular and maxillary alveolar bone augmentation. However, each graft material has various shortcomings, such as donor site morbidity and bone resorption after autogenous bone transplantation, infection risk and possible immune rejection response in allogenic and xenogenic material, and reduced bone forming capacity in allogenic, xenogenic and alloplastic graft materials [1,2]. To overcome these disadvantages, various approaches using osseous growth factors with or without stem cells have been tested [3-5].

Among the many growth factors, bone morphogenetic protein-2 (BMP-2) has been recognized as one of the most potent osteoinducers, as it can trigger the differentiation of mesenchymal stem cells (MSCs) to osteogenic cells for accelerated new bone formation, and it is now regarded as an important modulator in the formation and remodelling of bone tissue [6-9]. The application of BMP-2 has demonstrated that bone regeneration increases new bone formation in animal models [10,11] and in clinical applications for alveolar bone regeneration and maxillary sinus floor augmentation [12,13]. However, a higher dose of BMP-2 is required for enhanced bone formation in humans compared to animals [12,13], which can cause undesirable complications such as extensive swelling, seroma formation, and cystic bone lesion [14,15]. To avoid these complications, it is necessary to reduce the dose of BMP-2, which can decrease its bone forming capacity.

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Vascular endothelial growth factor (VEGF) can enhance osteogenesis through osteoblast differentiation and the transport of precursor mesenchymal cells to the mineralized region via newly formed vessels [16,17]. Therefore, an additional growth factor such as VEGF may provide helpful compensatory action in bone regeneration with low-dose BMP-2. Moreover, a synergistic effect of VEGF in combination with BMP-2 may be beneficial for enhanced bone regeneration, especially for early functional loading of dental implants after bone grafting. However, there is still controversy in animal studies whether the effect on osteogenesis of VEGF in combination with BMP-2 is synergistic or additive. Patel et al. [18] reported that combined delivery of VEGF and BMP-2 significantly enhanced osteogenesis in a rat critical-size defect model compared with BMP-2 alone. Zhang et al. [19] reported that biomaterial involving the growth factors BMP-2 and VEGF increased angiogenesis and new bone formation for maxillary sinus augmentation in experimental animals. However, a study by Young et al. [20] and Kempen et al. [21] showed that VEGF combined with BMP-2 did not have a synergistic effect on bone regeneration in rat bone calvarial or femoral defects. In a study by Kim et al. [22], bone regeneration at supra-alveolar peri-implant defects in the canine mandible was not significantly different between BMP-2 alone and BMP-2 combined with VEGF.

Currently, novel bone biomaterials incorporated with various osteogenic growth factors are being studied to replace bone-grafting materials. It has been shown that various biomaterials from the gelatine hydrogel complex [23], injectable hydrogel [19] or collagen sponge [12] incorporate with growth factors enhance bone regeneration in human and animal experiments. However, these osteogenic growth factors were difficult to maintain at defect sites for treatment *in vivo* [24]. Of the scaffolds for new bone formation, hydrogels plays a key role in new bone formation applications, such as osteogenic cell proliferation and the repair of bone defects. In particular, biomimetic hydrogels are a smart material, controlling degradability and growth factor release [3,19,25]. In addition, osteogenic effects of BMP-2 with collagen sponge have been extensively investigated, especially new bone formation and dental restoration [1,12,26]. Thus, the combination of hydrogel and collagen gel for bone regeneration could be more effective in bone regeneration than only a single osteoconductive scaffold.

Based on this knowledge, we used scaffolds of collagen sponge/hydrogel incorporated with BMP-2 and VEGF and compared the effects of BMP-2 alone and the combination of BMP-2 and VEGF growth factors on an alveolar bone defect dog model. Furthermore, based on the controversial results in relation to the effect of dual delivery of BMP-2 and VEGF, especially at the jaw bone with relatively abundant vasculature, we have attempted to evaluate the synergistic or additive effect of VEGF combined with BMP-2 delivered with collagen sponge/hyaluronic acid-based hydrogel (HAH) on maxillary alveolar bone regeneration in large animals in comparison with BMP-2 alone.

MATERIALS AND METHODS

Materials

Preparation of the hydrogel

MMP-sensitive hyaluronic acid (HA)-based hydrogel was prepared using acrylated HA, as previously described [27,28]. Briefly, acrylated HA (4wt%, 230 kDa) was dissolved in 0.3 M triethanolamine (TEA) buffered solution (pH 8). MMP-sensitive peptide (GCRDGPQGIWGQDRCG) was dissolved in 0.3 M TEA buffer and then added to acrylated HA solution with the same molar ratio of acryl and thiol groups. The reaction mixture was incubated at 37 for gelation. The HA-based hydrogel was formed via Michael-type addition reaction. VEGF (recombinant human VEGF165, R&D Systems, Minneapolis, MN, USA) and recombinant human BMP-2 (Novosis®-Dent, CGBio Inc., Seongnam, Korea) were incorporated into the HA-based hydrogels. These HA-based hydrogels were used for the *in vivo* experiments.

Preparation of the collagen sponge

Collagen matrices were prepared as described elsewhere with slight modifications [29]. Briefly, homogenized 1.5% collagen solution (w/v, pH 7.4) was poured into the mould and lyophilized. Freeze-dried collagen matrices were cross-linked with 20 mM EDC for 24 h. Residual EDC was washed out with autoclaved distilled water for 5 times. Rinsed collagen matrices were relyophilized and kept at 4°C until further use.

Animals

Alveolar bone defects at the upper first molar on both sides (n=14) in seven adult beagle dogs (1 year old, 10 kg) were used in this study. All the animals were treated and handled in accordance with the "Recommendations for Handling of Laboratory Animals for Biomedical Research" compiled by the Committee on the Safety and Ethical Handling Regulation for Laboratory Experiments at the School of Dentistry at Seoul National University. The animals were maintained in the animal facility, where a constant room temperature of 22°C was maintained. The animals were fed a soft diet during the first two postoperative weeks, otherwise, there was no restriction of food.

Surgery

The surgical design of this study involved the creation of an

alveolar bone defect sized 10 mm in anteroposterior width and 10 mm in height after extraction of the upper first molar on both sides and transplantation of scaffold, with or without growth factors. In all animals, maxillary sinus membrane was exposed. The scaffold was composed of one HAH sheet and three collagen sponge sheets, and growth factor was mixed with the HAH. Three layers of collagen sponge were placed on the cranial side to contact the maxillary sinus membrane, which was caudally covered by one layer of HAH sheet to contact with the mucoperiosteal flap on the oral side (Fig. 1). The control group (n=4) received only scaffolds (230 kDa 4wt% HAH sheet) without growth factors, and the experimental group I (n=5) contained only recombinant human bone morphogenetic protein (rhBMP) (80 μg/300 uL) in the 230 kDa 4wt% HAH sheet. In experimental group II (n=5), rhBMP-2 (80 μg/300 uL) and VEGF (20 μg/300 uL) were mixed in the 230 kDa 4wt% hydrogel. The animals were medicated with intramuscular injections of zoletil 50 (10 mg/kg, Virbac Korea, Seoul, Korea), xylazine hydrochloride (Rompen® 2 mg/kg, Bayer in Korea, Seoul, Korea) and atropine 0.05 mg/kg (atropine sulphate, 0.5 mg/mL, Jeil Pharm. Co., Seoul, Korea). Once an adequate depth of anaesthesia was established, an endotracheal tube was placed, and anaesthesia was maintained with O_2 and Enflurane (U.S.P) 2% (Gerolan soln® 100 mL/100 mL, Choongwae Pharm, Seoul, Korea).

After intraoral and extraoral disinfection with 10% betadine (Potadine®, Sam-Il Pharm., Seoul, Korea) and local infiltration anaesthesia with 2% lidocaine (Lidocaine HCL Inj®., Yuhan Corp., Seoul, Korea), a marginal gingival incision with releasing incision was made at the upper first molar with vertical releasing incisions. A full thickness mucoperiosteal flap was created. After exposing the buccal surface of the maxillary bone, a standardized alveolar bone defect sized 10×10 mm was formed between the first and third molar by using a dental rotary instrument under cooling with physiological saline after extraction of the upper first molar on both sides. The space created was filled with scaffolding. Finally, the mucoperiosteal flap was readapted and sutured with resorbable material (Vicryl 4.0, Ethicon, Cincinnati, OH, USA). During the pre- and postoperative periods, all animals were treated with prophylactic antibiotics (cefazolin sodium 1 g/vial, 20 mg/kg, Chongkeundang Pharma Co., Seoul, Korea). This prophylaxis was commenced during surgery and continued postoperatively for 3 days. To reduce postoperative pain associated with the surgical procedure, analgesic medication (acetaminophen tablet 15 mg/kg, Daewoo Pharma Co., Seoul, Korea) was administered after surgery for 3 days. The animals were euthanized after a six-week (n=5) latent period. They were perfused with 10% neutral buffered formalin solution, which was injected through the carotid artery. The maxillary bone at the first molar area (30×20 mm size) was removed from the skull for radiological and histological evaluation.

Micro-computed tomography analysis

The maxillary bone segment was fixed using a 10% formalin solution for one week. Micro-computed tomography (micro-CT) scans were taken for quantitative evaluation of new bone formation using the SkyScan 1172VR microfocus X-ray system (Bruker microCT, Kontich, Belgium). Micro-CT was taken under the following conditions: 80 kV source voltage, 124 μA current, 474 ms exposure time, 0.4° rotation angle and 3458 pixel size. The SkyScan 1172VR microfocus X-ray system is equipped with a microfocus X-ray tube with a focal spot of 2 μm, producing a cone beam that is detected by a 12-bit cooled

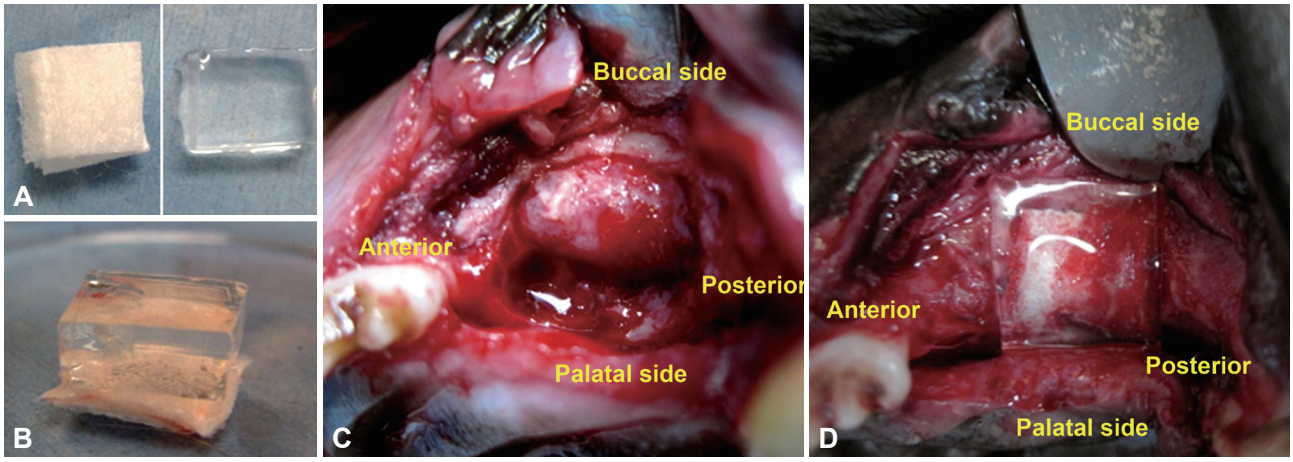

Figure 1. Application of scaffolding to maxillary alveolar bone defects. (A) Collagen sponge (left) and hyaluronic acid-based hydrogel (HAH) after overnight swelling according to the bone defect size. (B) Collagen sponge/HAH scaffold complex. (C) Maxillary alveolar bone defect (10 mm in width and 10 mm in height) created after extraction of maxillary first molar. (D) Application of HAH sheet over 3 layers of collagen sponges.

X-ray camera charge-coupled device fibre-optically coupled to a 0.5 mm scintillator. The resulting images were 1000×524-pixel square images with an aluminium filter used to produce optimized images. Reconstruction and analyses were performed using NRecon reconstruction and CTAn 1.8 software (Bruker micro-CT, Kontich, Belgium), respectively. To measure the osseous changes and new bone formation, a rectangular area was selected as the region of interest (ROI) in a two-dimensional image of the coronal plane of the maxilla at the centre of the first molar area in anteroposterior dimension. The pixel zone representing bone structure in the defined ROI was then reconstructed three-dimensionally by creating a volume of interest in the lower and upper ranges of the threshold using grayscale units. The bone defect volume was anteroposteriorly divided into three parts, namely P1 (anterior part), P2 (middle part), and P3 (posterior part) (Fig. 2). The total ROI was determined according to the maximum volume of new bone within the surgical bone defect (10×10×10 mm) and was less than the surgical bone defect. The defined ROI in each part was 3.3 mm in length, 4.5 mm in width and 4.2 mm in height. Then, microarchitecture parameters, including bone volume (BV), BV/tissue volume (TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp) were obtained using CTAn 1.8 software according to the manufacturer's instructions. To measure bone mineral density (BMD), attenuation data for the ROIs were converted into Hounsfield units and expressed as a value of BMD using phantom scans. BMD values were expressed in terms of grams per cubic centimetre of calcium hydroxyapatite in distilled water. A zero value for BMD corresponded to the density of distilled water alone (no additional calcium hydroxyapatite), and a value greater than zero corresponded to non-aerated biological tissue.

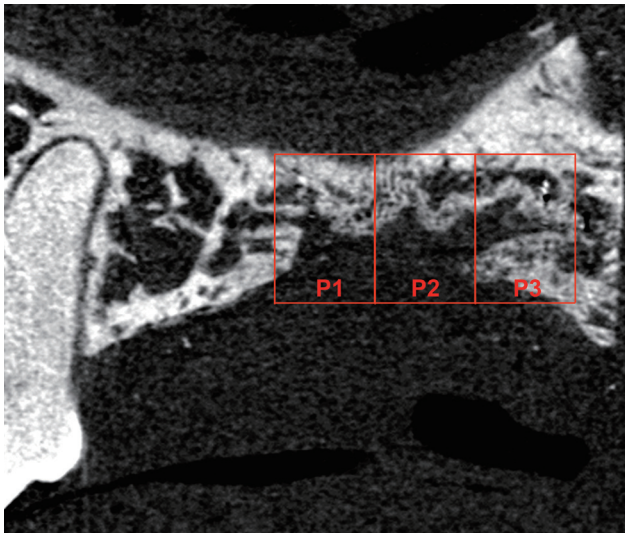

Figure 2. Evaluation of new bone formation using micro-CT at a maxillary alveolar bone defect that was anteroposteriorly divided into three parts. P1: anterior part, P2: middle part, P3: posterior part.

Histological evaluation

After micro-CT reconstruction, the specimens were decalcified in a solution of EDTA (7%, pH=7.0) for three weeks with a solution change every two days. The specimens were dehydrated in 70% ethanol, cleaned for 10 minutes with xylene, sectioned buccopalatally along the coronal plane of the maxilla, and embedded in paraffin. For histological analysis, 3-μm sections of each paraffin block were obtained and stained with haematoxylin and eosin stain and Masson's trichrome to evaluate bone healing status. Digital images of the stained sections were obtained using a transmission and polarized light Axioskop microscope, Olympus BX51 (Olympus Corporation, Tokyo, Japan).

Immunohistochemical staining

The specimens within paraffin blocks were sectioned buccopalatally along the coronal plane of the maxilla. The sectioning was done at two positions, namely, in the mid-position of whole specimens and in the mid-position of the posterior half specimens. The paraffin sections were cleaned for 10 min with xylene, and the deparaffinized sections were treated with undiluted serum solution for 30 min. The specimens were then incubated with anti-von Willebrand factor (vWF) (1:200; Abcam, Cambridge, UK) at 4°C overnight. After incubation, the sections were incubated with R.T.U biotinylated universal antibody using a Vectastatin kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Staining was detected with a NovaRED substrate kit (Vector Laboratories, Burlingame, CA, USA), and slides were mounted with Crystal/Mount (Biomeda Corp., Burlingame, CA, USA). Images of stained cells were captured by bright field microscopy. Positively stained cells on the vascular lumen structure were counted. The area of blood vessels with positive staining of vWF was measured using Image J (NIH, Bethesda, MD, US). It was expressed in ratio [positive area/total area (mm^2)×100 (%)].

Statistical analysis

All data are presented as the mean±standard error of the mean or standard deviation. Statistical analyses were performed with SPSS 21 (IBM Co., Armonk, NY, USA). Data between the two groups were evaluated with a 2-tailed Student's t-test, and the comparison of data in more than 2 groups was performed through a one-way analysis of variance in the animal studies according to the Bonfferoni method of post hoc testing. The results with values of $p<0.05$ were considered to be statistically significant.

RESULTS

Micro-CT analysis measurements of regeneration in the alveolar bone defect dog model

As shown in Figure 1, collagen sponge (Fig. 1A, left) and HAH (Fig. 1A, right) complex (Fig. 1B) incorporated with BMP-2 or combination of BMP-2 and VEGF were transplanted into maxillary alveolar bone defects (10 mm in with and 10 mm in height) after the extraction of canine upper first molars on both sides. Figure 2 displays the three parts (P1: anterior part, P2: middle part, P3: posterior part) of new bone formation depending on the anteroposterior location of the alveolar bone defect dog model. Figure 3 displays reconstructed three dimensional outer surface images of the new bone in the three groups. The regenerated new BV was clearly smaller than the surgically created bone defect, which would have resulted from compression of the scaffold by soft tissue tension from the direct closure of the gingival defect after molar extraction because the HAH and collagen sponge did not have enough physical strength to resist the soft tissue wound tension. Although new bone formation from the adjacent residual alveolar bone was observed in all groups, there was more in the two experimental groups than in the control group. Especially at the middle part, which was far from the adjacent residual bone, the new bone was clearly smaller in the control group than in the two experimental groups. As shown in Figure 4, in terms of the three parts (P1, P2, and P3), depending on the anteroposterior location of the alveolar bone defect, the two experimental groups (BMP-2 only and combination of BMP-2 and VEGF) showed significantly higher new bone formation than the control. At P1 and P3, the group with BMP-2 only (34.19±2.91 mm^3 at P1, $p<0.01$; 31.15±4.61 mm^3 at P3, $p<0.05$) and the group with BMP-2 and VEGF (31.50±2.33 mm^3 at P1, $p<0.01$; 27.86±3.35 mm^3 at P3, $p<0.05$) had significantly higher new bone formation than the control group (19.42±1.88 mm^3 at P1 and 17.84±3.64 mm^3 at P3).

At P1 and P3, the group with BMP-2 only (48.46±11.68% at P1, $p<0.05$; 43.05±11.41% at P3, $p<0.05$) and the group with BMP-2 and VEGF (48.26±7.97% at P1, $p<0.05$) had significantly higher ratio of BV/TV than the control group (29.74±5.77% at P1 and 27.32±11.16% at P3). At P1 and P3, the group with BMP-2 only (1.32±0.04 g/cm^3 at P1, $p<0.01$; 1.31±0.04 g/cm^3 at P3, $p<0.05$) and the group with BMP-2 and VEGF (1.33±0.03 g/cm^3 at P1, $p<0.01$; 1.31±0.05 g/cm^3 at P3, $p<0.05$) had significantly higher BMD than the control group (1.22±0.06 g/cm^3 at P1 and 1.23±0.05 g/cm^3 at P3). However, Tb.Th, Tb.N and Tb.Sp were no significant difference between two groups. As shown in Figure 5, BMD was significantly higher in the BMP-2 (1.268±0.002 g/cm^3 vs. 1.208±0.011 g/cm^3, $p<0.01$) and the combination of VEGF and BMP-2 groups (1.266±0.013 g/cm^3 vs. 1.208±0.011 g/cm^3, $p<0.05$) than in the control. However, there was no significant difference between the BMP-2 alone group and the combination of VEGF and BMP-2 group (1.266±0.013 g/cm^3 vs. 1.268±0.002 g/cm^3, p=NS). The ratio of BV/TV was significantly higher in the BMP-2 alone (34.991±0.496% vs. 21.747±2.79%, $p<0.01$) and combination of VEGF and BMP-2 (32.681±2.451% vs. 21.747±2.79%, $p<0.05$) groups than in the control. Tb.Th was significantly higher in the BMP-2 group (0.394±0.024 mm vs. 0.304±0.015 mm, $p<0.05$) than in the control. However, there was no

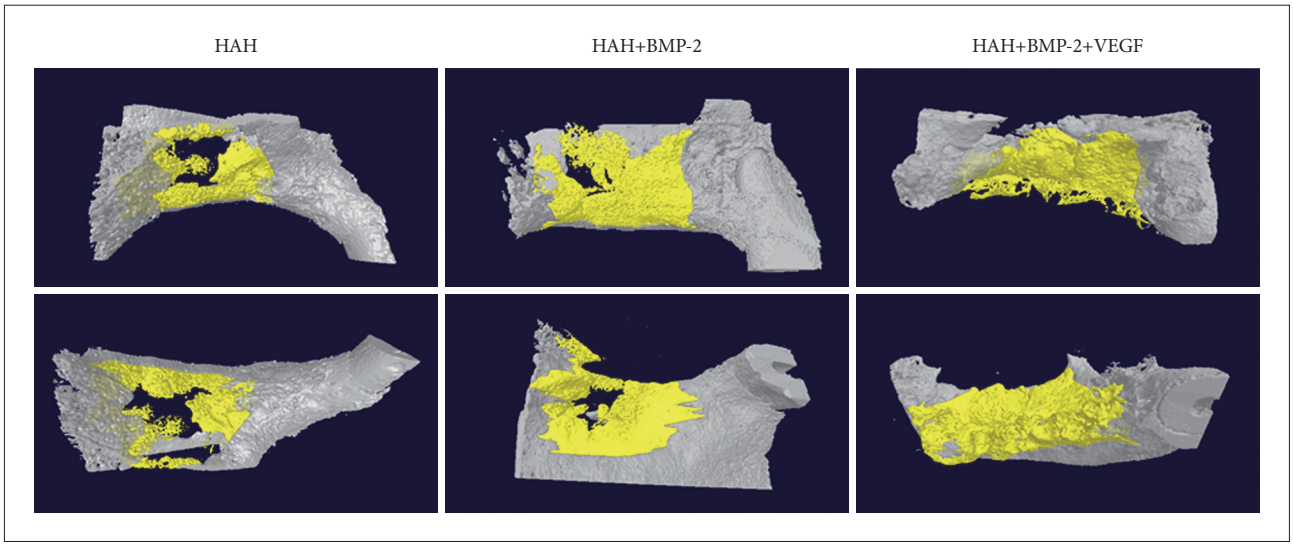

Figure 3. 3D image of outer surface of new bone at the maxillary alveolar bone defect in the three groups (HAH: hydrogel only group, HAH+BMP-2: hydrogel+BMP-2 group, and HAH+BMP-2+VEGF: hydrogel+BMP-2+VEGF group). Yellow color indicates new bone. HAH: hyaluronic acid-based hydrogel, BMP-2: bone morphogenetic protein-2, VEGF: vascular endothelial growth factor.

significant difference between the combination of VEGF and BMP-2 and the control groups (0.361±0.023 mm vs. 0.304±0.015 mm, *p*=NS). Tb.N (BMP-2 vs. control; 0.907±0.073 1/mm vs. 0.706±0.060 1/mm, *p*=NS, VEGF+BMP-2 vs. control; 0.909±0.071 1/mm vs. 0.706±0.060 1/mm, *p*=NS) and Tb.Sp (BMP-2 vs. control; 0.905±0.041 mm vs. 1.088±0.153 mm, *p*=NS, VEGF+BMP-2 vs. control; 1.096±0.128 mm vs. 1.088±0.153 mm, *p*=NS) did not significantly differ between the BMP-2, the combination of VEGF and BMP-2 and the control groups.

Histological data of bone formation in the maxillary sinus of the alveolar bone defect model

Figure 6 displays the histological analysis results after 6

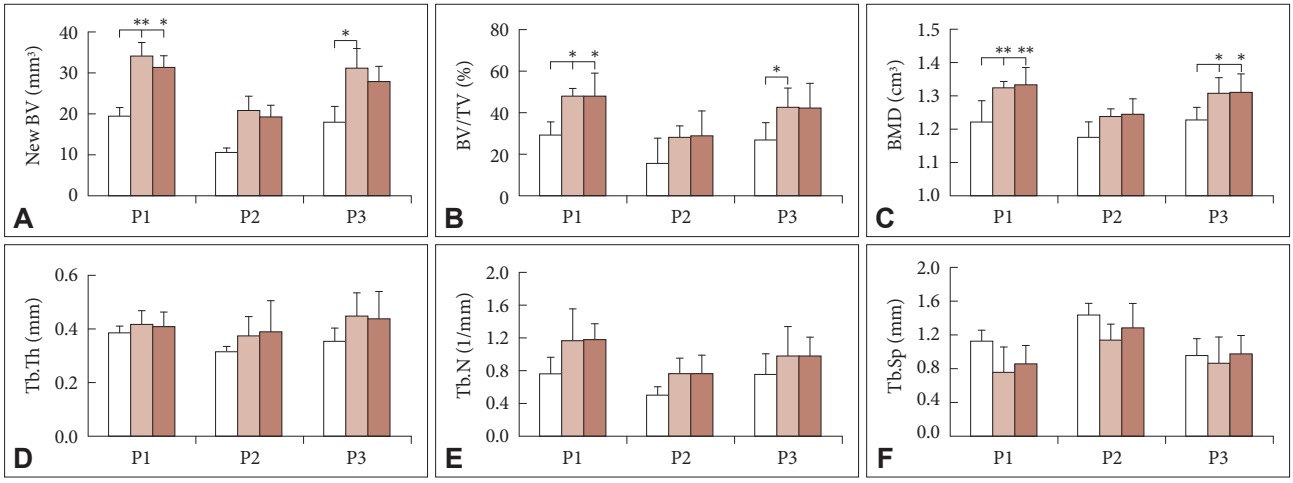

Figure 4. Analysis of new bone using micro-CT at a maxillary alveolar bone defect that was anteroposteriorly divided into three parts (P1: anterior part, P2: middle part, P3: posterior part). (A) New bone volume, (B) the ratio of bone volume to total volume (BV/TV), (C) bone mineral density (BMD), (D) trabecular thickness (Tb.Th), (E) trabecular number (Tb.N), and (F) trabecular separation (Tb.Sp) were calculated to assess the quantitative variables of newly formed bone in the three groups (gray color: hydrogel only group, dark gray color: hydrogel+BMP-2, and black color: hydrogel+BMP-2+VEGF). *p<0.05, **p<0.01. BMP-2: bone morphogenetic protein-2, VEGF: vascular endothelial growth factor.

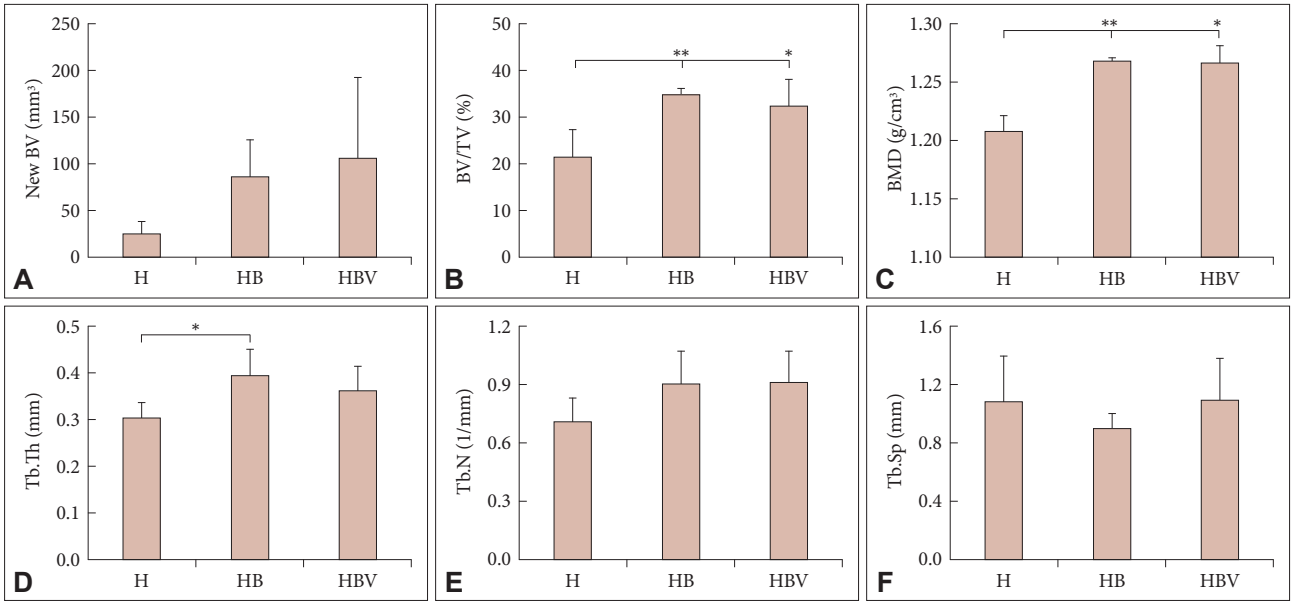

Figure 5. Analysis of new bone using micro-CT in the three groups (HAH: hydrogel only group, HAH+BMP-2: hydrogel+BMP-2 group, and HAH+BMP-2+VEGF: hydrogel+BMP-2+VEGF group). (A) New bone volume, (B) the ratio of bone volume to total volume (BV/TV), (C) bone mineral density (BMD), (D) trabecular thickness (Tb.Th), (E) trabecular number (Tb.N), and (F) trabecular separation (Tb.Sp) were calculated to assess the quantitative variables of newly formed bone in the three groups. *p<0.05, **p<0.01. HAH: hyaluronic acid-based hydrogel, BMP-2: bone morphogenetic protein-2, VEGF: vascular endothelial growth factor, H: hydrogel, HB: hydrogel+BMP-2, HBV: hydrogel+BMP-2+VEGF.

weeks. There were no inflammatory signs or wound healing disturbances in any of the cases. The control group demonstrated premature bone structure without or only with a small number of osteocytes. Only a small amount of lamellar bone could be observed near the residual host alveolar bone. In the experimental groups with BMP only or in combination with VEGF, relatively abundant new bone was observed, while the residual collagen sponge was reduced. Lamellar bone structure could be observed more in the group with BMP only than in the group with dual growth factors.

As shown in Figure 7, histological study with vWF at 6 weeks after scaffold transplantation revealed endothelial cell proliferation in alveolar bone defect model. The area with positive staining for vWF at the mid-position of bone defect was significantly more greater in the group with dual growth factors (13.27±5.18%, $p<0.05$) than in the group with BMP only (11.79±3.85%) and the control (4.58±1.34%) (Fig. 7B). At the mid-position of posterior half bone defect, vWF was significantly more

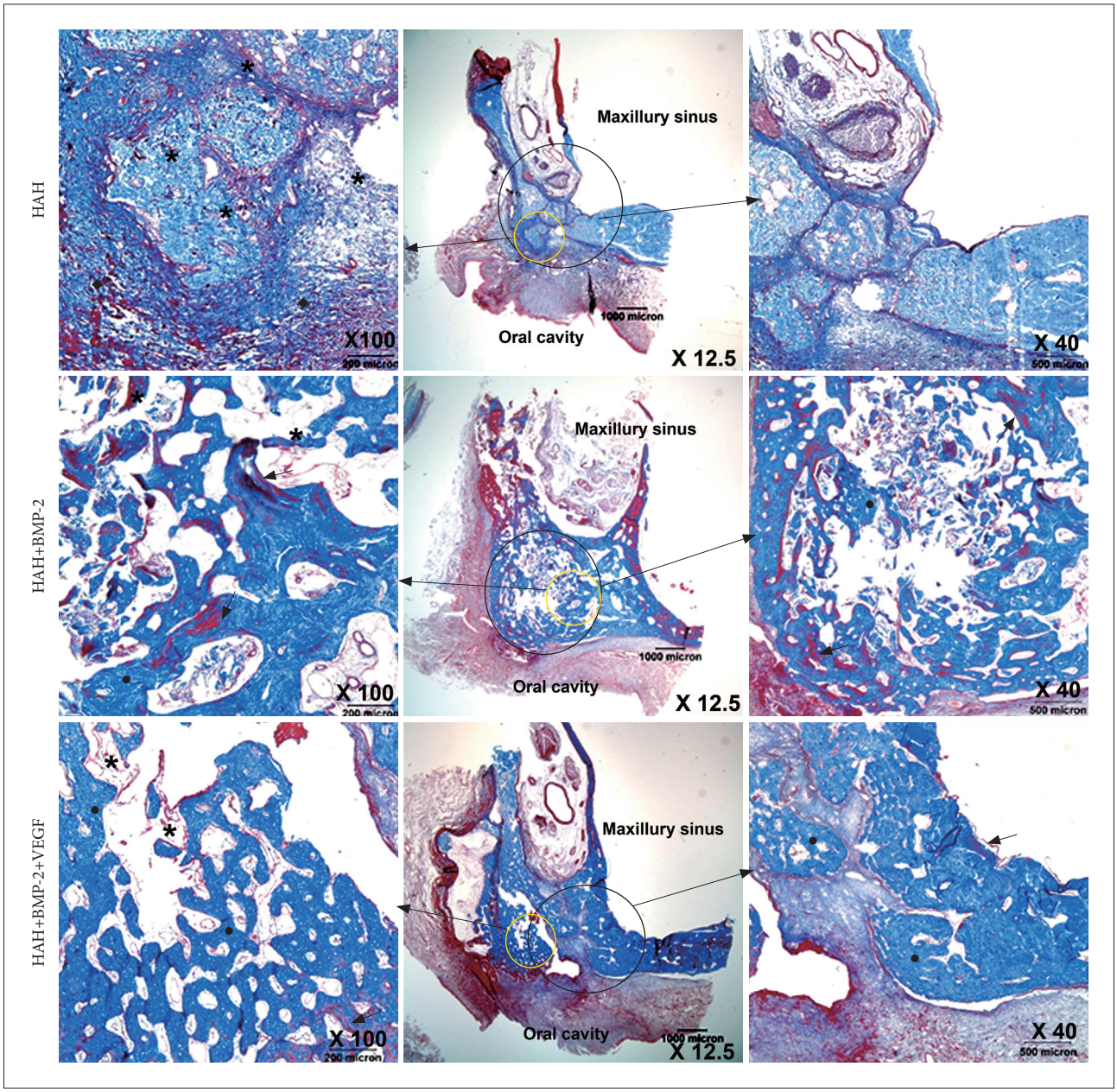

Figure 6. New bone formation detected by Masson's trichrome (MT) staining and histological images in three groups (HAH: hydrogel only group, HAH+BMP-2: hydrogel+BMP-2 group, and HAH+BMP-2+VEGF: hydrogel+BMP-2+VEGF group). ◆: premature bone tissue without bone cells, ●: new bone (woven bone), *: residual collagen sponge, ←: lamellar bone. HAH: hyaluronic acid-based hydrogel, BMP-2: bone morphogenetic protein-2, VEGF: vascular endothelial growth factor.

stained in the group with BMP-2 (22.20±2.17%, $p<0.01$) and the group with dual growth factors (24.22±1.88%, $p<0.01$) compared to the control (14.58±2.21%). However, there was no significant difference between the BMP only and dual growth factor groups (Fig. 7C).

DISCUSSION

Collagen sponge/hydrogel scaffold-incorporated osteogenic growth factors and osteogenesis

Various bone graft materials are currently being used for bone regeneration in the maxillary sinus. Several studies have demonstrated that osteogenic growth factors enhance new bone formation in maxillary sinus animal models and clinical applications in oral surgery following implantation using several biomaterials such as collagen sponge, hydrogel, gelatin/hydrogel complex and chitosan thermosensitive hydrogel [2,29,30,31]. In our study, the implantation of a collagen sponge/hydrogel scaffold delivery system incorporated with BMP-2 and VEGF growth factors was a relatively simple and non-invasive procedure. Hydrogel degraded *in vivo* has long been favourable for clinical use in humans as a material for delivery of growth factors to the defect region, and its safety has been well documented [3]. Park et al. [28] and Song et al. [32] reported that the growth factors release from the MMP-sensitive hydrogels were evaluated in the *in vivo* experiment. In addition, several animal and human studies have shown rapid new bone formation by using collagen sponge incorporated with osteogenic growth factors [33,34]. For these reasons, we used a collagen sponge/hydrogel scaffold incorporated with osteogenic growth factors for new bone formation in an alveolar bone defect dog model. In addition, bone regeneration procedures for maxillary sinus augmentation utilize various bioactive factors [35,36]. In our study, collagen sponge/hydrogel scaffold incorporated with osteogenic growth factors increased the ability of the collagen sponge and hydrogel to mediate bone regeneration in the maxillary sinus region of the alveolar bone defect dog model. In particular, collagen sponge/hydrogel scaffold incorporated with BMP-2 en-

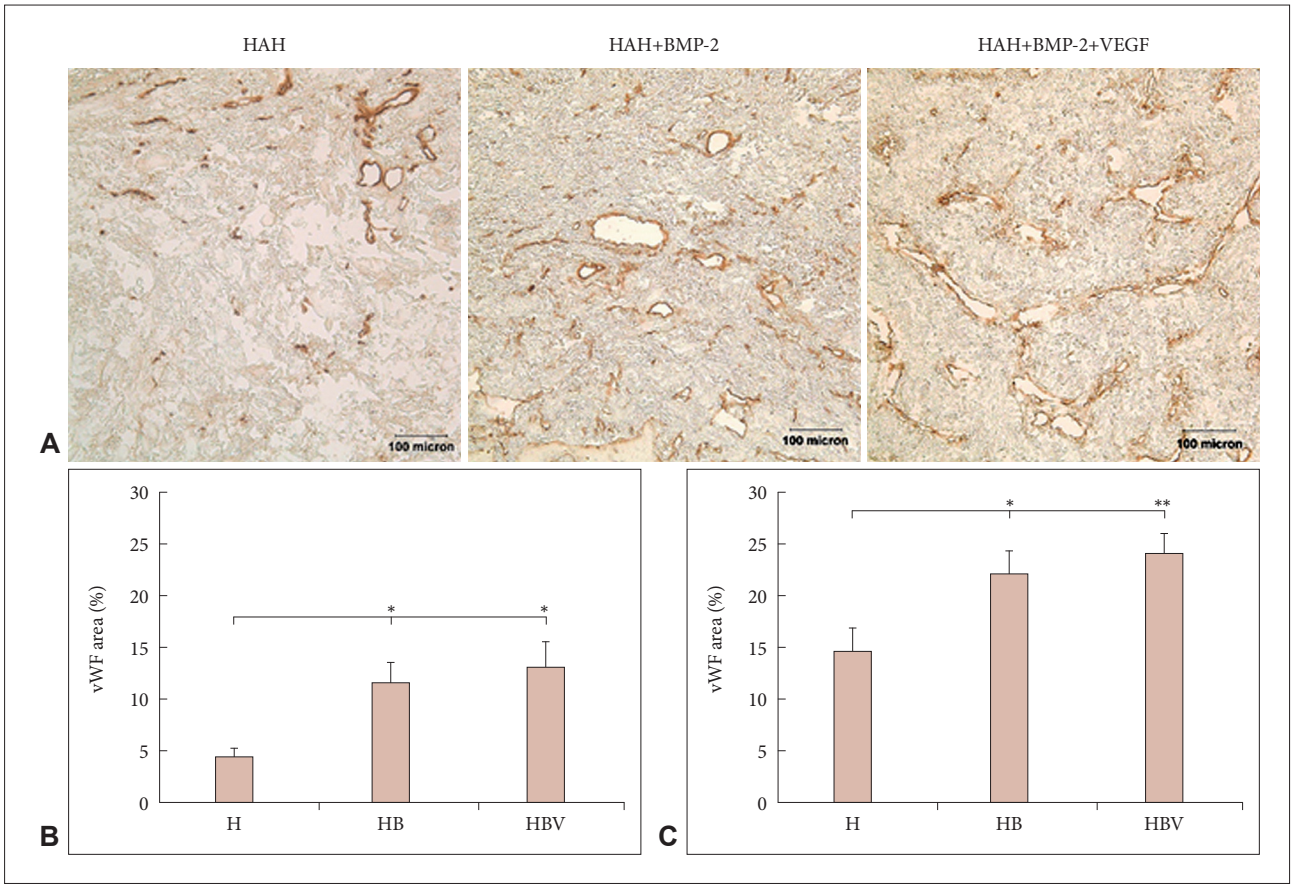

Figure 7. Immunohistochemical staining with von Willebrand factor (vWF) at the the mid-position of whole specimens and at the mid-position of posterior half specimens. vWF was seen significantly more in the BMP-2 combined with VEGF group than in other groups at the mid-point of whole specimens, and more in the BMP-2 only and BMP-2 in combination with VEGF groups compared to the control at the mid-point of posterior half specimens. *p<0.05, **p<0.01. HAH: hyaluronic acid-based hydrogel, BMP-2: bone morphogenetic protein-2, VEGF: vascular endothelial growth factor, H: hydrogel, HB: hydrogel+BMP-2, HBV: hydrogel+BMP-2+VEGF.

hanced osteogenesis in the alveolar bone defect dog model.

Assessment of the effects of BMP-2 and VEGF growth factors on new bone formation in the alveolar bone defect dog model

This study evaluated the additive effect of VEGF combined with BMP-2 delivered with HAH for maxillary alveolar bone regeneration in dogs. With a low dose of BMP-2 (80 μg/300 uL), new bone formation was observed at the maxillary alveolar bone of the dogs compared to the control. The combination of VEGF and BMP-2 also induced new bone formation compared to the control. However, in this group (VEGF combined with BMP-2), the new bone formation was similar to or slightly lower than that in the BMP-2 only group.

As a potent angiogenic factor, VEGF in bone formation regulates bone homeostasis, bone angiogenesis and bone development [37]. VEGF is known to participate in key steps of the molecular cascades in the bone repair process. It has been reported that VEGF can be used to stimulate new bone formation for maxillary sinus augmentation in animal experiments [36,38]. Several studies have demonstrated that BMP-2 enhances local bone formation in maxillary sinus animal models and clinical applications in oral surgery following implantation using a collagen sponge. For these reasons, BMP-2 and VEGF were adopted for use in this study. Zhang et al. [19] demonstrated that silk hydrogel loaded with the combination of BMP-2 and VEGF promotes bone regeneration for maxillary sinus floor augmentation in irregular bony cavities. Amirian et al. [39] reported that VEGF and BMP-2 loaded on osteoconductive scaffold could enhance bone regeneration both *in vitro* and *in vivo*. Moreover, the combination of an angiogenic (VEGF), an osteogenic factor (BMP-2) and MSCs enhances bone regeneration [40]. In contrast, previous studies have demonstrated that a synergistic effect of the dual delivery of VEGF and BMP-2 does not affect new bone formation. Patel et al. [18] showed that the combination of VEGF and BMP-2 increase early bone regeneration at 4 weeks. However, at 12 weeks, dual growth factors fail to affect new bone formation. This suggests that a synergistic effect of both growth factors may be dependent on the treatment duration of new bone formation. It has been reported that differential dose-dependent effects of VEGF combined with BMP-2 are associated with bone regeneration in a rat critical-size defect model. Young et al. [20] demonstrated that differential dose-dependent treatment with dual growth factors decreased new bone formation. These findings suggest that effective new bone formation may require the ability of a bone healing environment through the controlled release of multiple differential dose-dependent growth factors. Moreover, in an ectopic defect site, the combination of VEGF and BMP-2 significantly enhanced ectopic bone formation compared to BMP-2 alone. However, VEGF and BMP-2 did not increase bone formation in an orthotopic defect site. Kim et al. [22] demonstrated that the combination of BMP-2 and VEGF did not enhance the synergistic effect of bone regeneration compared with the single use of BMP-2 in the alveolar bone defect dog model. Similarly, in our results, BMP-2 accelerated new bone formation in the maxillary sinus. Although the combination of BMP-2 and VEGF growth factors also facilitated new bone formation, the combination treatment did not show synergistic effects in the alveolar bone defect model. Consequently, the BMP-2 only group showed substantially more new bone formation than the combination of BMP-2 and VEGF group. Therefore, one possible explanation is that the soft and hard tissues at the oral and maxillofacial areas have better blood supply with abundant vasculature, as is well known [41,42]. For these reasons, in our study, the addition of VEGF may not have a synergistic effect on new bone formation at alveolar bone defects in large animal models. A second possible explanation is that there is no standardization of the optimum concentration range of BMP-2 and VEGF growth factors for new bone formation.

The concentration of BMP-2 inducing the most effective new bone formation still remains unclear, and the activity of this growth factor is influenced by various factors including the target region, release kinetics and several types of delivery systems [43]. Furthermore, regarding the amount of BMP-2, 450 μg/300 uL BMP-2 shows increased new bone formation over a long term period [19]. In contrast, it has been reported that a low dose of 30 μg/300 uL or 45 μg/300 uL of BMP-2 induces the early stage of new bone formation in both *in vitro* and *in vivo* studies [44,45]. In our study, we used the rhBMP-2 concentration of 80 μg/300 uL to facilitate new bone formation in the alveolar bone defect dog model. This finding suggests that collagen sponge/hydrogel incorporated with 80 μg/300 uL of BMP-2 may be effective in generating sufficient osteogenic bone to cover the implant in the alveolar bone defect dog model in our study. Moreover, it has been reported that a high dose of VEGF has been proven to have adverse effects on new bone formation including vessel leakage and the induction of massive non-physiological endothelial cells forming multiple channels [46]. A third possible explanation is that the combination of BMP-2 and VEGF promotes angiogenesis but suppresses the terminal differentiation of osteoblasts. Song et al. [47] reported that a balancing regulation of BMP-2 and VEGF has a critical role in bone regeneration. BMP-2 and VEGF are necessary factors for osteoblasts and bone vascular endothelium cells. These cell-to-cell communications, depending on the balanced regulation of BMP-2 and VEGF growth factor concentrations, could

be crucial to the development and regeneration of bone. As mentioned above, the reasons for this is unclear, but these non-standardized concentrations of growth factors may have undesired consequences such as the creation of new bone formation in the combination of BMP-2 and VEGF group.

The use of the collagen sponge/hydrogel complex scaffold incorporated with osteoconductive growth factors has the advantages of minimal invasion and a short operation time. In the alveolar bone defect dog model, the creation of new bone formation was increased in the BMP-2 group compared to the control group. However, the combination of osteogenic and angiogenic growth factors (BMP-2 and VEGF) did not increase the new bone formation compared to the BMP-2 group. Thus, these results indicate that the complex biomaterial of collagen sponge/hydrogel sheets involving osteogenic growth factor (BMP-2) promotes bone regeneration, and it serves as an alternative bone grafting material for the production of new bone formation in the maxillary sinus alveolar defect dog model.

Limitations

Within the limited results of this study using collagen sponge/hydrogel incorporated with BMP-2 and VEGF, it suggests that this scaffold delivery system incorporated with only BMP-2 growth factor may provide favourable bone formation at the early stage. However, the combination group did not show good efficacy for new bone formation in the alveolar bone defect dog model. Thus, further study should clarify the effectiveness of various concentrations of growth factors for the combination of BMP-2 and VEGF groups. In addition, we need to evaluate the release of these growth factors from the scaffold over a long experimental period.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was approved by the Committee on the Safety and Ethical Handling Regulation for Laboratory Experiments at the School of Dentistry at Seoul National University.

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