

## Research Article



# Four-week histologic evaluation of grafted calvarial defects with adjunctive hyperbaric oxygen therapy in rats

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## ABSTRACT

**Purpose:** The aim of this study was to characterize the healing in the grafted calvarial defects of rats after adjunctive hyperbaric oxygen therapy.

**Methods:** Twenty-eight male Sprague-Dawley rats (body weight, 250–300 g) were randomly divided into two treatment groups: with hyperbaric oxygen therapy (HBO; n=14) and without HBO (NHBO; n=14). Each group was further subdivided according to the bone substitute applied: biphasic calcium phosphate (BCP; n=7) and surface-modified BCP (mBCP; n=7). The mBCP comprised BCP coated with *Escherichia coli*-derived recombinant human bone morphogenetic protein-2 (ErhBMP-2) and epigallocatechin-3-gallate (EGCG). Two symmetrical circular defects (6-mm diameter) were created in the right and left parietal bones of each animal. One defect was assigned as a control defect and received no bone substitute, while the other defect was filled with either BCP or mBCP. The animals were allowed to heal for 4 weeks, during which those in the HBO group underwent 5 sessions of HBO. At 4 weeks, the animals were sacrificed, and the defects were harvested for histologic and histomorphometric analysis.

**Results:** Well-maintained space was found in the grafted groups. Woven bone connected to and away from the defect margin was formed. More angiogenesis was found with HBO and EGCG/BMP-2 ( $P<0.05$ ). None of the defects achieved complete defect closure. Increased new bone formation with HBO or EGCG/BMP-2 was evident in histologic evaluation, but it did not reach statistical significance in histometric analysis. A synergic effect between HBO and EGCG/BMP-2 was not found.

**Conclusions:** Within the limitations of this study, the present findings indicate that adjunctive HBO and EGCG/BMP-2 could be beneficial for new bone formation in rat calvarial defects.

**Keywords:** Biphasic calcium phosphate; BMP-2; Bone substitute; EGCG; Hyperbaric oxygen therapy

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**Conflict of Interest**

No potential conflict of interest relevant to this article was reported.

**INTRODUCTION**

It has been proposed that increased plasma oxygen levels have favorable effects on the healing of bone and soft tissue [1]. The oxygen level is 6%–9% in healthy bone, but this decreases to 1%–3% in necrotic bone. Hyperbaric oxygen (HBO) therapy can increase tissue levels of oxygen: the partial pressure of oxygen in irradiated tissues, which is normally 5–15 mmHg, can increase to 20–35 mmHg following HBO therapy [2,3]. HBO therapy stimulates the differentiation of circulating stem cells and the growth of new blood vessels from local endothelial cells, and promotes the production of reactive oxygen species, which play important roles as signaling molecules for various growth factors, cytokines, and hormones [3]. There have been several reports on the ability of HBO therapy to improve osteogenic potential [4-6], and prophylactic and therapeutic effects have been demonstrated clinically [1,2]. HBO therapy is often applied for improving healing capacity in healing-impaired tissues such as irradiated bone, and the effect of HBO on bone formation after irradiation has been demonstrated in animal studies with rabbits [7,8]. HBO therapy is also applied for improving healing capacity in normal tissues without healing impairment. It has been shown that bone regeneration in ungrafted rabbit calvarial defects was significantly greater following the application of HBO [4], and that bone regeneration in grafted rabbit calvarial defects was improved with HBO therapy [6].

There are various types of bone substitute, including allograft, xenograft, and alloplastic grafts, which show great variability in their osteoconductivity and osteoinductivity [9]. Alloplastic grafting has been used widely with no evidence of associated critical infection or disease transmission. Biphasic calcium phosphate (BCP) is an alloplastic material that is structurally similar to the inorganic phase—hydroxyapatite (HA) and beta-tricalcium phosphate ( $\beta$ -TCP)—of human bone tissue. The insolubility of HA allows the maintenance of the defect form and structure, while  $\beta$ -TCP is dissolved into calcium and phosphate ions and is ultimately replaced by newly formed bone. The optimum conditions for new bone formation can be obtained by controlling the ratio of HA to  $\beta$ -TCP. However, better clinical outcomes will be achievable with improvements in the osteoinductivity of this alloplastic material [10].

The osteoinductive characteristics of BCP could potentially be improved by modifying its surface [11,12]. One such surface-modifying material is recombinant human bone morphogenetic protein-2 (rhBMP-2) [13], which has been shown to enhance new bone formation. However, its use is associated with several side effects, such as the formation of an ectopic bone void and the swelling of soft tissue, which remain of concern and limit its general application. It is recommended that bone morphogenetic protein-2 (BMP-2) not be used at high concentrations [14]. Another potential surface-modifying agent is epigallocatechin-3-gallate (EGCG), which is the most abundant catechin in green tea and is reported to have antioxidant, anti-inflammatory, and anticarcinogenic properties. It is also known that EGCG improves osteoblastic activity, suppresses osteoclastic activity, and influences bone metabolism [15,16]. Few side effects of EGCG have been reported. Therefore, using a combination of EGCG and a low concentration of BMP-2 could be considered for modifying the surface of BCP to improve its osteoinductivity [14].

The purpose of this study was therefore to determine the effect of 4 weeks of HBO on the healing of grafted calvarial defects in rats.

## MATERIALS AND METHODS

Twenty-eight male Sprague-Dawley rats (body weight, 250–300 g) were maintained in plastic cages in a room with a 12-hour day/night cycle, an ambient temperature of 21°C, and free access to water and standard laboratory food pellets. The animal selection, management, and preparation, and the surgical protocol followed routines approved by the Institutional Animal Care and Use Committee of Yonsei Medical Center, Seoul, Korea (IACUC number 2011-0056-2).

The animals were divided into two groups according to whether or not they were given HBO therapy: with (HBO) and without (NHBO). Each group was then subdivided further according to the type of bone substitute applied (BCP and mBCP) (Table 1). The bone substitute used for the BCP group consisted of HA and  $\beta$ -TCP (a ratio of 70:30) while that used for the mBCP group was BCP coated with EGCG (5 mg/mL, purity >90%; Sigma, St. Louis, MO, USA) and ErhBMP-2 (0.05 mg/mL; Cowellmedi, Busan, Korea). The BCP surface-coating process comprised three steps. First, the hydroxide ion (OH<sup>-</sup>) of HA was combined with a silane coupling agent (3-aminopropyltriethoxysilane [APTES] [Sigma]). Second, a bifunctional cross-linker (N-succinimidyl-3-maleimidopropionate [SMP] [Sigma]) was combined with amino radicals. Finally, EGCG and ErhBMP-2 were combined with SMP. Following the coating procedure, the graft material was lyophilized, frozen, and then stored at -45°C for 3 hours. It was then subjected to primary drying (2 hours in a pressure chamber at 7–10 mTorr), and secondary drying (more than 2 hours at -20°C to 20°C). The mBCP was then subjected to gas sterilization with ethylene oxide [14].

All surgical procedures were performed under general anesthesia with an intermuscular injection of 1 mL/kg of xylazine (Rompun, Bayer Korea, Seoul, Korea) and zolazepam (Zoletil 50, Virbac, Carros, France). The surgical site was isolated by shaving and then sterilizing with povidone-iodine solution, and then locally anesthetized with 2% lidocaine with 1:100,000 epinephrine by infiltration. Two bilateral calvarial defects (6 mm in diameter) were created on the parietal bones of each rat. One defect was filled with bone substitute (BCP or mBCP), while the other defect was left unfilled (the control defect). The skin/periosteum was sutured with 4-0 polyglactin 910 suture material (Vicryl, Ethicon, Somerville, NJ, USA). During the healing period, animals assigned to the HBO group were provided with 5 sessions of HBO therapy (2.4 ATM, 1 hour/day, for 5 days/week) in a high-pressure oxygen chamber. All of the animals were sacrificed after a healing period of 4 weeks following the surgical procedure by carbon dioxide asphyxiation.

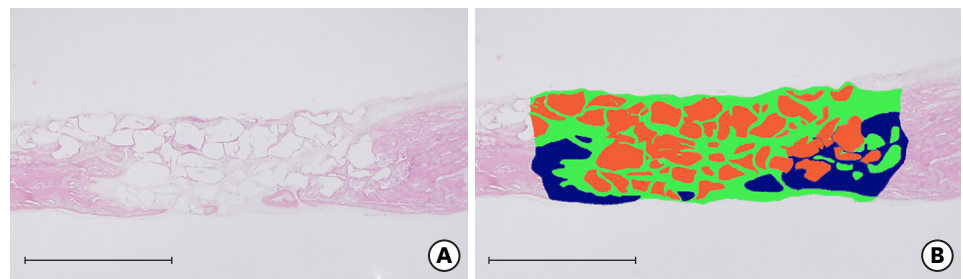
### Histologic and histometric analysis (H/E staining)

The calvarial defects were harvested and then fixed, decalcified, embedded in paraffin, and stained with hematoxylin-eosin. A series of 4- $\mu$ m-thick sections was prepared and examined histomorphometrically with an optical microscope at 12.5 $\times$  magnification. The histometric measurements included the areas of new bone and soft tissue, which were obtained using the

**Table 1.** Groups defined according to HBO therapy and graft materials

| Groups       | N | Materials/conditions               |
|--------------|---|------------------------------------|
| NHBO-BCP     | 7 | BCP without HBO therapy            |
| NHBO-mBCP    | 7 | BCP+EGCG/BMP-2 without HBO therapy |
| NHBO-control | 7 | No graft without HBO therapy       |
| HBO-BCP      | 7 | BCP with HBO therapy               |
| HBO-mBCP     | 7 | BCP+EGCG/BMP-2 with HBO therapy    |
| HBO-control  | 7 | No graft with HBO therapy          |

HBO, hyperbaric oxygen therapy; BCP, biphasic calcium phosphate; EGCG, epigallocatechin-3-gallate.



**Figure 1.** (A, B) Histometric analysis (bar=2 mm). New bone, graft particles, soft tissue. Each slide was painted using Photoshop CS4 (Adobe, San Jose, CA, USA). Navy blue, new bone; Orange, graft particles; Green, soft tissue.

Tomoro ScopeEye 3.6 system (Techsan, Seoul, Korea). The percentage of new bone area (NB) was calculated as total new bone area/[soft tissue + remaining graft (RG) + new bone area] (see Table 1). The ratio of NB to RG was also calculated (Figure 1). Measurements within groups were pooled and used to calculate the mean and standard deviation (mean±SD) values.

### Histologic and histometric analysis (immunohistochemical staining)

Microvessels and the presence of vascular endothelial growth factor (VEGF) were identified by immunohistochemical staining with monoclonal antibodies raised against endothelial cell adhesion molecule-1 (CD31; sc-1506-R, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and VEGF antibody (ab28775, Abcam, Cambridge, UK). After incubation with the primary antibodies, the sections were further incubated for 30 minutes with horseradish-peroxidase-labeled anti-rabbit immunoglobulin antibody (Dako, Glostrup, Denmark). After rinsing in Tris-buffered saline, the sections were incubated at room temperature for 30 minutes, and then stained with diaminobenzidine (DAB) for 15 minutes. An optical microscope was used to evaluate the distribution and localization of stained endothelial cells in the defects. An overview image of the specimens was obtained at a magnification of 200×, and DAB staining was explored on both defect sides within a 1-mm-wide strip.

### Statistical analysis

Statistical analysis was performed using SPSS ver. 18.0 (IBM Corp., Armonk, NY, USA). The Mann-Whitney *U* test was used to compare the difference between control and experimental group, as well as the difference according to use of hyperbaric oxygen therapy. The level of significance was set at  $P < 0.05$ .

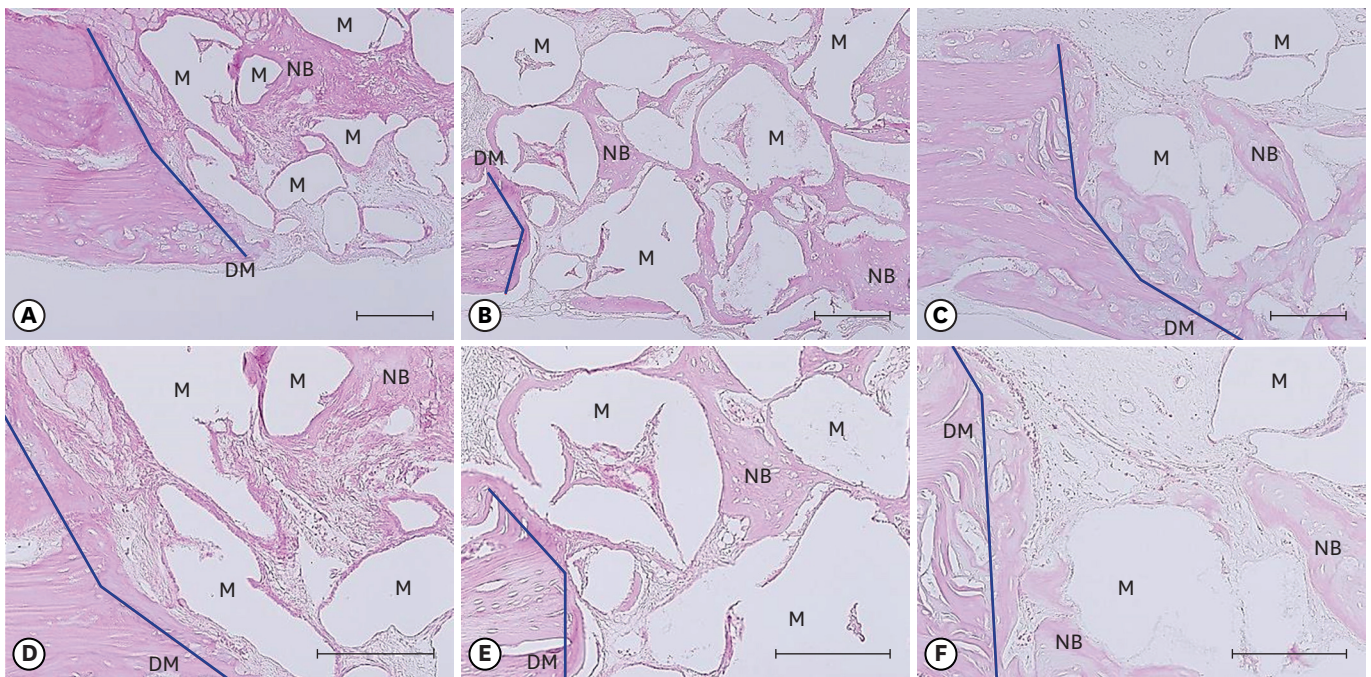
## RESULTS

### Histologic analysis

#### HE staining

After the 4-week healing period, complete defect closure was not observed in any of the study groups. A limited amount of new bone formation was observed from the defect margin in the control groups, together with dimensional shrinkage of the defect (Figure 2A and D). The dimension of the defect was well maintained in all of the grafted groups. New bone formation close to and away from the defect margin and the BCP or mBCP particles was observed. There was no sign of inflammation or infection in any of the groups. Increased new bone formation around bone substitute particles was evident in the HBO groups (Figure 3C





**Figure 2.** Histologic evaluation around the defect margin (bar=25 and 100  $\mu$ m). It shows the osteogenic effect of HBO and BMP-2/EGCG. (A, D) NHBO-BCP subgroup, (B, E) NHBO-mBCP subgroup, (C, F) HBO-BCP subgroup. New bone formation was rarely present in (A) and (D). However, new bone in contact with the defect margin (C, F) and between particles (B, E) was increased. DM, defect margin; M, material; NB, new bone.

and F) when bone substitute was applied.

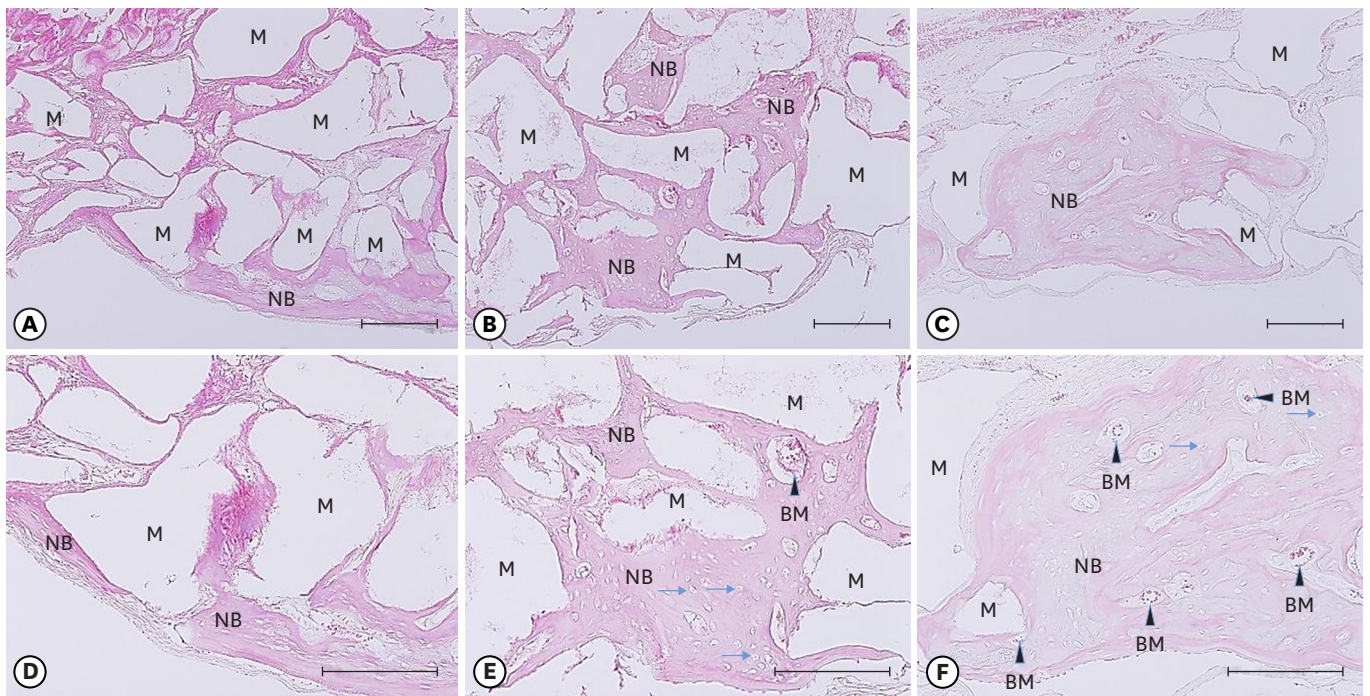
At the defect margin, without adjunctive therapy (HBO or EGCG/BMP-2), new bone formation was rarely found (Figure 2A and D). New bone in contact with the defect margin or between particles was hardly found. However, new bone in contact with the defect margin and between particles was increased with HBO therapy (Figures 2C, 2F, 3C, and 3F). New bone between particles was increased with application of EGCG/BMP-2 (Figure 3B and E). However, new bone in contact with the defect margin was not increased with EGCG/BMP-2. It could be assumed that EGCG/BMP-2 has a greater effect on new bone formation between particles than at the defect margin (Figure 2B and E).

At the defect base close to periosteum (the lower part of each slide) (Figure 4), there was more new bone formation than in the upper part. This is the area away from the defect margin where most of the healing occurs. It could be assumed that the healing at the area away from the defect margin was enhanced by HBO therapy and EGCG/BMP-2. A relatively large amount of new bone was found in the HBO-BCP group (Figure 4B). This new bone formed around particles and connected each other.

#### Immunohistochemical staining

To compare the vascularization of each group, the CD31 biomarker was used. In the NHBO control group, a diminished number and smaller size of vessels was found than in the HBO-control or NHBO-mBCP groups (Figure 5). Blood vessels relatively large in size were found in the HBO-control group. A relatively greater number of blood vessels were found with NHBO-mBCP. The increase in size/number of blood vessels with HBO and EGCG/BMP-2 could be estimated based on this comparison.





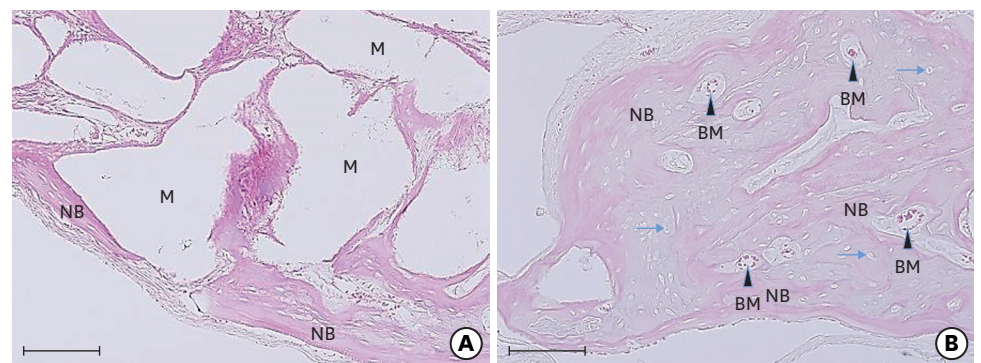
**Figure 3.** Histologic evaluation at the middle of defect (bar=25 and 100  $\mu$ m). (A, D) NHBO-BCP subgroup, (B, E) NHBO-mBCP subgroup, (C, F) HBO-BCP subgroup. In the experimental groups, osteocytes (arrow) and bone marrow (arrow head) were observed. M, material; NB, new bone; BM, bone marrow.

The mBCP group also showed more blood vessels than the control or BCP groups (Figure 6). However, an increase or decrease in the number of blood vessels with HBO therapy in the mBCP group could not be found. It could be assumed that the effect of HBO therapy is minimal in the mBCP group.

### Histometric analysis

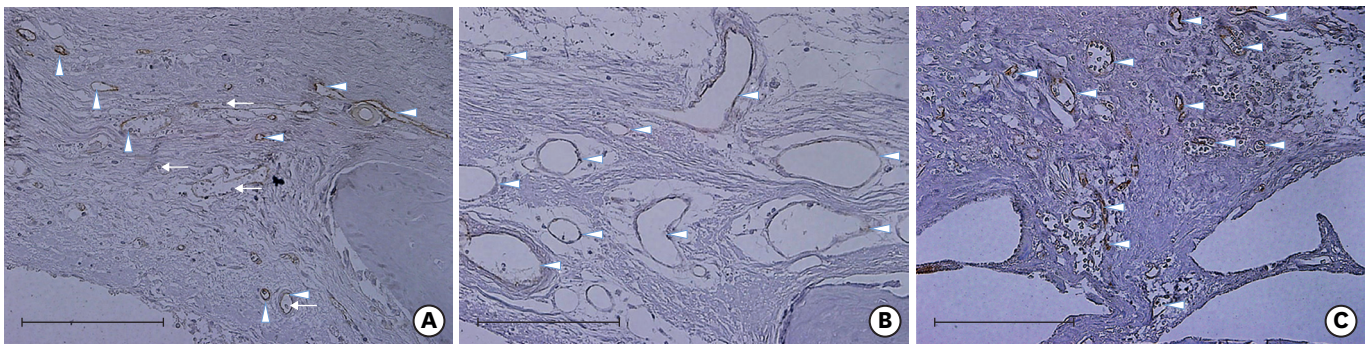
The percentage of new bone area

A statistically significant difference in the percentage of new bone area was not found among groups.



**Figure 4.** Histological evaluation at the middle of the defect close to the defect base showing the osteogenic effect of HBO (H/E, bar=25  $\mu$ m). (A) NHBO-BCP group, (B) HBO-BCP group. M, material; NB, new bone; BM (arrow head), bone marrow; Arrow, osteocyte.





**Figure 5.** Histologic evaluation (CD31 immunohistochemical staining, bar=50  $\mu$ m) showing the angiogenic effect of HBO and BMP-2/EGCG. Blood vessels were observed in all groups. The CD31-positive endothelial cells were generally replaced (arrow head). (A) NHBO-control group, (B) HBO-control group, (C) NHBO-mBPC group.

Arrow head, vessel; Arrow, red blood cell.

#### The ratio of NB to RG

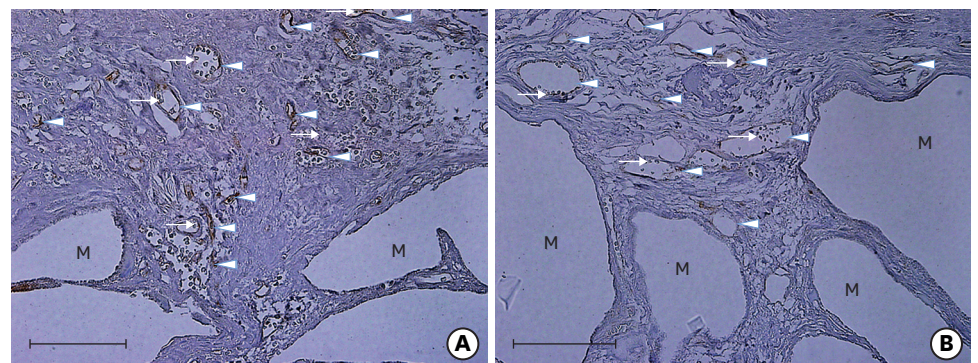
The ratio of NB to RG was calculated (Table 2). Among the NHBO-treated defects, the mBPC group exhibited a higher NB:RG ratio (0.34) than the BCP group (0.22). In the HBO-treated defects, the NB:RG ratio was higher for the BCP group (0.36) than for the mBPC group (0.24). Comparison of the NHBO and HBO groups revealed that the BCP+HBO group had a higher ratio (0.36) than the BCP+NHBO group (0.22). This ratio was lower for the mBPC+HBO group (0.24) than for the mBPC+NHBO group (0.34).

#### Blood vessel count

The blood vessel count (BVC) was obtained by immunohistochemical staining with a CD31 monoclonal antibody. The BVC of the NHBO-mBPC group was higher than the NHBO-control group ( $P<0.05$ ). The BVC of the HBO-control group was higher than the NHBO-control group ( $P<0.05$ ).

#### VEGF immunostaining

The NHBO-mBPC group ( $70.86\pm 16.98$ ) showed higher levels of VEGF immunostaining than the NHBO-control group ( $33.00\pm 7.79$ ) ( $P<0.05$ ).



**Figure 6.** Immunohistochemical analysis showing the synergic effect of HBO with BMP-2/EGCG (CD31 staining, bar=50  $\mu$ m). (A) NHBO-mBPC subgroup, (B) HBO-mBPC subgroup.

M, material; Arrow head, vessel; Arrow, red blood cell.

**Table 2.** NB (%), NB/RG ratio, BVC, VEGF

| Measurement | NHBO        |                           |                            | HBO         |             |                           |
|-------------|-------------|---------------------------|----------------------------|-------------|-------------|---------------------------|
|             | BCP         | mBCP                      | Control                    | BCP         | mBCP        | Control                   |
| NB (%)      | 29.85±12.43 | 29.90±12.66               | 13.64±8.01                 | 35.35±15.33 | 27.61±17.02 | 18.75±6.03                |
| NB/RG ratio | 0.22        | 0.34                      |                            | 0.36        | 0.24        |                           |
| BVC         | 58.57±39.00 | 81.57±35.31 <sup>a)</sup> | 35.57±8.10 <sup>a,b)</sup> | 60.68±33.09 | 66.86±33.86 | 53.86±22.83 <sup>b)</sup> |
| VEFG        | 47.43±27.34 | 70.86±16.98 <sup>c)</sup> | 33.00±7.79 <sup>c)</sup>   | 58.57±27.69 | 63.14±32.60 | 48.29±18.33               |

Values are presented as mean±standard deviation.

NB, new bone; NB/RG ratio, new bone/remaining graft ratio; BVC, blood vessel count; VEGF, vascular endothelial growth factor; BCP, biphasic calcium phosphate.

<sup>a)</sup>Significantly different between NHBO-control and NHBO-mBCP groups ( $P<0.05$ ); <sup>b)</sup>Significantly different between NHBO-control and HBO-control groups ( $P<0.05$ ); <sup>c)</sup>Significantly different between NHBO-control and NHBO-mBCP groups ( $P<0.05$ ).

## DISCUSSION

Previous studies have found that wound healing is enhanced by HBO therapy, such that beneficial effects in both soft- and hard-tissue healing were seen in HBO-treated groups relative to NHBO-treated groups [4,6]. In the present study, an increase in the BVC with HBO and BMP-2/EGCG was found in the histometric analysis. An increase in the BVC with HBO and BMP-2/EGCG was also found in the histologic analysis (Figure 5). An increased size and number of blood vessels was found with HBO (Figure 5B) and an increased number of blood vessels was found with BMP-2/EGCG (Figure 5C). The angiogenic effects of HBO and BMP-2/EGCG seemed to be different, but a beneficial effect during the early phase of bone healing could be similarly expected.

Increased new bone formation with HBO or EGCG/BMP-2 could be found with histologic analysis, but not with histometric analysis. Increased and more mature new bone formation with HBO was found in the area close to the defect margin. Similarly enhanced new bone formation with HBO and BMP-2/EGCG was also found between particles in the center of the defect. The HBO therapy seemed to enhance healing at the defect margin and at the middle of the defect. The application of BMP-2/EGCG seemed to increase healing potential between particles, but not around the defect margin. Therefore, minor effects from BMP-2/EGCG could be expected at the defect margin without any bone graft. BMP-2/EGCG should be applied with a proper carrier, such as BCP. However, no difference in new bone formation between groups was found with histometric analysis. This finding could also be attributed to the healing period implemented in the present study. A healing time of 4 weeks might not be sufficient to reveal an effect with EGCG/BMP-2 and HBO. In a previous study [17] using the rabbit calvarial defect model, a healing time of 6 weeks was thought to be insufficient to observe the effect of HBO. Those authors suggested that a healing period of more than 12 weeks was necessary, corresponding to a healing period of 8 weeks in a rat. It is possible that a healing period longer than 4 weeks would yield different results than those presented here.

Synergic effects of EGCG/BMP-2 and HBO could not be found in regard to blood vessel increase or new bone formation in the present study. This finding might also be explained based on the findings of previous studies. During bone healing, osteoprogenitor-cell differentiation is modulated by endothelial cells through the BMP-2 signaling pathway. The endothelial-cell proliferation is stimulated by VEGF-producing osteoblasts [18,19]. There is thus a strong correlation between VEGF and BMP-2. The HBO-induced increase in VEGF is known to result in an increase in endothelial-cell proliferation, which could enhance bone healing. Therefore, in the present study, both BMP-2 and VEGF were applied to the bone defect. One previous study found that BMP-2 could amplify the angiogenic activity of VEGF [20]. However, there is an ideal and specific ratio of BMP-2:VEGF for new bone formation



[21]. The present finding could thus be explained by a sub-optimal concentration of BMP-2 for additional new bone formation when adjunctive HBO was applied.

EGCG improves osteoblastic activity, suppresses osteoclastic activity, and influences bone metabolism [15,16]. It was therefore used together with BMP-2 for the surface modification of mBCP with BMP-2, with the expectation that it would help to increase the osteoblastic activity of BMP-2, allowing a lower concentration of BMP-2 to be used [13,14], and thus preventing the unwanted side effects observed with supraphysiological concentrations of BMP-2 [13,22]. However, EGCG has been shown to inhibit VEGF expression in a dose-dependent manner. Therefore, in the present study, HBO and BMP-2 stimulated VEGF expression and angiogenic activity, while EGCG likely inhibited them [23]. Although it has been demonstrated elsewhere that EGCG increases osteoblastic activity when used with HBO, no additional osteogenic effect was found with surface modification of BCP using EGCG in the present study.

Within the limitations of this study, adjunctive HBO therapy and BMP-2/EGCG could be considered to improve angiogenesis and osteoinductive potential when BCP is grafted for a bone defect.

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