Bone Formation and Remodeling of Three Different Dental Implant Surfaces with *Escherichia Coli*–Derived Recombinant Human Bone Morphogenetic Protein 2 in a Rabbit Model

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Purpose: *The objective of this study was to analyze orthotropic bone formation and remodeling of three different dental implant surfaces with and without recombinant human bone morphogenetic protein 2 derived from* Escherichia coli *(ErhBMP-2) in a rabbit model.* ***Materials and Methods:*** *Resorbable blasting media (RBM); sandblasted, large-grit, acid-etched (SLA); and magnesium-incorporated oxidized (MgO) implant surfaces were coated with ErhBMP-2 (1.5 mg/mL). The implants were placed into the proximal tibia in six New Zealand White rabbits. Each rabbit received six different implants (three coated with ErhBMP-2 in one tibia and three uncoated implants in the other tibia), and the sites were closed, submerging the implants. The animals received alizarin (at 2 weeks), calcein (at 4 weeks), and tetracycline (at 6 weeks) fluorescent bone markers, and were euthanized at 8 weeks for histomorphometric analysis.* ***Results:*** *The amount of ErhBMP-2 coating was 9.6 ± 0.4 μg per MgO implant, 14.5 ± 0.6 μg per RBM implant, and 29.9 ± 3.8 μg per SLA implant. Clinical healing was uneventful. Mean bone-to-implant contact (± standard deviation) for the ErhBMP-2/RBM (35.4% ± 5.1%) and ErhBMP-2/MgO (33.4 % ± 13.2%) implants was significantly greater compared with RBM (23.6% ± 6.2%) and MgO (24.9% ± 2.7%) implants (*$P < .05$*). Considering the mean bone-to-implant contact in cortical bone, ErhBMP-2/SLA implants (32.9% ± 7.8%) showed lower bone-to-implant contact in cortical bone than all other implant variations (range, 39.9% ± 18.1% to 51.3% ± 9.2%;* $P < .05$*). There were no remarkable differences in new bone area, with minor differences between implants.* ***Conclusions:*** *Within the limits of study, it was found that the absorbed ErhBMP-2 dose varied with implant surface characteristics, influencing local bone formation and remodeling.* Int J Oral Maxillofac Implants 2013;28:424–430. doi:10.11607/jomi.2751

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Pure titanium cannot promote new bone formation at the early stage of osseointegration. Therefore, numerous attempts have been made to enhance osseointegration and reduce the healing time by improving implant biocompatibility and modifying the surface characteristics mechanically, chemically, and/or biologically using methods such as blasting, plasma spraying, sandblasting and etching, micro-arc oxidation, and growth factor application.[1–5]

Several growth factors have been shown to improve osteoblast differentiation and matrix mineralization, such as bone morphogenetic proteins (BMPs), insulin-like growth factor 1, and basic fibroblast growth factor. Among them, BMPs are powerful inducers of osteoblast differentiation and bone formation.[6] More than 20 different isoforms of BMP have been described, but BMP-2 and BMP-7 are thought to play the most important roles in the skeletal system.[6,7]

The results of recent studies suggest that BMPs have an affinity to titanium, and hence, titanium implants have been considered as potential carriers for BMPs.[8–11] Several studies have evaluated the possibility of developing a load-bearing implant that could deliver recombinant human BMP-2 (rhBMP-2) for oral and maxillofacial reconstruction. Hall et al[12] reported that rhBMP-2-coated titanium porous oxide (TPO) implants exhibited osteoinductive effects in a rat ectopic model, including bone contact to the implant surface. Wikesjö et al[13,14] showed that in a critical-size, supra-alveolar, peri-implant defect model, rhBMP-2-coated implants induced new bone formation and osseointegration following an 8-week healing period. Similar accelerated local bone formation has been observed for implants coated with rhBMP-2 placed into type 2 bone in dogs and type 4 bone in nonhuman primates.[14,15] However, Leknes et al[16] and Wikesjö et al[10] reported that high concentrations of rhBMP-2 induce undesirable implant displacement. They concluded that the application of rhBMP-2 at appropriate doses may induce clinically relevant local bone formation, including vertical augmentation of the alveolar ridge and osseointegration, whereas higher concentrations were associated with undesirable effects.

Previously, most rhBMP-2 was obtained from mammalian cells, such as Chinese hamster ovary (CHO) cells.[17,18] However, the low yield (ng/mL) of rhBMP-2 production in a well-established eukaryotic protein expression system has been considered a major problem for clinical applications. One possible method of solving this problem is to use rhBMP-2 derived from *Escherichia coli* (ErhBMP-2), which can be produced at a low cost.[19,20] Bessho et al[20] demonstrated that the bone-inducing ability of ErhBMP-2 was similar to that of CHO-cell–derived rhBMP-2 (CrhBMP-2).

While a few studies have shown improved bone responses of CrhBMP-2-coated TPO implants, little attention has been paid to ErhBMP-2-coated surface-modified implants. Therefore, the purpose of this study was to analyze bone formation and remodeling resulting from three different dental implant surfaces with and without ErhBMP-2 using a rabbit model in vitro and in vivo.

MATERIALS AND METHODS

In Vitro Study

Preparation of the ErhBMP-2-Coated Implants. Dental implants with three different surface characteristics were used: a resorbable blasting media (RBM) (3.5 mm in diameter and 8.5-mm long; GS III, Osstem Implants); sandblasted large-grit, acid-etched (SLA) (3.5 mm in diameter and 8.5 mm long; TS III, Osstem Implants); and magnesium-incorporated oxidized (MgO) implants (3.3 mm in diameter and 8.0-mm long; Shinhung Implant, Shinhung).

The implants were coated with ErhBMP-2 (Cowellmedi) at a concentration of 1.5 mg/mL. In a previous study, concentrations of 1.5 mg/mL were able to stimulate local bone formation.[10,11] Each implant was immersed three times in protein solution for 5 seconds and lyophilized, freeze dried at –40°C, and vacuum dried at a maximum of 20°C.[11]

Thirty-six dental implants in six groups were prepared: ErhBMP-2/RBM, ErhBMP-2/MgO, ErhBMP-2/SLA, RBM, MgO, and SLA. Three random implants were selected from the coated groups and the amount of coating was calculated using the Bradford protein assay (Bio-Rad) to react to absorbance at 595 nm.[21] The surface morphologies of the uncoated and ErhBMP-2-coated implants were evaluated by scanning electron microscopy (JSM-5800, JEOL).

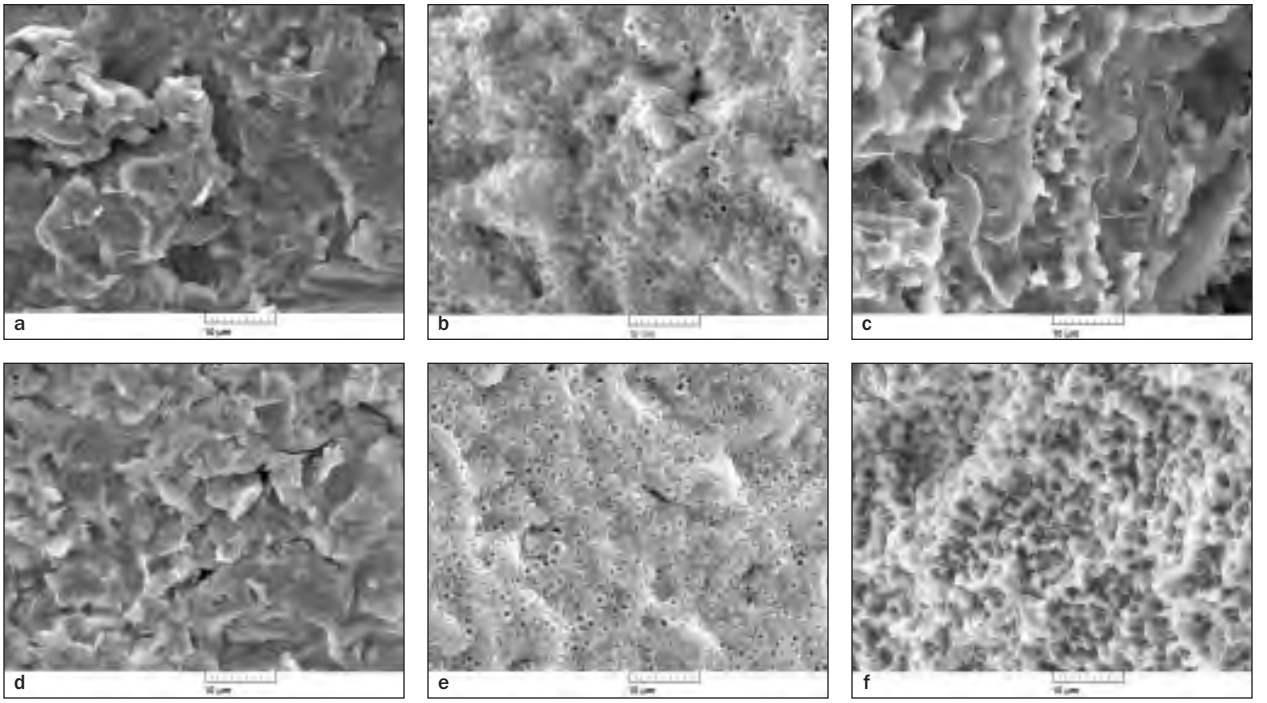
In Vivo Study

Animals. The protocol of this study was approved by the Ethical Committee on Animal Research of the Institute of Gangneung-Wonju National University (IACUC 2010-1). Six New Zealand White rabbits weighing 3,450 ± 180 g (mean ± SD) were used in this study. The animals were housed in separate cages and fed a standard diet. Before surgery, general anesthesia was induced by an intramuscular injection of Zoletil at 0.4 mL/kg (Virbac Laboratories) and Rompun at 0.1 mL/kg (Bayer). Prior to surgery, the operative sites were shaved and carefully washed with iodine solution. Local anesthesia was induced by injecting 1.8 mL of 2% lidocaine with 1:100,000 epinephrine (Huons) at the location of the tibia where the incision was planned. After surgery, all rabbits received 4 mL/kg gentamicin (Kukje Pharmacy) intramuscularly. The animals were allowed to recover to full weight-bearing capacity after surgery.

Surgical Procedures. A skin incision was made along the proximal one-third of the tibia using a sterile surgical technique. After full-thickness flap reflection, three holes were drilled approximately 7 mm apart with copious irrigation. The drilling procedures followed the manufacturer's instructions.

In total, 36 implants were surgically placed. Each rabbit received six different implants (three in each tibias in random circulating order into the left and right sides of the tibia to ensure unbiased comparisons). The middle third of each implant was engaged by the upper cortical bone only.

Fluorochrome Labeling. The polyfluorochrome sequential labeling process was used to evaluate the postoperative bone formation and remodeling.[22] After implantation, all rabbits received a subcutaneous injection of polyfluorochrome label with an interval of 2 weeks. The polyfluorochrome labels used in the



Figs 1a to 1f Representative scanning electron microscopy images of uncoated and ErhBMP-2-coated implants (original magnification ×5000): *(a)* ErhBMP-2/RBM, *(b)* ErhBMP-2/MgO, *(c)* ErhBMP-2/SLA, *(d)* RBM, *(e)* MgO, *(f)* SLA.

present study were alizarin red (30 mg/kg Sigma-Aldrich), calcein green (10 mg/kg, Sigma-Aldrich), and tetracycline (60 mg/kg, Sigma-Aldrich).

Specimen Preparation. The rabbits were sacrificed by an excess dose of sodium pentobarbital at 8 weeks after inserting the implants, and specimens comprising implants and surrounding tissues were removed en bloc from the tibia. The samples were fixed by immersion in a 10% neutral-buffered formalin solution (Accustain, Sigma-Aldrich) for 1 day, then dehydrated in a graded series of ethanol solutions, and embedded in methyl methacrylate resin (Technovit 7200 VLC, Heraeus Kulzer). After dehydration, the specimens were polymerized using a light-based polymerization unit (Exakt System, Exakt). The implants were cut midaxially in a buccolingual plane into 200-µm-thick sections using a band saw with a diamond blade (Exakt Cutting-Grinding System, Exakt). The final section was ground to no thicker than approximately 20 µm using an Exakt microgrinder and polished to an optical finish utilizing the cutting-grinding technique described by Donath and Breuner.[23]

All sections were first examined by immunofluorescence microscopy (Leica Microsystems), and then stained with 1% toluidine blue solution and examined by optical microscopy (BX-50, Olympus).

Specimen Analysis. One blinded examiner analyzed the histomorphometric measurements by optical microscopy. Histomorphometric analyses were performed to obtain additional information on the quality of the implant-tissue interface. The data were quantified as the percentage of the bone-to-implant contact (BIC) for *(1)* the bone contact in each/all threads and *(2)* the bone contact with the cortical bone. The percentage of the total mineralized bone tissue within the threads (referred to as the bone area [BA]) in the cortical region was also calculated. The new bone area (NBA) within the implant threads in the endosteal region was quantified.

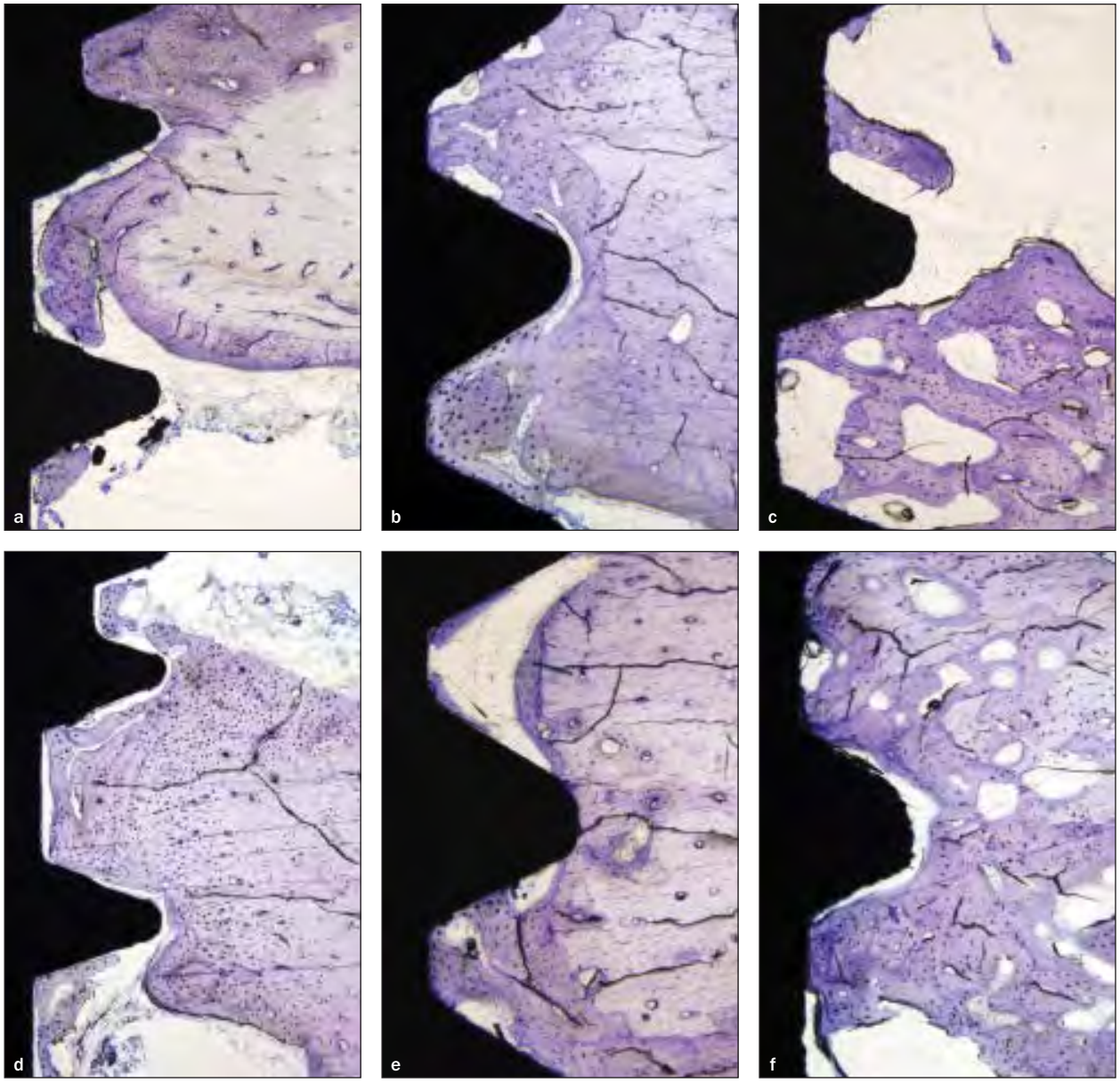
Statistical Analysis

One-way analysis of variance was used to analyze the differences in bone formation between all groups, followed by individual post hoc comparisons using the Duncan test. Statistical significance was established at a 95% confidence level. SPSS (version 18, IBM) was used for data analysis.

RESULTS

ErhBMP-2-Coated implants

The amount of ErhBMP-2 coating on each implant was 9.6 ± 0.4 µg for the MgO implant, 14.5 ± 0.6 µg for the RBM implant, and 29.9 ± 3.8 µg for the SLA implants. Figures 1a to 1f show scanning electron images of the uncoated and ErhBMP-2-coated implants.



Figs 2a to 2f Histologic images of representative implants after 8 weeks of healing in the tibia (1% toluidine blue staining; original magnification ×100). The BIC of cortical bone appears to be highest in panel *(b)*, followed by panels *(e, d, f, d, a,* and *c)*. *(a),* ErhBMP-2/RBM, *(b)* ErhBMP-2/MgO, *(c)* ErhBMP-2/SLA, *(d)* RBM, *(e)* MgO, *(f)* SLA.

Clinical Findings

Postoperative healing was uneventful in all rabbits, with no cases of implant exposure or loss. No clinical differences were detected between the six groups.

Histologic Analysis

After 8 weeks, all implants were histologically in direct contact with the surrounding cortical bone along the threads (Figs 2a to 2f). In some specimens, there was overgrowth of cortical bone, and this was greatest in ErhBMP-2/RBM and ErhBMP-2/MgO implants.

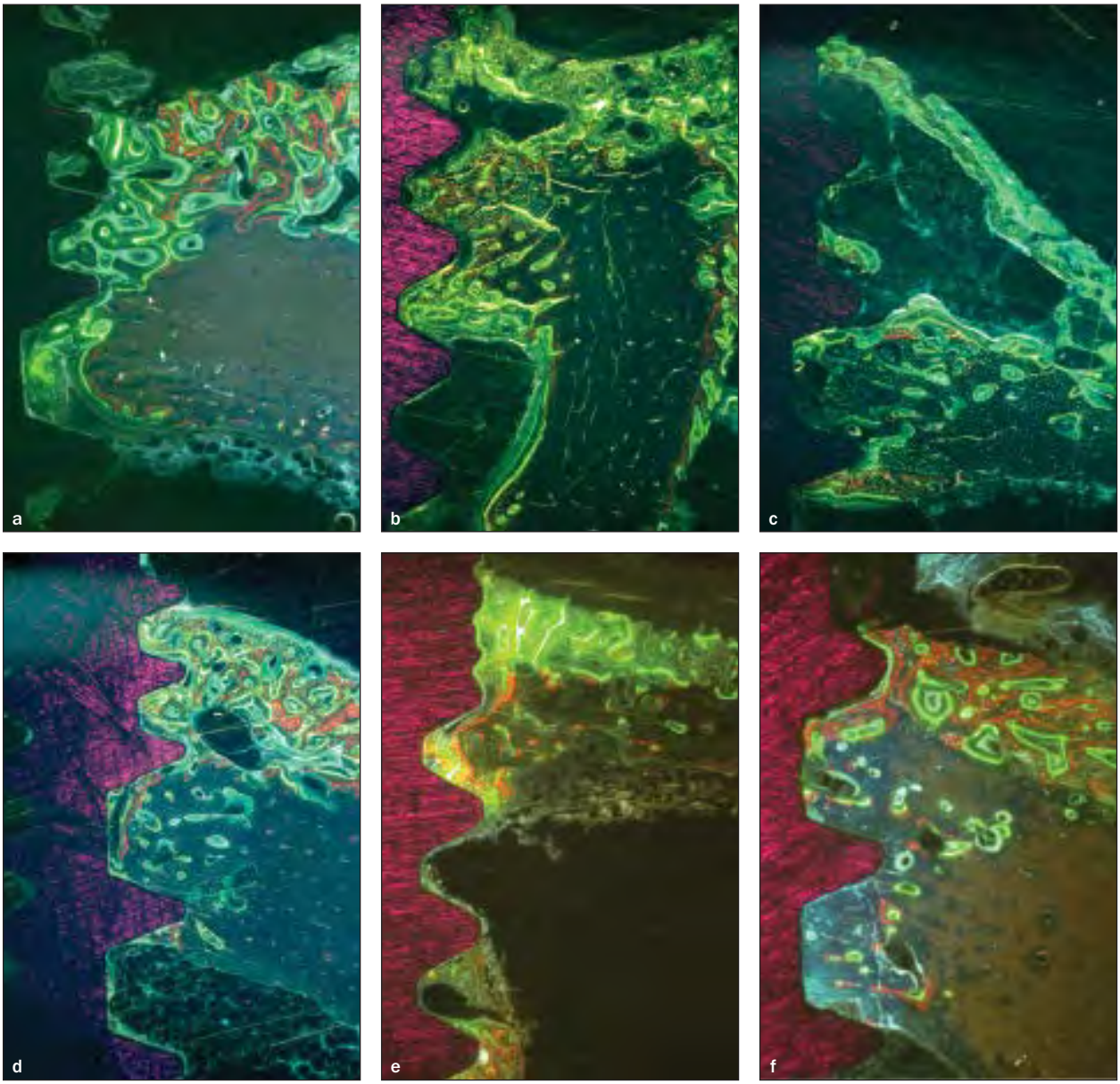
Histomorphometric Analysis

Table 1 lists the results of the histomorphometric measurements. Considering the entire implant, mean BIC (± standard deviation) for the ErhBMP-2/RBM (35.4% ± 5.1%) and ErhBMP-2/MgO (33.4% ± 13.2%) implants was significantly greater compared with RBM (23.6% ± 6.2%) and MgO (24.9% ± 2.7%) implants ($P < .05$). ErhBMP-2/SLA implants (19.1% ± 7.2%) showed slightly lower BIC compared with SLA implants (23.4% ± 3.8%; $P > .05$). Considering mean BIC in cortical bone and bone area within the threads, there were

Table 1 Histomorphometric Analysis*

Group	BIC (%)	BIC of cortical bone (%)	BA (%)	NBA (%)
ErhBMP-2/RBM	35.4 (5.1)[a]	39.9 (18.1)[a]	71.0 (14.8)[a]	51.7 (1.6)[a]
ErhBMP-2/MgO	33.4 (13.2)[a,b]	51.3 (9.2)[a]	83.3 (5.8)[a]	50.5 (10.5)[a]
ErhBMP-2/SLA	19.1 (7.2)[c]	32.9 (7.8)[b]	52.6 (14.2)[b]	39.1 (7.1)[b]
RBM	23.6 (6.2)[b,c]	45.7 (14.3)[a]	83.1 (5.7)[a]	47.8 (8.8)[a,b]
MgO	24.9 (2.7)[b,c]	50.8 (11.6)[a]	80.5 (12.3)[a]	49.9 (9.9)[a]
SLA	23.4 (3.8)[b,c]	45.7 (18.4)[a]	69.8 (22.7)[a]	42.2 (2.3)[a,b]

BA = bone area; BIC = bone-to-implant contact; NBA = new bone area.
*The same superscript letters indicate values that are not significantly different ($P > .05$).



Figs 3a to 3f Fluorochrome-labeled bone at 8 weeks after implant placement (original magnification ×40): *(a)* ErhBMP-2/RBM, *(b)* ErhBMP-2/MgO, *(c)* ErhBMP-2/SLA, *(d)* RBM, *(e)* MgO, *(f)* SLA. Alizarin, calcein, and tetracycline are represented by red, green, and yellow color bands, respectively. Bone remodeling appears to be greatest in panel *(a)*, followed by panels *(b)* and *(d)*. Panel *(c)* exhibits inhibition of bone remodeling. The ErhBMP-2-coated implants *(a)* and *(b)*, except for ErhBMP-2/SLA *(c)*, exhibit bone remodeling not only in the periosteum but also in the surface bone in contact with the implant threads. However, uncoated implants exhibit bone remodeling in the periosteum area.

no significant differences between ErhBMP-2-coated and uncoated RBM and MgO implants. ErhBMP-2/SLA implants (32.9% ± 7.8%) showed lower BIC than all other implant variations (range 39.9% ± 18.1% to 51.3% ± 9.2%; $P < .05$). Similarly there were no remarkable differences in new bone area, with minor differences between implants.

Fluorochrome Labeling Analysis

Figures 3a to 3f show fluorochrome-labeled bone observed under a fluorescence microscope. The different colors indicate continuing osteogenesis. The fluorochrome labels revealed that the patterns of osteogenesis and remodeling differed between the ErhBMP-2-coated and uncoated implants. Bone remodeling occurred in the periosteum area in the uncoated implants (RBM, MgO, and SLA) but was minimal in the regions in contact with the implant surface. However, in the ErhBMP-2/RBM and ErhBMP-2/MgO implants, bone remodeling occurred not only in the periosteum but also in the contacting bone area with the implant threads.

DISCUSSION

This study was designed to evaluate the bone response to ErhBMP-2 on three different surface-modified commercial implants. Despite successful clinical trials of rhBMP-2, which have led to its clinical use, the dose, delivery technologies, and conditions that would optimize the stimulation of bone growth are not fully understood.[5,7,16,24] Hypothetically, dental implants coated with rhBMP-2 would stimulate local bone formation and osseointegration in sites of poor bone quality or in need of augmentation. Sykaras et al[25] observed that the mean bone growth and BIC was greater in experimental implants (hollow chamber implants filled with 20 µg of rhBMP-2 with a bovine collagen carrier) than in control implants. Huh et al[11] described that the ErhBMP-2 coated and anodized implant may stimulate vertical bone augmentation, which significantly increases implant stability on completely healed alveolar ridges. All of these studies have shown that rhBMP-2 can improve alveolar repair, regeneration, and dental implant healing, which is in agreement with the results obtained in the present study.

Previous studies have mainly evaluated TPO implants coated with CrhBMP-2,[12,14,15,24] whereas the present study used three commercial dental implants with different surfaces (RBM, MgO, and SLA) with and without ErhBMP-2 (1.5 mg/mL). The concentration of ErhBMP-2 was determined based on previous studies' findings that it can stimulate local bone formation.[10,11] Wikesjö et al[10] demonstrated in a mongrel dog model that sites receiving TPO implants coated with rhBMP-2 at 0.75 or 1.5 mg/mL showed local bone formation including vertical augmentation. However, sites receiving TPO implants coated with rhBMP-2 at 3.0 mg/mL exhibited more immature trabecular bone formation, seroma formation, and peri-implant bone remodeling, resulting in undesirable implant displacement. Huh et al[11] observed in a beagle dog model, implants coated with ErhBMP-2 at 0.75 and 1.5 mg/mL exhibited significant vertical bone formation and increased implant stability compared with the control group; the amounts of ErhBMP-2 coated in these groups were 10 and 20 µg, respectively. No adverse effects were reported.

The experimental hypothesis was that the amount of ErhBMP-2 coating would be varied with the implant surface morphology and surface roughness, and this might affect local bone formation and remodeling. All of the implants in this study were immersed three times in ErhBMP-2 solution (1.5 mg/mL) for 5 seconds and then lyophilized, which resulted in 9.6 ± 0.4 µg, 14.5 ± 0.6 µg, and 29.9 ± 3.8 µg of ErhBMP-2 being coated on the MgO, RBM, and SLA implants, respectively. The difference in the amounts coated—despite using the same concentration of ErhBMP-2 and the same procedure in each implant—was probably due to the surface morphology and roughness variables of the groups; for example, the surface was more irregular and rougher for the SLA implant than for the RBM and MgO implants (see Figs 1a to 1f). Rougher surfaces have enlarged surface area for ErhBMP-2 absorption.

In this study, the mean BIC values for ErhBMP-2-coated implants other than the ErhBMP-2/SLA implant (35.4% for ErhBMP-2/RBM and 33.4% for ErhBMP-2/MgO) were significantly higher than those for uncoated implants (23.6% for RBM, 24.9% for MgO, and 23.4% SLA). The mean BIC value was lower for the ErhBMP-2/SLA implant (19.1%) than for the SLA implant. The BIC value for cortical bone and the bone area did not differ significantly between the ErhBMP-2-coated and uncoated RBM and MgO implants; however, the values for the SLA implants were lower in the ErhBMP-2-coated implant than in the uncoated implant. The new bone area did not differ significantly between the ErhBMP-2-coated and uncoated implants (Table 1).

The results suggest that coating the implant surface with ErhBMP-2 can increase the initial growth of new bone around an endosseous implant and promote bone remodeling around the implant threads, although its effect on cortical bone is minimal. However, an overdose of ErhBMP-2 inhibits bone formation, as does the BMP-2/SLA implant. This concurs with results found in previous studies.[10,16] In this study, although the same concentration of ErhBMP-2 (1.5mg/mL) was used on each implant, experimental results showed differences according to the implant surface. This difference was probably due to the different dental implants' surface

topographies and different experimental animals (dog versus rabbit) used compared to previous studies. However, it is uncertain whether the loading amount of ErhBMP-2 used in the present study is optimal. Further research should be performed using various loading amounts depending on different experimental animals.

The results obtained using the fluorochrome labeling method showed that the pattern of osteogenesis and remodeling differed between the ErhBMP-2-coated and uncoated implants. In the ErhBMP-2-coated implants other than ErhBMP-2/SLA (ie, ErhBMP-2/RBM and ErhBMP-2/MgO), mineralization occurred not only in the periosteum but also in the surface bone in contact with the implant threads. However, in the uncoated implants (RBM, MgO, and SLA), mineralization occurred mainly in the periosteum area (see Figs 3a to 3f). These two ErhBMP-2-coated implants (ie, other than ErhBMP-2/SLA) showed stronger fluorochrome labeling. Remodeling near the periosteum reflects mainly new bone formation, whereas remodeling of the bone surface in contact with the implant thread is thought to promote osseointegration. Therefore, the presence of ErhBMP-2 at appropriate concentrations will help to promote osseointegration.

CONCLUSION

Within the limits of study, absorbed ErhBMP-2 dose varies, with implant surface characteristics in turn influencing local bone formation/remodeling.

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