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Research Paper
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Effects of anodized implants coated with *Escherichia coli*-derived rhBMP-2 in beagle dogs

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Abstract. This study evaluated the effects of *Escherichia coli*-derived rhBMP-2 (ErhBMP-2) coated onto anodized implants to stimulate bone formation, osseointegration and vertical bone growth in a vertical bone defect model. Six young adult beagle dogs were used. After a 2-month bone healing period, anodized titanium implants (8 mm in length) were placed 5.5 mm into the mandibular alveolar ridge. Eighteen implants coated with ErhBMP-2 (BMP group) and another 18 uncoated implants (control group) were installed using a randomized split-mouth design. The implant stability quotient (ISQ) values were measured. Specimens were fabricated for histometric analysis to evaluate osseointegration and bone formation. The ISQ values at 8 weeks after implant placement were significantly higher in the BMP group than in the control group ($p < 0.05$). Histological observations showed that the changes in bucco-lingual alveolar bone levels were higher in the BMP group than in the control group ($p < 0.05$). The ErhBMP-2 coated anodized implants can stimulate bone formation and increase implant stability significantly on completely healed alveolar ridges in dogs. Further studies evaluating the effects of ErhBMP-2 on osseointegration in the bone–implant interface are warranted.

Key words: rhBMP-2; coating; anodized implant; osseointegration; bone growth.

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Urist et al.[1] introduced the 'bone induction principle' which states that protein extracts from bone induce ectopic and orthotopic cartilage and bone formation. Recently, further attempts have been made to apply bio-activating elements like recombinant human bone morphogenetic protein (rhBMP-2), a growth factor, onto the implant surface. Only a few studies evaluating the effect of the rhBMP-2 on osseointegration and alveolar bone growth have been completed.[2,3] Wikesjö et al.[4,5] examined the clinical effect of rhBMP-2 around the implant immediately after tooth extraction as well as after considerable bone absorption. This study attempted to determine whether rhBMP-2 coating on an anodized implant is effective in promoting alveolar bone growth on a completely healed alveolar ridge.

BMP, a protein derived from a subgroup of the transforming growth factor β family, accelerates ossification by controlling the essential factors of the bone induction cascade, resulting in the proliferation of osteoblasts from mesenchymal stem cells and the biosynthesis of bone matrix.[6–11] Since BMP-2 possesses high osteoinductive capacity,[12] it has been considered as a prime candidate among

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growth factors for coating titanium implants. BMP-2 has proved to be beneficial and can be used in various medical treatments.[13] It has been reported that rhBMP-2, which is produced by the gene recombination technique, can exert an osteoinductive effect when coated on the implant surface.[2,3,5] In particular, Hall et al.[2] concluded that an osteoinductive effect, including bone contact with the implant surface, is an advantage of titanium porous oxide surfaces (anodized surfaces) yielding the most bone at a low rhBMP-2 dose. Leknes et al.[3] radiologically evaluated the clinical outcomes of implants coated with rhBMP-2 in terms of local bone formation when the implants were placed 5 mm into the alveolar ridge following extraction of the premolar teeth and reduction of the alveolar ridge.

Recently, rhBMPs have been produced by BMP gene-transfected mammalian cell (Chinese hamster ovary (CHO)) cultures,[14,15] and rhBMP-2 and BMP-7 (rhBMP-7/osteogenic protein-1 (OP-1)) are now commercially available for the treatment of bony defects.[16,17] One of the problems associated with the clinical application of CHO-cell-derived rhBMP-2 (CrhBMP-2) is its high cost due to the need for use of high doses for effective treatment. One possible way of solving this problem is to produce monomer rhBMPs from BMP-gene-transfected *Escherichia coli* (*E. coli*), which has a high efficiency of production and low cost. Bessho et al.[18] examined the bone-inducing ability of an *E. coli*-derived rhBMP-2 (ErhBMP-2) variant with an N-terminal sequence and compared it with CrhBMP-2. A quantitative analysis indicated that the activity of ErhBMP-2 was similar to that of CrhBMP-2. It is unclear whether the characteristics of ErhBMP-2 are appropriate for clinical application. In particular, the outcomes and effects of ErhBMP-2 on osseointegration have not yet been determined.

In a previous study by Wikesjö et al.,[19] bilateral, critical-size, supra-alveolar defects were created in the mandibular premolar region, which exposed a wide area of myeloid tissue resulting in high cell activity and easy flap manipulation. In a clinical situation, implants are installed at the completion of physiological healing after tooth extraction. There have been a few studies evaluating the effect of implants coated with ErhBMP-2 on bone augmentation in alveolar bones physiologically completely healed after tooth extraction.

This study aimed to evaluate the effects of *E. coli*-derived rhBMP-2 (ErhBMP-2) coated onto anodized implants to stimulate bone formation, osseointegration and vertical bone growth in a model of vertical bone defects, which are formed in physiologically completely healed alveolar bones after tooth extraction.

Materials and methods

36 implants (8.0 mm in length, 4.0 mm in diameter; Cowellmedi Co, Busan, Korea) were fabricated. All treated implants were made of pure titanium and were designed with microthreads on the upper part and broader threads on the lower part. The implant surface was treated by the anodizing method (Cowellmedi Co), and half of the implants were processed with the ErhBMP-2 coating agent (Cowellmedi Co.). To coat with ErhBMP-2, each implant was immersed 3 times in a protein solution (1.5 mg/ml concentration) up to the microthreads and freeze dried under sterile conditions (freeze drying at −40 °C, followed by vacuum drying at maximum 20 °C). The amount of ErhBMP-2 coated on the surface was about 20 μg.

The surface morphologies of anodized implants without ErhBMP-2 (control group) and anodized implants with ErhBMP-2 (BMP group) were investigated by a scanning electron microscope (SEM, S2300, Hitachi, Japan). The substrates were coated with gold using a sputter coater (Eiko IB, Japan). The SEM was operated at 15 kV.

In vitro ErhBMP-2 release study

Only the anodized surface was used for this in vivo study, but the authors wanted to determine the merits of anodized surfaces regarding release mechanics. The anodized surface coated with ErhBMP-2 was compared with a pristine titanium surface coated with ErhBMP-2 in a release study.

To evaluate the amount of ErhBMP-2 released from the pristine titanium surface coated with ErhBMP-2 and the anodized titanium surface coated with ErhBMP-2, each sample was immersed in 50 ml conical tube containing 1 ml of PBS buffer (pH 7.4) and gently shaken at 100 rpm at 37 °C. At predetermined time intervals of 1 h, 3 h, 6 h, 10 h, and 1, 3, 5, and 7 days, the supernatants were collected and replaced with fresh PBS. All samples were was stored at −20 °C until analysis. The amount of ErhBMP-2 released from the surface was evaluated with an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions using a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm. Cumulative release of ErhBMP-2 was determined as a percentage of initial loading concentration.

Experimental animals and surgery

The animal selection management and the surgical protocol were approved by the Ethics Committee on the Animal Experimentation of Chonnam National University (IACUC-YB-R-2010-10). Six 3-year-old beagle dogs, approximately 10–15 kg in weight, were used for the study and were allowed to acclimatize for 2 weeks. The animals were fed a soft diet and had free access to water.

During the first surgery, the premolars and molars of the upper and lower jaws were extracted. The dogs were premedicated with atropine sulfate (0.05 mg/kg intramuscular injection; Dai Han Pharm Co., Seoul, Korea) and anaesthesia was maintained using isoflurane (Choongwae Co., Seoul, Korea). 1 ml of a mixture of lidocaine (Yu-Han Co., Gunpo, Korea) and 1:100,000 epinephrine was infiltrated into the mucosa at the surgical sites. Each premolar and molar was separated into two pieces, the mesial and distal roots. Care was taken to preserve the buccal, lingual, and lateral walls of the alveolar socket. The two pieces were carefully extracted without causing any damage to the extraction site. The extraction site was sutured using 4-0 nylon (Mersilk, Ethicon Co., Livingston, UK) to enhance healing. The extraction sites were allowed to heal for 2 months.

The second surgery was performed 2 months after complete healing of the extraction socket. Local infiltration and general anaesthesia were performed in the same way as in the first surgery. Experimental implants were installed into the edentulous mandibular alveolar ridges 2 months after surgical extraction. All 36 anodized implants with ErhBMP-2 (BMP group) or without ErhBMP-2 (control group) were installed using the Cowellmedi (Busan, Korea) implant system. Three experimental implants were installed in the right and left edentulous mandibular alveolar ridge areas each using a split-mouth design. Treatments were randomized between the left and right jaw quadrants in all dogs. Then, 5.5 mm implants were placed within the reduced alveolar ridges to the depth of the reference notch, creating 2.5 mm, supra-alveolar, peri-implant defects (Fig. 1a). In order to ensure symmetrical placement of implants on both sides, the planned implant placement sites along the exposed

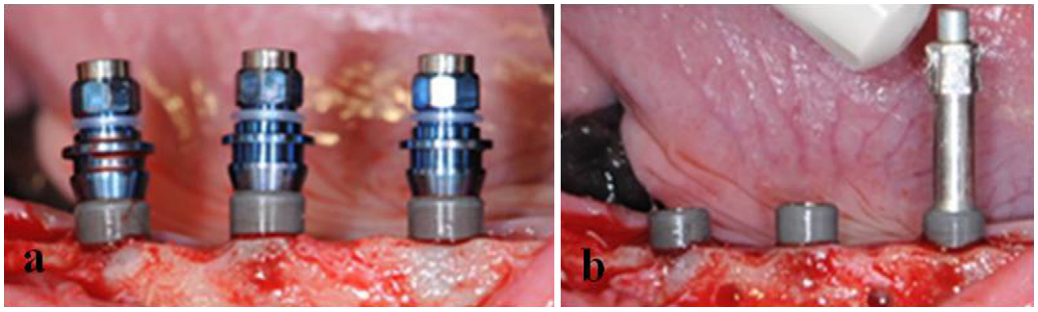

Fig. 1. (a) Alveolar bone was flattened without exposure of cancellous bone. 5.5 mm implants were placed within the reduced alveolar ridge to the level of the reference notch, creating 2.5 mm, supra-alveolar, peri-implant defects. (b) The ISQ was measured for each implant to evaluate stability at the time of implant placement.

bone were marked with a ruler. The implant stability quotient (ISQ) value was measured for each implant to evaluate stability at the time of implant placement (Fig. 1b). Each implant had a cover screw. The mucoperiosteal flaps were advanced, adapted and sutured, leaving the implants submerged.

Immediately after surgery, broad-spectrum antibiotics (penicillin G procaine and penicillin G benzathine) were administered and again after 48 h via an intramuscular injection (1 ml per 5 kg). Plaque control was maintained by daily flushing of the oral cavity with 2% chlorhexidine gluconate until completion of the study. The implant placement sites were examined every day to check for mucosal health, maintenance of suture line closure, oedema, and evidence of tissue necrosis or infection until suture removal. The sutures were removed 1 week after the placement of implants. The animals were given a soft diet for 2 weeks, followed by a conventional regular diet. At 8 weeks after surgery, the animals were anesthetized and killed by an intravenous injection of concentrated sodium pentobarbital (Euthasol, Delmarva Laboratories Inc., Midlothian, VA, USA). Following death, block sections including implants, alveolar bone and surrounding mucosa were collected.

Assessment of implant stability

In the implants placed in the mandible, the ISQ values were measured by Osstell Mentor® (Integration Diagnostics Ltd., Göteborg, Sweden) immediately after surgery and at 8 weeks after surgery. ISQ values were recorded 5 times for each implant, and the middle 3 values, excluding the minimum and maximum values, were used to calculate the mean and SD for the evaluation of changes in implant stability.

Histological analysis

The specimens were fixed in neutral buffered formalin (Sigma–Aldrich, St Louis, MO, USA) for 2 weeks and dehydrated in ascending concentrations of ethanol (70%, 80%, 90% and 100%). The dehydrated specimens were embedded in Technovit 7200 resin (Heraeus KULZER, South Bend, IN, USA). Each block of the polymerized specimen was sectioned longitudinally from the centre of each implant by an EXAKT diamond cutter (KULZER EXAKT 300, EXAKT, Norderstedt, Germany). The 30-μm final slides were prepared from the initial 400 μm slides by grinding the sections with an EXAKT grinding machine (KULZER EXAKT 400CS, EXAKT). The specimens were stained with Goldner's Trichrome. The images were captured using a computer connected light microscope (Olympus BX, Tokyo, Japan) attached to a CCD camera (Polaroid DMC2 digital Microscope camera; Polaroid Corporation, Cambridge, MA, USA). All measurements were made using SPOT Software V4.0 (Diagnostic Instruments Inc., Sterling Heights, MI, USA). The percentage of bone-to-implant contact (BIC, %) was measured, and the ratio of the area of bone formation on intra-threads of the implant to overall threads was calculated for the measurement of intra-thread bone density (ITBD, %). The height of newly formed marginal bone induced by the installed implant was measured. The overall specimen images were captured at a magnification of 12.5×. A magnification of 40× was used for histometric analysis, and a 100× magnification lens was used for a precise assessment of BIC and ITBD. The images captured at a magnification of 100× were analyzed histometrically.

Statistical analysis

All analyses were performed using computer-based statistical software (SPSS software, ver.12.0). The mean and SD of the ISQ values, BIC and ITBD were calculated for the histologic specimens in each group. Comparisons of the ISQ values between the BMP group and the control group were made using the Mann–Whitney *U*-test. Statistical analyses for BIC and ITBD were performed using ANOVA (SPSS Inc., Chicago, IL, USA). Post hoc comparison test analysis was used for determining the relationship between bone density changes over time and ErhBMP-2 application. Statistical significance was established at the 95% confidence level.

Results

Clinical observations

Healing was uneventful. In general, the implants remained submerged during the 8-week healing period, but in 1 dog most of the coronal aspects of the implants were exposed. The buccal side of 3 implants in the BMP group was also exposed due to failure of wound closure. One implant in the BMP group was not osseointegrated; this implant was excluded from the study. The three exposed implants in the BMP group did not show any evidence of osseointegration failure, but they were excluded from the study because they might be able to contribute much to the effect of ErhBMP-2.

Surface morphology

The surface morphologies of each group were confirmed via SEM. The SEM image (200×) showed that an ErhBMP-2 coating layer was formed between the threads in the BMP group (Fig. 2a and b).

In vitro ErhBMP-2 release study

As shown in Fig. 3, 54.4% of ErhBMP-2 and 36% of ErhBMP-2 was released from

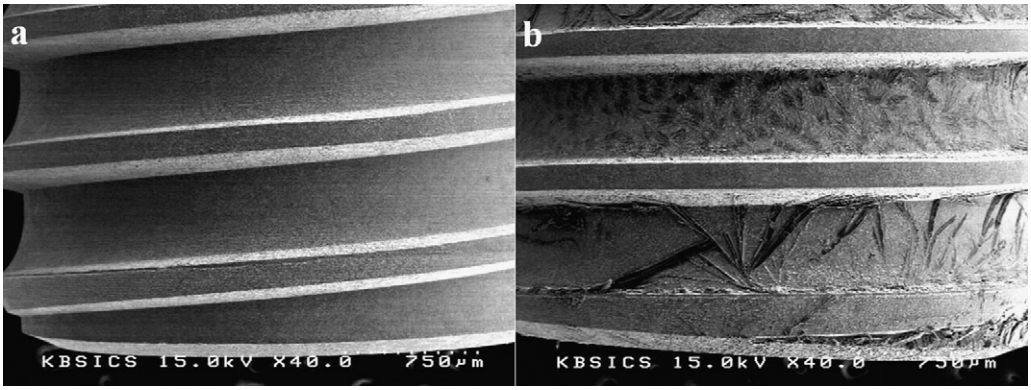

Fig. 2. SEM images of (a) control group and (b) BMP group.

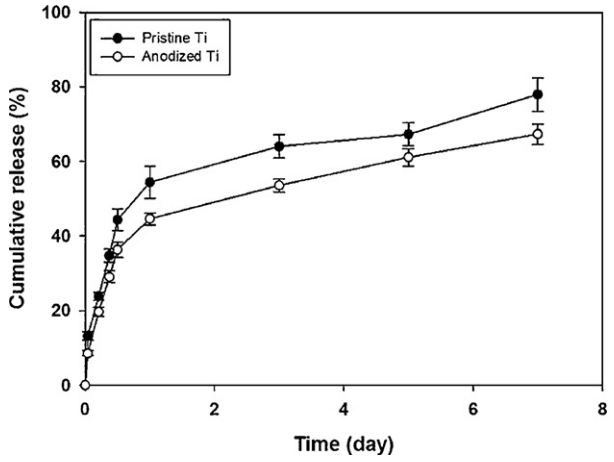

Fig. 3. Cumulative release ratio of coated ErhBMP-2 in pristine and anodized titanium surfaces.

a pristine titanium surface coated with ErhBMP-2 and an anodized titanium surface coated with ErhBMP-2, respectively, during the first day. Over the next week, 78% and 67% of ErhBMP-2 was released, respectively. These results indicate that ErhBMP-2 was released quickly from the pristine titanium surface compared with the anodized titanium surface.

Stability evaluation

Immediately after surgery, the overall ISQ value was higher in the control group than in the BMP group, but the difference was not statistically significant. In the BMP group, at week 8 after surgery, the ISQ values were relatively higher than the baseline value, whereas in the control group, at week 8 after surgery, the ISQ values were similar or relatively lower than the baseline values. The increase in the ISQ value was significantly higher in the BMP group than in the control group ($p < 0.05$) (Table 1).

Histological analysis

Jaw quadrants in the BMP group exhibited robust bone formation approaching the microthreads and bone remodelling around the implants (Figs. 4–6). In the histological observation of bucco-lingual alveolar bone levels, the mean bone level change was 0.82 ± 0.61 mm in the BMP group and -1.03 ± 0.83 mm in the control group. There was about 1.85 mm greater bone loss in the control group than in the BMP group ($p < 0.05$). The mean percentages (±SD) of BIC and ITBD within macrothreads at 8 weeks after surgery were $41.88 \pm 22.71\%$ and $59.12 \pm 31.02\%$, respectively, in the BMP group and $40.16 \pm 23.77\%$ and $57.78 \pm 32.79\%$, respectively, in the control group, but the differences were not statistically significant (Table 2).

Table 1. ISQ value according to groups and observation intervals.

	At surgery	At 8 weeks	ISQ change
Control group	74.00 ± 4.45	74.27 ± 6.67	0.27 ± 8.57
Experimental group	70.49 ± 7.91	$79.21 \pm 3.11^{*}$	$9.29 \pm 8.17^{*}$
p value	0.072	0.041	0.011

$^{*}p < 0.05$ compared with the control group.

Discussion

One problem with the clinical use of BMPs is their high cost of production because they are originally derived from mammalian CHO cells.[20] To solve this problem, a novel method was developed to produce rhBMP-2 derived from *E. coli* and to convert BMP monomers into biologically active dimmers (ErhBMP-2). ErhBMP-2 has an osteoinductive activity similar to that of CHO-derived rhBMP-2, in vivo and in vitro.[21–23] Only the results for ErhBMP-2-induced ectopic bone formation were included in these reports. The aim of the present study was to determine whether anodized implants coated with ErhBMP-2 could achieve better osseointegration and bone formation than non-coated implants in a completely cured alveolar model in dogs.

Son and Zhu[24] have reported the role of the oxidized layer in activating cells on a titanium surface, which has been widely used in clinical practice. Becker et al.[25] have demonstrated various applications of rhBMP-2 for modifying osseointegration. A rat ectopic model has been established to test the effects of rhBMP-2-coated titanium porous oxide implants on bone formation.[2,3]

When the oxidized layer of the implant is coated by genetically engineered rhBMP-2, it acts as an effective carrier for rhBMP-2.[2,5] A standard technique for the application of BMP on the implants is yet to be determined, and the effects of BMP-2 coated on an anodized surface are not clearly known. Owing to the short half-life of BMP, it becomes difficult to

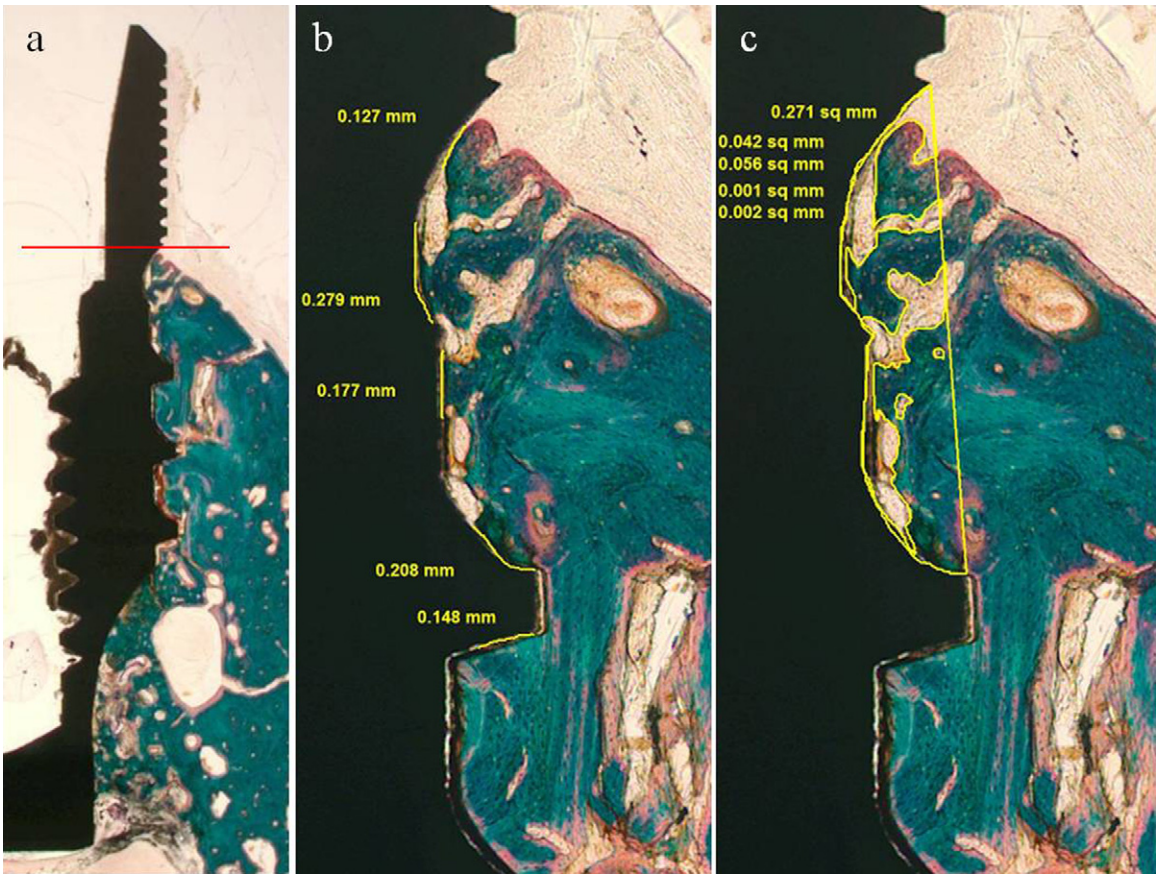

Fig. 4. Histological specimens of the control group. (a) There was no pronounced peri-implant bone re-modelling and vertical bone growth. (b, c) In existing bone area, BIC and ITBD had good quality but there was a small decline in marginal bone level. The red lines are the level of insertion. The specimens were stained with Goldner Trichrome. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

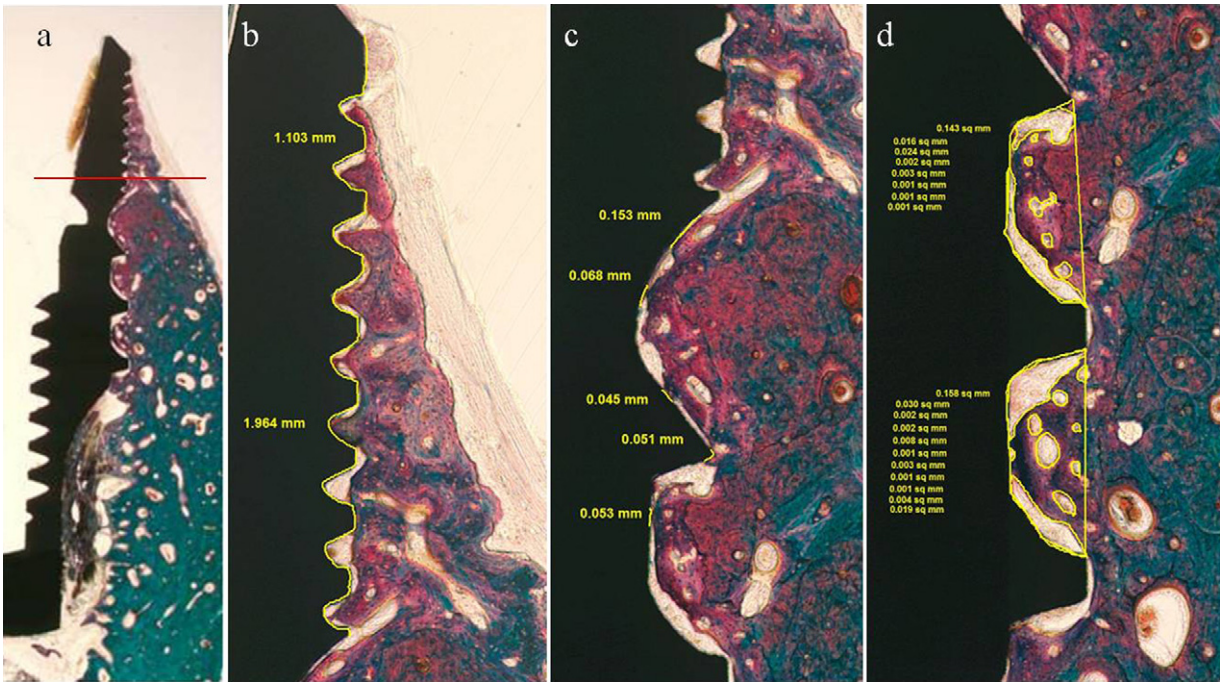

Fig. 5. Histological specimens of the BMP group. (a, b) Note pronounced peri-implant bone re-modelling and vertical bone growth in the BMP group. The red lines are the level of insertion. (c, d) In the existing bone area, marginal bone was well preserved. The specimens were stained with Goldner Trichrome. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

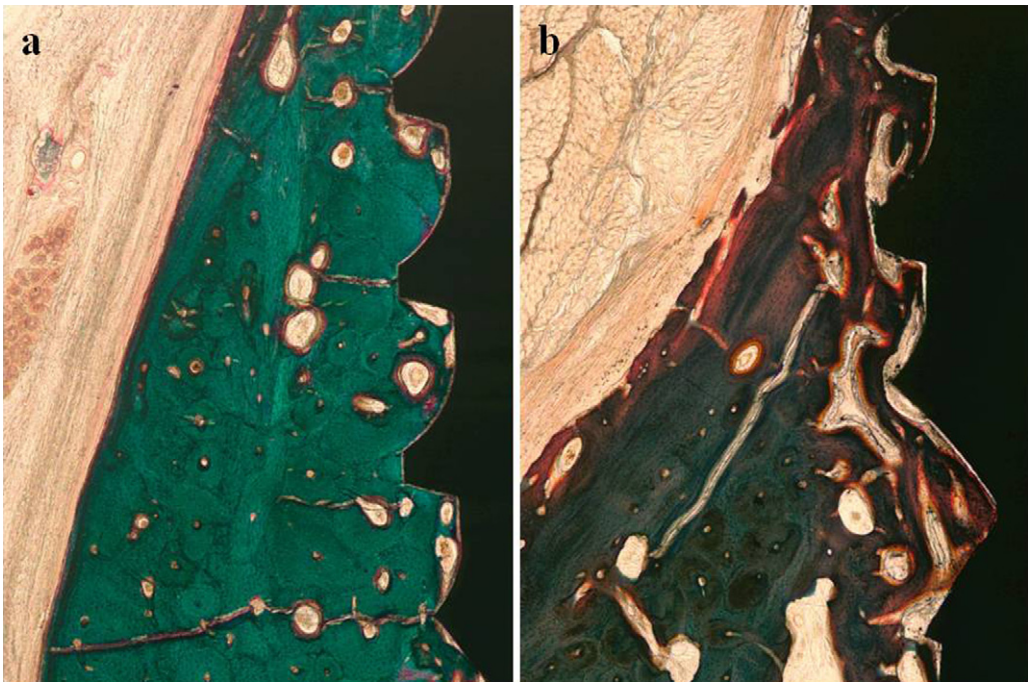

Fig. 6. New aspect of bone remodelling in the BMP group. (a) Implants in the control group and (b) ErhBMP-2 coated implants in the BMP group (40× magnification). Note pronounced peri-implant bone re-modelling actively in the BMP group.

use it in clinical practice, and continuous release of BMP from the implant surface cannot be expected.[26] In this study, ErhBMP-2 was coated onto the anodized surface that was likely to release ErhBMP-2 more slowly and it was thought that ErhBMP-2 could coat the anodized surface more effectively than the machined surface due to its porous structure, although, it could not induce slow release of ErhBMP-2. In addition, by using the freeze–drying process, the delivery and storage of ErhBMP-2 improved. In similar studies conducted previously,[3,4] implants were pretreated with the BMP solution before their installation.

Compared to the previous method, the method used in this study is likely to be more practical. In addition, the in vitro ErhBMP-2 release experiment conducted in this study confirmed that ErhBMP-2 was released more slowly from the anodized surface than from the machined surface. Although a large amount of ErhBMP-2 was released during the first day, approximately 67% of the ErhBMP-2 was found to be released over 1 week, which showed that a significant amount of ErhBMP-2 remained on the anodized surface even after its early release.

Leknes et al.[3] reported the effects of 3 different concentrations of rhBMP-2 on bone formation. They observed adequate bone formation at concentrations of 0.75 mg/ml and 1.5 mg/ml and robust bone formation as well as implant dislocation and multi-lobular radiolucent voids at a concentration of 3 mg/ml. Based on these results, in the present study the implants coated with ErhBMP-2 at 1.5 mg/ml were used. It is uncertain whether this concentration of ErhBMP-2 would be effective for bone formation. Further studies are needed to determine the ideal concentration of ErhBMP-2.

In the present study, the ISQ values in the BMP and control groups were similar immediately after surgery, but 8 weeks later the ISQ values were significantly higher in the BMP group than in the control group ($p < 0.05$). The higher ISQ values in the BMP group may be due to newly formed bone at the peri-implant area. At 8 weeks after surgery, implant stability was higher in the BMP group than in the control group ($p < 0.05$). Implant stability was lower in the BMP group immediately after surgery but it was much higher at 8 weeks after surgery. This could be because of the effects of ErhBMP-2. ErhBMP-2 activated osteoclasts on bone healing, resulting in stronger bone formation at 8 weeks after surgery. The similar ISQ values between the BMP and control groups immediately after surgery may be due to compression on the cortical bone, but the stability may have decreased over time due to the remodelling process in the cortical bone.

Histological examination showed that in the BMP group implants were integrated with the remaining alveolar bone with limited new bone formation. Eight weeks after surgery, the mean value of regeneration of bucco-lingual alveolar bone was 0.82 ± 0.61 mm in the BMP group, whereas the mean bone loss was -1.03 ± 0.83 mm in the control group ($p < 0.05$). There was a significant difference of 1.85 mm in the mean bucco-lingual alveolar bone level between the two groups. BIC and ITBD values were similar between the BMP and control groups, which corresponds well with the results reported by Wikesjö et al.[5] They also stated that these results were due to bone remodelling. They demonstrated clinically significant bone formation (height and area) in implants

Table 2. Bucco-lingual bone level, bone to implant contact ratio (BIC) on macrothread, and bone density on macrothreads 8 weeks after surgery.

	Bone level	BIC	Intra-thread bone density
Control group	-1.03 ± 0.83	40.16 ± 23.77	57.78 ± 32.79
Experimental group	$0.82 \pm 0.61^{*}$	41.88 ± 22.71	59.12 ± 31.02
p value	0.033	0.102	0.095

$^{*}p < 0.05$.

coated with rhBMP-2 and proved the effects of rhBMP-2 on bone formation. In their study, both the height and area were greater in rhBMP-2-coated implants than in controls, but bone quality significantly differed according to the different rhBMP-2 concentrations. Since the studies performed by Sigurdsson et al.[27] evaluating the concentration of rhBMP-2 for bone formation in dogs, more studies have been conducted to determine the ideal concentration of rhBMP-2 for bone formation. Kato et al.[28] demonstrated that higher concentrations of BMP led to better and faster bone formation. Tatakis et al.[29] reported that there were no significant differences in bone formation with rhBMP-2 at concentrations of 0.05, 0.1, and 0.2 mg/ml. On the other hand, rhBMP-2 concentrations of 0.75 and 1.5 mg/ml have been shown to be safe for implant coating.[19] The osteogenic potential of rhBMP-2-coated implants appeared to be significantly higher ($p < 0.05$), and these implants induced a limited amount of bucco-lingual bone formation or, at least, prevented bone loss.[19] In the present study, one implant in the BMP group failed to osseointegrate. The failed implant showed slow bone absorption during the study period. This osseointegration failure may be attributed to surgical trauma, bacterial infection or implant surface characteristics as reported by Esposito et al.[30] Further histological studies are needed to confirm the definite cause of osseointegration failure of the implant.

The present study has some limitations regarding the ErhBMP-2 concentrations and the ErhBMP-2-release system. Numerous articles have been published recently on the significance of rhBMP-2 concentrations. The results of this study warrant further investigation using ErhBMP-2-coated anodized implants for evaluating outcomes when various concentrations of ErhBMP-2 are applied. Further studies are also needed to determine the ideal healing period required for achieving implant stability.

In conclusion, the results of the present study suggest that ErhBMP-2 coated anodized implants can stimulate bone formation and increase implant stability significantly on completely healed alveolar ridges in dogs. Further studies evaluating the effects of ErhBMP-2 on osseointegration in the bone-implant interface are warranted.

Funding

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Competing interests

None declared.

Ethical approval

The Ethics Committee on Animal Experimentation of Chun Nam University, Kwangju, Republic of Korea (Approval number: CNU IACUC-YB-2010-10).

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