Research Article

Effects of fibrin-binding oligopeptide on osteopromotion in rabbit calvarial defects

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Purpose: Fibronectin (FN) has been shown to stimulate bone regeneration in animal models. The aim of this study was to evaluate the capacity of bovine bone mineral coated with synthetic oligopeptides to enhance bone regeneration in rabbit calvarial defects.

Methods: Oligopeptides including fibrin-binding sequences of FN repeats were synthesized on the basis of primary and tertiary human plasma FN structures. Peptide coated and uncoated bone minerals were implanted into 10 mm calvarial defects in New Zealand white rabbits, and the animals were sacrificed at 4 or 8 weeks after surgery. After specimens were prepared, histologic examination and histomorphometric analysis were performed.

Results: At 4 weeks after surgery, the uncoated groups showed a limited amount of osteoid formation at the periphery of the defect and the oligopeptide coated groups showed more osteoid formation and new bone formation in the center of the defect as well as at the periphery. At 8 weeks, both sites showed increased new bone formation. However, the difference between the two sites had reduced.

Conclusions: Fibrin-binding synthetic oligopeptide derived from FN on deproteinized bovine bone enhanced new bone formation in rabbit calvarial defects at the early healing stage. This result suggests that these oligopeptides can be beneficial in reconstructing oral and maxillofacial deformities or in regenerating osseous bone defects.

Keywords: Fibrin, Fibronectins, Oligopeptides.

INTRODUCTION

Various graft materials have been widely used in the reconstruction of maxillofacial deformities and alveolar bone defects. Ideal grafts should promote the repair by ingrowth of new reparative tissue and should allow the delivery of biological factors to stimulate bone regeneration. Such substitutes should also provide physical strength and protection of the underlying structures during healing and should prevent the ingrowth of soft tissue.

Although autogenous bone is regarded as the gold standard in osseous reconstruction procedures, donor site morbidity, complications, hospital stays, cost, unpredictable graft resorption, and limited volume availability have been considered disadvantages [1-4].

Allografts, xenografts, and synthetic materials have been shown to promote osseous defect filling in humans [5]. Xenografts consist of bone mineral from animals or bone-like minerals derived from corals or algae [6], and deproteinized bovine bone (DBB) has a long history of successful use in filling in bone defects and maxillary sinus floor grafting [7-12]. As proteins in bovine bone have been extracted to avoid immu-

nologic rejection, DBB acts just as an osteoconductive scaffold. However, using an osteoconductive grafting material may prolong the healing period, which may cause clinical shortcomings. Therefore, reliable bone regeneration requires the additional application of suitable bioactive substances to the surface of the grafted material in order to enhance osteoblast differentiation and proliferation.

In order to accelerate new bone formation, there have been many attempts to combine DBB with proteins or cytokines such as recombinant human bone morphogenic protein-2 and growth factors [13,14]. However, usage of whole natural protein imposes many disadvantages, including instability from enzymatic degradation, difficulties in obtaining stable attachment, nonspecific binding to other types of cells, immunogenecity, reduced material availability, relatively high cost, and sterilization problems [15,16]. Therefore, synthetic oligopeptides, which contain the active binding sequences of the natural protein, are regarded to be attractive alternatives, due to their ease of synthesis, handling convenience, and the reduced immunogenicity.

Fibronectin (FN), a major component of extracellular matrix (ECM), mediates a wide variety of cellular interactions with the ECM and plays important roles in cell adhesion, migration, growth, and differentiation [17]. It is a heterodimeric glycoprotein and interacts with cells and transmits signals through many receptors. FN has a remarkably wide variety of functional activities besides binding to cell surfaces through integrins. It binds to a number of biologically important molecules that include heparin, collagen/gelatin, and fibrin, and these interactions are mediated by several distinct structural and functional domains.

The FN subunit contains two fibrin binding sites. Among these two sites, the major site close to the amino terminus serves as a transglutamination site for activated factor XIII, crosslinking FN to various other proteins including fibrin, fibrinogen, and other FN [18]. The N-terminal site binds first due to its higher rate/affinity of fibrin binding, followed by the C-terminal site, which may then strengthen the fibrin-binding interactions of FN. Interaction of FN with fibrin mediated by factor XIII transglutaminase is thought to be important for cell adhesion or cell migration into fibrin clots. After tissue injury, a blood clot formation serves the dual role of restoring vascular integrity and serving as a temporary scaffold for the wound healing process. Fibrin and plasma FN, the major protein component of blood clots, are vascular essentials to these functions [19]. In blood clotting systems, after fibrin deposition, plasma FN-fibrin matrix is covalently crosslinked, and then it promotes fibroblast adhesion, spreading, and migration into the clot [20,21]. Fibroblast migration leads to deposition of cellular FN, which serves as a temporary scaffold for further recruitment of cells and granulation tissue formation in the wound healing process [22]. Therefore, crosslinked FN is an absolute requirement for the wound healing process. FN can also be cross-linked to collagen, a reaction that is potentially important for collagen fibrillogenesis [23].

In this study, we used DBB coated with synthetic oligopeptides including fibrin binding sites from amino-terminal FN fragments to evaluate the osteopromotive effect of this peptide in rabbit calvarial defects.

MATERIALS AND METHODS

Preparation of peptide-coated deproteinized bovine bone

Oligopeptides including fibrin-binding sequences of FN repeats were designed on the basis of primary and tertiary human plasma FN structures (FINC-HUMAN: P02751 [24]). A solid-phase peptide synthesizing system was used to prepare these oligopeptides. The amino acid sequence was as follows: CTSRNRCNDQ.

Deproteinized cancellous bovine bone particles (OCS-B, NI-BEC, Seoul, Korea) ranging from 0.2 to 1.0 mm were soaked in 3.1 mM synthetic peptide in distilled water. They were kept for 18 hours at room temperature after gentle shaking to ensure equilibration of the peptide with all exposed surfaces of the microporous bone. After freeze drying, the bone particles were sterilized by γ-radiation.

Surgical procedures

Twelve New Zealand white male rabbits weighing 2.1 to 2.5 kg were used. Two circular defects were made in the parietal bone of each rabbit. The surgical procedures for this investigation were performed according to recognized techniques approved by the Institute of Laboratory Animal Resources, Seoul National University. Each animal was premedicated according to their weight with an intramuscular injection of tiletamine/zolazepam (0.2 mg/kg body weight) and xylazine (5 mg/kg body weight). After the surgical site was disinfected with betadine, local anesthesia was provided using a 2% lidocaine solution. An incision was made along the midline of the scalp from a point midway between the bases of the ears to approximately 5 cm anteriorly through the full thickness of the skin. Sharp subperiosteal dissection reflected the pericranium from the outer table of the cranial vault exposing the parietal bones. An electric drill with an 8 mm inner diameter trephine bur was used to create bilateral full thickness calvarial defects. The defect sizes that resulted ranged from 9.5 to 10 mm. The peptide coated bone mineral was implanted on one side, and uncoated bone mineral was implanted on the other side served as a control (Fig. 1). Care was taken to prevent displacement of the bone particles into the other defect
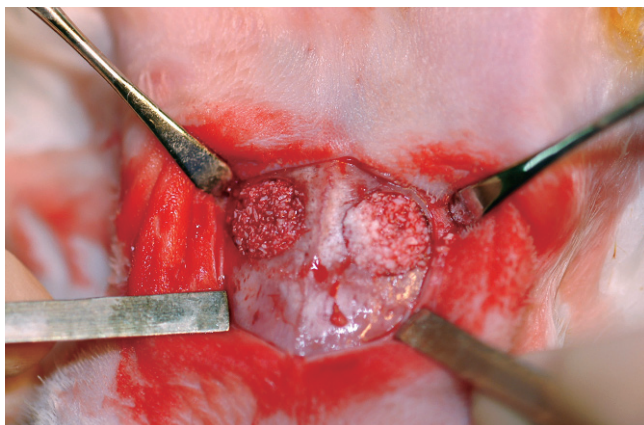
Figure 1. Bilateral calvarial defects were created in parietal bones.

to prevent a spill-over effect. The pericranium and skin were closed with 5-0 chromic gut and 4-0 silk sutures, respectively. Six of the animals were euthanized at 4 weeks, and the others at 8 weeks. No specimen showed any evidence of infection or foreign body reaction, and all wounds healed unevenfully.

Histologic analysis

Each animal was euthanized at 4 or 8 weeks. The cranial vault was carefully removed from each animal and embedded in the media (Technovit 7200, Exakt, Hamburg, Germany) after 10% neutral buffered formalin treatment with a series of ethanol solutions of increasing concentrations. The embedded specimens were mounted on acrylic glass slabs and sections were cut from the middle of each specimen. The sections were grounded and polished to a final thickness of 40 μm. The thin sections were stained with hematoxylin-eosin for quantification of the amount of bone regeneration under light microscopy.

Histomorphometric analysis

Computer-assisted histomorphometric analysis was done after conventional microscopic examination. Measurements of newly formed bone and graft particles were obtained using a software package (TDI Scope Eye, Techsan, Seoul, Korea) coupled with a digital camera on a light microscope (BH-2, Olympus Optical, Osaka, Japan). The proportion of new bone and DBB particles over the entire surface was measured. The total perimeter of DBB particles and the portion of the perimeter of DBB particles in contact with bone tissue were also measured, and the degree (%) of new bone-DBB contact was calculated. Two sections were obtained from each biopsy and visual fields from each biopsy were measured.

Statistical analysis

Data were reported as mean±SD with a significance level
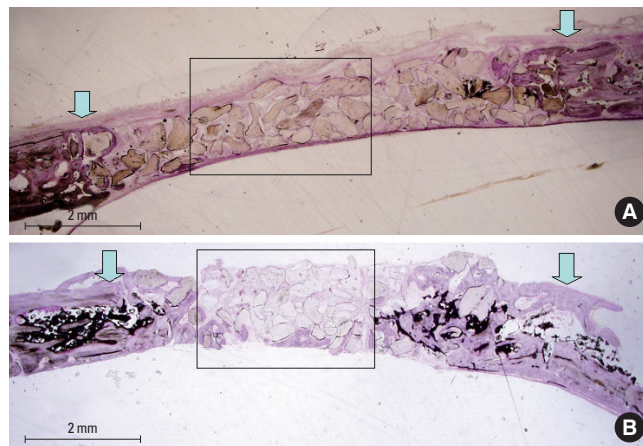
Figure 2. (A) Histologic view of uncoated bone group after 4 weeks. Newly formed bone was found at the periphery of the defect; however, there was little, if any, new bone formation in the central part of the defect. Arrows indicate the margin of the bone defect. (B) Histologic view of the peptide coated bone group after 4 weeks. New bone formation was obvious and observed in the central part of the defect. Arrows indicate the margin of the bone defect (H&E staining).
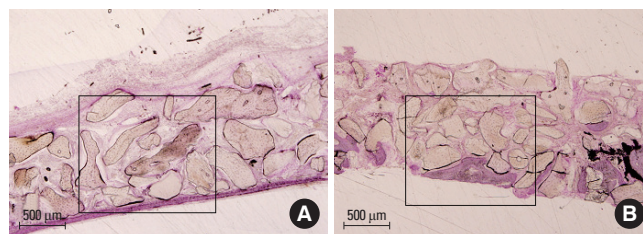
Figure 3. (A) Histologic view of the uncoated bone group at 4 weeks. Higher magnification of the area marked in Fig. 2A. A very limited amount of bone formation around the graft material is noted. (B) Histologic view of the coated bone group at 4 weeks. Higher magnification of the area marked in Fig. 2B. New bone formation was observed in the central area of the defect (H&E staining).

of $P<0.05$. A Mann-Whitney U test was used to compare data from histomorphometric analyses.

RESULTS

Histologic evaluation

At 4 weeks after surgery, in the sites with uncoated bone, the defect area was primary occupied by the graft material and the connective tissue (Fig. 2A). Few immature osteoid formations were observed in the central part of the defect and newly formed bone was near the defect border (Figs. 3A and 4A). On the other hand, the new bone formation in the site with peptide coated bone was more enhanced than that of the control sites (Fig. 2B). Most of the specimens showed new bone formation extending to the central part of the defect (Fig. 3B). Evidence of osteoid formation adjacent to the graft
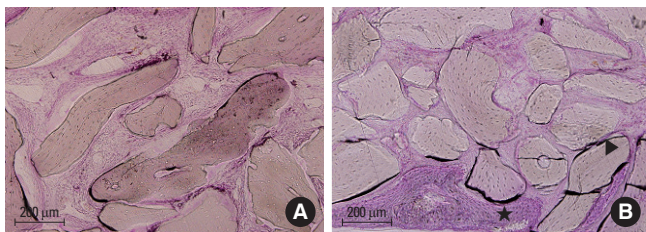
Figure 4. (A) Higher magnification of Fig. 3A. A very limited amount of immature osteoid formation around the graft material was noted. (B) Higher magnification of the area marked in Fig 3B. Osteoid formation was noted around the graft material (▶). The newly generated bone seems well integrated with the graft material (★)(H&E staining).
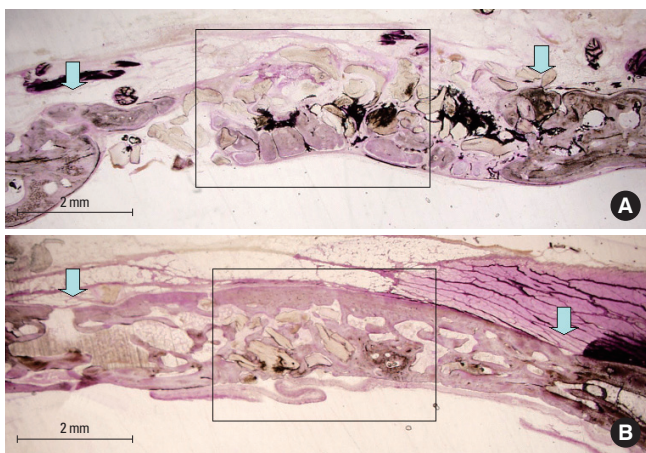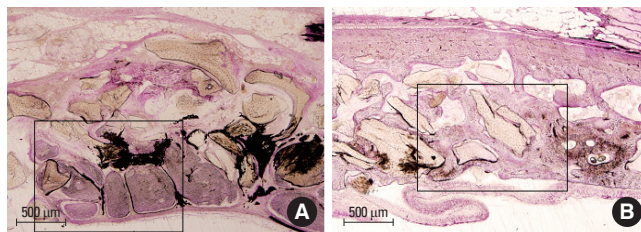

Figure 5. (A) Histologic view of the uncoated bone group after 8 weeks. New bone formation was noted throughout the defect. Arrows indicate the margin of the bone defect. (B) Histologic view of the peptide coated bone group after 8 weeks. New bone formation was obvious, and the defect tended to coalesce with new bone. Arrows indicate the margin of the bone defect (H&E staining).

Figure 6. (A) Histologic view of the uncoated bone group at 8 weeks. Higher magnification of the area marked in Fig. 5A. New bone formation was observed in the central area of the defect. (B) Histologic view of the coated bone group at 8 weeks. Higher magnification of the area marked in Fig. 5B. New bone formation was observed evenly throughout the defect with highly cellular marrow spaces, osteoids, and osteoblsts (H&E staining).

Figure 7. (A) Higher magnification of Fig. 6A. Multiple bony islands fill the central part of the defect (⇒). (B) Higher magnification of Fig. 6B. The grafted bone is integrated fully with the new bone (◀) and shows a more advanced stage of remodeling and consolidation (▶)(H&E staining).

Table 1. The ratio of the area of the grafted particles to the original defect (mean±SD).

Group	4 weeks (%)	8 weeks (%)
Uncoated bone	33.40±3.19	28.06±6.16
Peptide coated bone	30.37±5.66	27.63±5.49

There was no significant difference between the groups ($P>0.05$).

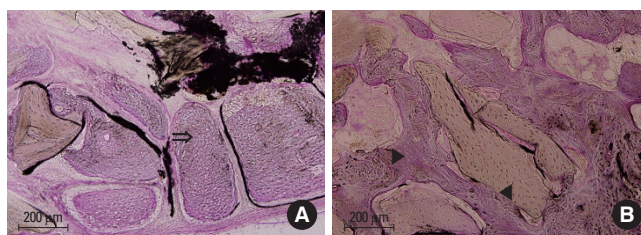material was found and coalescence of the new bone with graft material was observed (Fig. 4B).

At 8 weeks, the amount of the new bone was greater than that observed at 4 weeks in both groups. New bone was deposited evenly around the graft material and extended to the central area of the defects (Fig. 5). The bone mineral was integrated with the new bone and newly formed bones had connected to each other (Fig. 6), especially in the peptide coated groups. In higher magnification, multiple bony islands filled the central part of the defect (Fig. 7A) and connected each other. In the peptide coated group, new bone showed a more mature pattern (Fig. 7B). The particles of DBB were easily identified. Neither resorption lacunae nor active osteoclasts were found in specimens at 4 and 8 weeks.

Histomorphometric evaluation

The average areas occupied by grafted materials are shown in Table 1. There was no significant difference between the groups at 4 and 8 weeks ($P>0.05$). The average areas occupied by new bone was 15.29±1.93% for the control group and 18.84±1.75% for the experimental group at 4 weeks. At 8 weeks, the average areas were 18.14±2.81% and 19.00±2.08%, respectively (Table 2, Fig. 8). There was a significant difference in the regeneration areas between the control and experimental group at 4 weeks ($P<0.05$). At 8 weeks, no statistical difference was observed ($P>0.05$).

The average bone-DBB contact ratios were 14.50±3.53% for the control group and 19.33±3.37% for the experimental group at 4 weeks. At 8 weeks, the average contact ratios were 17.36±3.54% and 19.03±2.79%, respectively (Table 3, Fig. 9). While there was a significant difference in the contact ratios between control and experimental groups at 4 weeks ($P<0.05$), but no statistical difference in 8 weeks ($P>0.05$).

Table 2. The ratio of the area of newly formed bone to the original defect (mean±SD).

Group	4 weeks (%)	8 weeks (%)
Uncoated bone	15.29±1.93	18.14±2.81[b]
Peptide coated bone	18.84±1.75[a]	19.00±2.08

[a]There was a significant difference between the peptide coated bone group and uncoated bone group after 4 weeks (P<0.05).
[b]There was a significant difference between the 4-week and 8-week peptide-coated mineral groups (P<0.05).
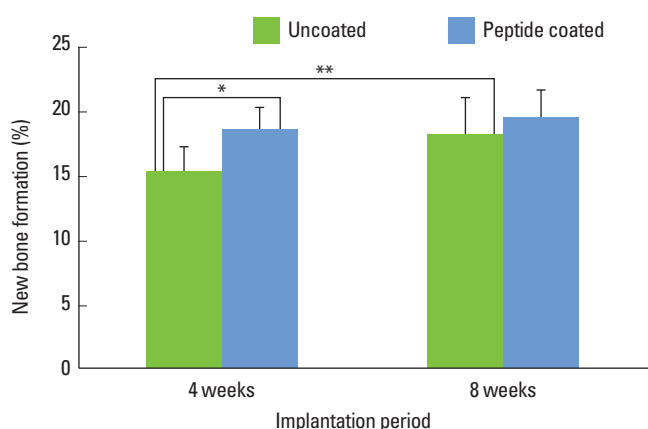
Figure 8. Percentages of newly formed bone after implantation for different periods using histomorphometric analysis. *There was a significant difference between the peptide coated bone group and uncoated bone group at 4 weeks (P<0.05). **There was a significant difference between the 4-week and 8-week uncoated bone group (P<0.05).

DISCUSSION

In this study, fibrin-binding synthetic oligopeptide derived from FN was used to coat the DBB, and the effect was evaluated. Histologic observation showed that no adverse response was induced by graft bone, with or without peptide. At 4 weeks after surgery, the uncoated bone groups showed evidence of osteoid formation near the defect periphery area; however, at the center area, osteoid formation was not observed. On the other hand, in the coated bone groups, new bone formation was observed in the defect center as well as the defect periphery. Among the 4-week models, new bone formation was more evident in the coated bone group than in the uncoated group. After 8 weeks, new bone formation was observed throughout the defect area in both cases. Newly formed bones around the DBB were connected to each other and showed a more advanced stage of remodeling and maturation. In the histomorphometric analysis, the percentage gain of bone regeneration was calculated as the ratio of the area of newly formed bone to that of the original defects. A significant finding in this study is the accelerated formation of new bone

Table 3. The degree of bone-DBB contact (mean±SD).

Group	4 weeks (%)	8 weeks (%)
Uncoated bone	14.50±3.53	17.36±3.54
Peptide coated bone	19.33±3.37[a]	19.03±2.79

DBB: deproteinized bovine bone.
[a]There was a significant difference between the peptide coated bone group and uncoated bone group after 4 weeks (P<0.05).
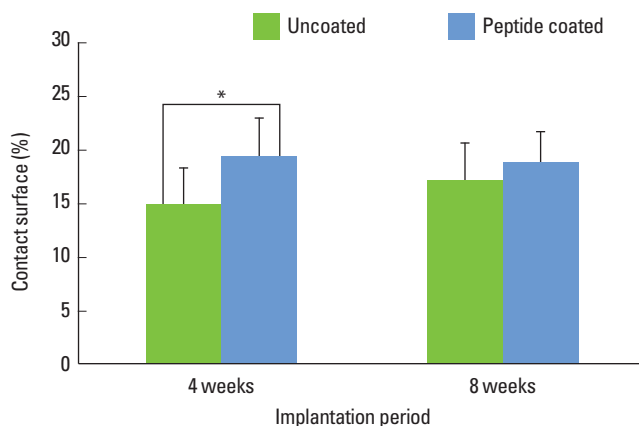
Figure 9. Percentages of newly formed bone-deproteinized bovine bone contact after implantation for different periods using histomorphometric analysis. *There was a significant difference between the peptide coated bone group and uncoated bone group after 4 weeks (P<0.05).

observed within the experimental groups at 4 weeks. In the 4-week model, new bond formation in the coated group was 18.84±1.75% compared with 15.29±1.93% in the uncoated group, and there was a statistical difference between the two cases (P<0.05). By 8 weeks, the difference had decreased and a statistically significant difference was not observed. The new bone ratio in the control group was 18.14±2.81% and in the experimental group was 19.00±2.08%. The ratio of the contact surface showed a similar pattern. In the 4-week models, the contact surface ratio was 14.50±3.53% and 19.33±3.37%, and in the 8-week models the ratio was 17.36±3.54% and 19.03±2.79%, for the control and experimental groups, respectively. There was no statistically significant difference in the 8-week models (P>0.05). Okazaki et al. [25] found that DBB grafted in rabbit calvarial defects showed gradual increases in the proportion of newly formed bone at 4 and 8 weeks and the proportion of newly formed bone-graft particle contact length showed significant increases at 4 weeks and no significant changes at 8 weeks, which are consistent with our finding that fibrin-binding synthetic oligopeptide derived from FN coated on demineralized bovine bone enhanced new bone formation in rabbit calvarial defects, especially in the early healing stage.

FN is a major glycoprotein in the ECM, which binds to in-

tegrins and regulates cell survival, growth, motility, and differentiation. Many attempts have been made to promote bone regeneration using FN; however, the results have been controversial. In 2001, Camargo et al. [26] evaluated the ability of FN to augment the regenerative effects of a bovine-derived bone in human periodontal defects. They found that a significant difference between the regenerative treatment modalities could not be demonstrated. On the other hand, Lekovic et al. [27] used bovine porous bone mineral in combination with an autologous fibrinogen/FN system in the treatment of intrabony periodontal defects in humans. They found that the autologous fibrin/fibrinogen system used in combination with bovine porous bone mineral has some effect in promoting probing depth reduction, clinical attachment gain, and defect fill when employed as regenerative therapy for intraosseous lesions in humans. Di Bella et al. [28] reported that surface treatment with FN promotes bone formation within the polylactic acid scaffold in a critical-sized defect rabbit model with or without autologous adipose-derived mesenchymal stem cells in combination. In 2008, Schonmeyr et al. [29] found in vitro attachment and proliferation of bone-forming cells on hydroxyapatite is significantly increased by pretreatment with FN/fetal calf serum, but this difference is less profound and not significant *in vivo*. This controversy could be due to the common shortcomings of whole protein usage, including instability to enzymatic degradation, unexpected effects of other domains, nonspecific binding to other types of cells, difficulty in obtaining stable attachment, and even integrin blocking effects [15,16]. Therefore, small synthetic peptides with active binding sequences of natural proteins have been proposed as alternatives. In our previous study, fibrin-binding synthetic oligopeptide showed marked enhancement in periodontal ligament cell proliferation, attachment, and mineralization [30]. These results can be compared with those obtained from previous studies conducted by other researchers using similar animal models. Augmentation of the maxillary sinus using DBB with or without recombinant human bone morphogenic protein (rhBMP)-7 was performed by Terhyeden et al. [31] using DBB in minipigs. A significant increase in bone formation was found (38.6-80.0%) when rhBMP-7 was added to the graft. In 2007, Park et al. [32] demonstrated that the use of DBB with a synthetic rhBMP-7 seems to be a more beneficial material for bone regeneration in the early healing period. They detected a significant difference in the percentage of new bone formation between peptide-coated group and peptide-uncoated group: 6.60±0.89% and 23.72±1.73% at 2 weeks, 34.77±3.31% and 42.27±2.35% at 4 weeks, respectively. On the contrary, Gordjestani et al. [33] investigated the influence of osteopontin on bone repair in rabbit calvarial defects. There were no statistically significant differences in total bone formation between defects filled with osteopontin-hydroxyapatite and those with hydroxyapatite only. The differences identified in the amount of reparative tissue seen in our study and previous other studies can be explained by the biomaterials used for each study. Compared with other studies, our study observed a lower percentage of new bone gain, but fibrin-binding synthetic oligopeptide derived from FN might have some effect as a DBB coating material, especially in the early bone healing stage.

The creation of nonunions in animals within the calvaria and mandible was size dependent. A critical size defect (CSD) was defined as the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal [34]. It was known that the size of a CSD was dependent on the breed and age of the experimental animal model used as well as the location of the defect. Frame [35] described the healing of 15-mm diameter defects in the calvaria of rabbits. After 24 and 36 weeks, the 15 mm diameter defects had healed by the formation of fibrous connective tissue. Also, Hollinger and Kleinschmidt [36] confirmed that untreated 15-mm cranial defects created in New Zealand white rabbits showed less than 10% bone regeneration after 48 weeks. However, Ascherman et al. [37] used 10-mm diameter defects as a CSD in their study of the 6-month histology of a quick-setting hydroxyapatite cement, with and without absorbable plates. In 2007, Pripatnanont et al. [38] used a bicortical 10×10 mm skull defect model in their study examining the osteoconductive ability of 3 bovine hydroxyapatites. After 8 weeks, the controls showed bone formation around the marginal bone, showing projection in a centripetal direction. In the upper and lower parts of the defect, woven bone was projected from the edges of the defects, whereas in the central part, only fibrous connective tissue occupied the defect.

Resorption and complete remodeling into new bone are the ideal outcomes of a grafting material. In this study, the ratio of grafted DBB did not change much according to the type of grafted material or healing period. The resorption of DBB has been a controversial subject in many studies. McAllister et al. [39] detected a decrease in the percentage of DBB, from 19±14% to 6±3% over a time period of 7.5-18 months in a chimpanzee study. Klinge et al. [40] found that DBB particles in defects of rabbit calvarial skulls had almost completely disappeared after 14 weeks of healing, as the effect of multinucleated cells. Based on these results, they suggested that DBB had been resorbed and replaced by vital bone. On the other hand, Schlegel [41] could identify DBB particles after a healing period of up to 7 years and Skoglund et al. [42] also suggested that DBB particles were slowly degraded and remained 44 months after augmentation of a maxillary alveolar

ridge. Yildrim et al. [43] suggested that such a difference in bony healing of DBB is mainly influenced by the healing response of the individual patient and is less dependent on the healing time of the augmentation material. From a biologic point of view, resorption requires adhesion molecules (arginine-glycine-aspartic acid [RGD] sequence) for the attachment of osteoclastic cells to plasma and ECM, including FN, fibrinogen, vitronectin, type I collagen, osteopontin, and bone sialoprotein [44]. As DBB is free of proteins, osteoclastic resorption can probably not occur. However, Schwartz et al. [12] observed transforming growth factor-β and BMP-2 in DBB particles, which might explain the lacunae found in some DBB particles. Rooney et al. [45] interpreted possible signs of resorption as evidence of osteoclastic resorption mediated by later surface adsorption of proteins containing RGD sequences, such as FN or fibrinogen. In this study, we used fibrin binding sequences of FN as DBB coating material; therefore, the RGD sequence of FN is not included and results of the initial healing stage, that is, post-surgery 2 weeks, were not included. Further investigation about DBB resorption is needed.

In this study, the peptide mentioned above was used to coat DBB, and the effects on bone regeneration were evaluated. The study was done at 4-week and 8-week periods to evaluate bone formation in the early and late healing period. Histologic observation showed that no adverse response was induced by the graft, with or without peptide. The amount of new bone at sites with peptide-coated bone mineral was greater than at sites with uncoated bone. The new bone was deposited evenly around the graft material, and the bone mineral was integrated fully with the new bone in experimental sites. By histomorphometric analysis, the percentage gain in bone regeneration was calculated as the ratio of the area of newly formed bone to the area of the original defect. A significant finding in this study is the accelerated formation of new bone observed at experimental sites compared to control sites at the early healing stage. However, more studies are required to determine the quantity of peptide suitable for optimal bone regeneration and DBB coating method.

These results suggest that the formation of new bone and the normal healing process occur more rapidly when synthetic oligopeptide is used.

CONFLICT OF INTEREST

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

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