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The Effects of Hydroxyapatite Coated with β-Tricalcium Phosphate in One-Wall Intrabony Defects in Dogs

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The purpose of this study was to evaluate the effect of hydroxyapatite coated with β-tricalcium phosphate (HCT) in surgically prepared one-wall periodontal intrabony defects in dogs. Four beagle dogs were used as the subjects. One-wall intrabony defects were prepared surgically at the mandibular second and fourth premolars in both right and left jaw quadrants. The experimental groups were divided into two, according to the kind of graft material used. In the HCT group, the defect was filled with HCT. In the HCT/ACS group, the defect was filled with HCT and then covered by an absorbable collagen sponge (ACS). The control group was treated by root planing only. Histologic and histometric analyses revealed statistically significant bone formation in the HCT and HCT/ACS groups, compared with the control group. New bone was observed along the root surface or in close contact with the residual graft particles in the HCT and HCT/ACS groups. The HCT/ACS group showed statistically greater bone formation than the HCT group. It can be concluded that HCT provides osteoconductivity in one-wall intrabony defects. In addition, HCT in one-wall intrabony defects provide new cementum formation and periodontal ligament fiber formation with neither root resorption nor ankylosis.

Key words: hydroxyapatites, beta-tricalcium phosphate, bone regeneration, bone substitute, histology

Introduction

Periodontal osseous defects can be treated by various procedures, including guided tissue regeneration,[1)] bone grafting,[2)] root surface conditioning,[3)] and the use of growth factors such as platelet-derived growth factor[4)] for regeneration of destroyed periodontal tissue. Many investigations have demonstrated that bone grafting reduces the probing depth and gains periodontal tissue attachment and the filling of defects in clinical situations.[5)] Among the available bone graft materials, autogenous bone is the first choice because of its osteogenic effect and the lack of host immune response. However, the use of autogenous grafts is limited by the amount of available host tissue, and it is associated with external root resorption and ankylosis.[6)] To overcome these disadvantages, the use of synthetic bone grafts has been proposed for the treatment of periodontal defects.

Hydroxyapatite (HA) is well known for its biocompatibility and osteoconductivity; it acts as a scaffold.[7)] It has been used clinically for the treatment of periodontal intrabony defects.[8-9)] However, disadvantages of its use have been reported, such as a low rate of biodegradability and low mechanical strength.[10-11)] Although β-tricalcium phosphate (β-TCP) shows greater resorbability than HA and permits replacement with bone in animal and human experiments,[12-13)] because of its rapid rate of resorption, which exceeds that of autogenous bone, it demonstrates an unpredictable rate of degradation and osteoconductivity.[14-16)] An ideal synthetic bone substitute should provide a scaffold for bone formation and have resorptive properties that allow replacement with new bone.[17)] Accordingly, many studies have suggested the use of so-called biphasic calcium phosphate (BCP), which combines the scaffolding properties of HA and the resorbability of β-TCP.[18)]

Currently, BCP is widely used as an alternative to autogenous bone for ridge augmentation[19)] and maxillary sinus grafting[20-21)] in dentistry, because it does not require production of an additional surgical site and can be supplied in unlimited amounts. In addition, it has demonstrated favorable osteoconductivity in clinical and animal studies.[22-23)]

It has been reported that the biologic activity of BCP, manifest by the dissolution rate and osteoconductivity, differs according to its HA/β-TCP ratio and porosity.[24-25)] In an effort to improve the osteoconductivity of BCP, various manufacturing methods that change the combination ratio, porosity, and crystallinity of BCP have been evaluated. Conventional BCP is

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generally prepared by mixing HA and β-TCP and then sintering it at 700°C or above.[26] A novel method has been developed recently for the fabrication of BCP, which involves coating the HA scaffold surface with β-TCP by the sol-gel technique. The HA is coated with β-TCP in order to provide convenient and stable environment for cell attachment, proliferation. This is caused by an increased release of ions owing to the resorption of β-TCP during the early implantation phase *in vivo*. Ioku *et al*.[27] have reported the behavior *in vivo* of porous HA coated with β-TCP, which showed higher compressive strength than the original porous HA. In addition, resorption of coated β-TCP resulted in precipitation of apatite on the porous HA. It has been reported that a coating of calcium phosphate on a porous scaffold resulted in enhanced cell proliferation and differentiation.[28] Kim *et al*.[22] also demonstrated that HA coated with β-TCP led to successful bone regeneration in a human sinus graft procedure.[22] On the basis of previous studies, we assumed that HA coated with β-TCP (HCT) would be favorable to bone formation. However, there is a lack of histologic and histometric studies that evaluate the regeneration of HCT in a well-established animal model.

The purpose of this study was to evaluate the regenerative effect of HCT in surgically prepared one-wall intrabony defects in dogs.

Materials and Methods

Animal and Materials

Four male beagle dogs, each weighing 10-15 kg and approximately 2 years old, were used. Animal selection, management, preparation, and the surgical protocol followed the routine procedures approved by the Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

The BCP that was investigated in this study was HA coated with β-TCP (HCT) (Osteon™; Genoss, Suwon, Korea). The HCT is composed of 70% HA and 30% β-TCP. It has a total porosity of approximately 77% and a particle size of 0.5-1.0 mm. To prepare HCT, porous HA was produced by a stretched polymeric sponge method. Subsequently, the HA was soaked in calcium/phosphate sol-gel, followed by sintering at 1000°C.[29-30] An absorbable collagen sponge (ACS) (Collatape®, Zimmer Dental, USA) was used to cover the bone substitute in the intrabony defects.

Surgical Protocol

Surgery was performed under general anesthesia induced by intravenous injection of atropine (Kwang-myung Pharmaceutical Ind., Seoul, Korea) 0.05 mg/kg and intramuscular injection of a combination of xylazine (Rompun, Bayer Korea, Seoul, Korea) 2 mg/kg and ketamine (Ketalar, Yuhan, Seoul Korea) 10 mg/kg, followed by inhalation anesthesia (Gerolan, Choongwae Pharmaceutical, Seoul, Korea). Local infiltrative anesthesia (2% lidocaine HCl with epinephrine 1:100 000, Kwangmyung Pharm., Seoul, Korea) was used at the surgical sites. Two months prior to surgery, both the mandibular first and third premolars had been extracted in order to create surgical sites. After complete healing of the extraction sockets, the mucoperiosteal flaps were reflected and one-wall intrabony defects (4 × 4 mm) were prepared surgically at the distal aspect of the second and the mesial aspect of the fourth mandibular premolars in both right and left jaw quadrants (Figure 1(B)).[31] Careful debridement and root planing was performed to ensure complete removal of the cementum from above the base of the defect. A reference notch was made on the root surface at the bottom of the intrabony defect using a round bur. The unilateral mandibular defects each underwent one of the two experimental protocols: implantation of HCT (HCT group) or HCT/ACS (HCT/ACS group). The contralateral mandibular defect received root planing only (control group). The experimental protocols were rotated between the defect sites in subsequent animals. The remaining

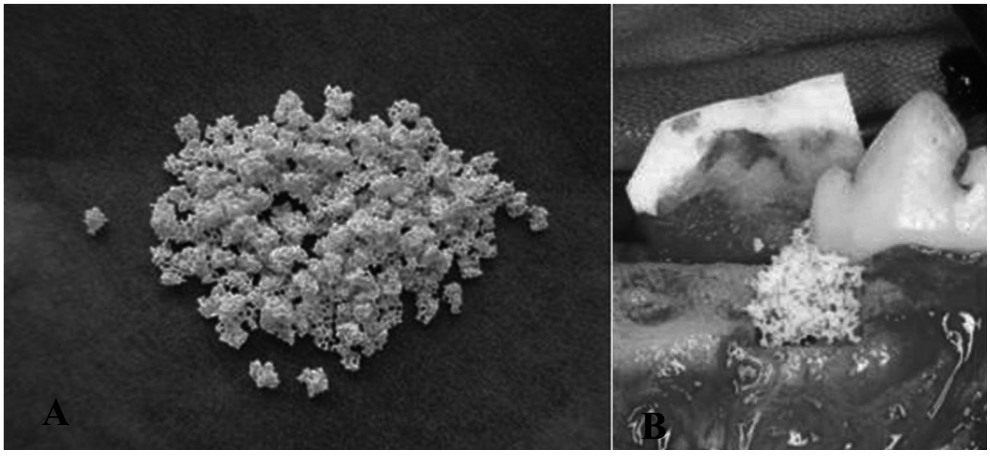

Figure 1. (A) HA coated with β-TCP (HCT), (B) Clinical photograph showing a surgically prepared one-wall intrabony defect of the mesial fourth premolar filled with HCT before covering with ACS.

defects were not included in this study. The defects were filled with the experimental implants to the level of the alveolar crest. In the HCT/ACS group, the defects were filled with HCT and then covered by ACS. After treatment of the defect, the flaps were sutured with 5-0 resorbable suture material (Polyglactin 910, braided absorbable suture, Ethicon, Johnson & Johnson Int., Edinburgh, U.K.). Postsurgical treatment included intramuscular antibiotics for 3 days and daily dressing with 0.2% chlorhexidine solution (Hexamedin, 2% chlorhexidine, Bukwang Pharm., Seoul, Korea) for 7 days. The sutures were removed after 10 days. The animals were killed 8 weeks after surgery by an overdose of pentobarbital sodium (90~120 mg/kg; i.v.). Samples were collected from the defect sites and block sections were prepared for histologic and histometric evaluation.

Histologic Analysis

The block sections were fixed in 10% buffered formalin and decalcified with 5% nitric acid for 14 days. Paraffin wax blocks were made and sectioned in the mesio-distal direction with a thickness of 4 μm. Each section was stained with hematoxylin-eosin and observed with a light microscope.

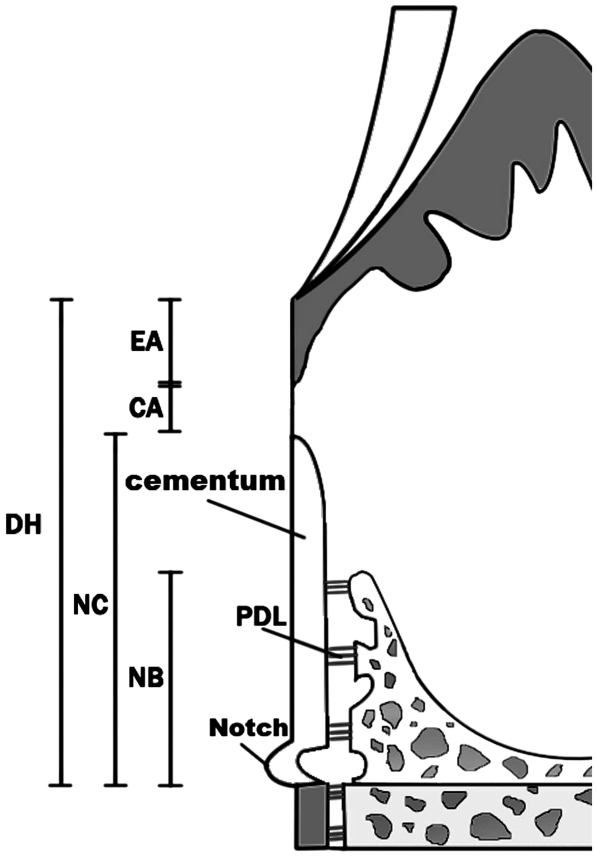

Figure 2. Schematic drawing describing the landmarks and parameters used in the histometric analysis. DH: defect height, EA: epithelial attachment, CA: connective tissue attachment, NC: newly formed cementum, NB: newly formed bone height, PDL: periodontal ligament.

Histometric Analysis

Histometric analysis was performed using a software program (Image-Pro Plus®, Media Cybernetics, Silver Spring, MD, USA). The cement–enamel junction and the apical notch were used as reference points. The histometric parameters were as follows: defect height (DH), epithelial attachment (EA), connective tissue attachment (CA), newly formed cementum (NC), and newly formed bone height (NB) (Figure 2).

Statistical Analysis

The mean and standard deviation were calculated for each group. The significance of the differences among the groups was determined by the Kruskal–Wallis test ($p < 0.05$). The Bonferroni test was used to analyze statistical significance at the 5% level.

Results

Clinical Findings

During the postoperative period, there were no adverse effects on healing in any group. There were no signs of inflammation or wound exposures.

Histologic Observations

Control Group

The histologic examination showed limited formation of new bone and cementum above the reference notch (Figure 3(A)). Inflammatory cell infiltration occurred minimally at the surgical sites. There were no signs of ankylosis or root resorption in any of the specimens.

HCT Group

Histologic analysis of the HCT group demonstrated more regeneration of bone and cementum compared with the control group. Regenerated bone was observed prominently along the root surface (Figure 3(B)). The remaining graft material was surrounded by immature woven bone and connective tissue. Regenerated cementum and the periodontal ligament space were also observed along the root surface, with no root resorption or root ankylosis.

HCT/ACS Group

Greater new bone formation and cementum regeneration were observed in this group than in both the control and the HCT groups. As shown in (Figure 4(A)), the regenerated bone was primarily formed of woven bone and contained many osteocytes. The remaining graft particles were closely contacted to the regenerated bone or were surrounded by loose connective tissue. Osteoblast-like cells were arranged along the regenerated woven bone. New bone also could be observed at the inner portion of remaining graft particles and vascular structures could be seen within the loose connective tissue.

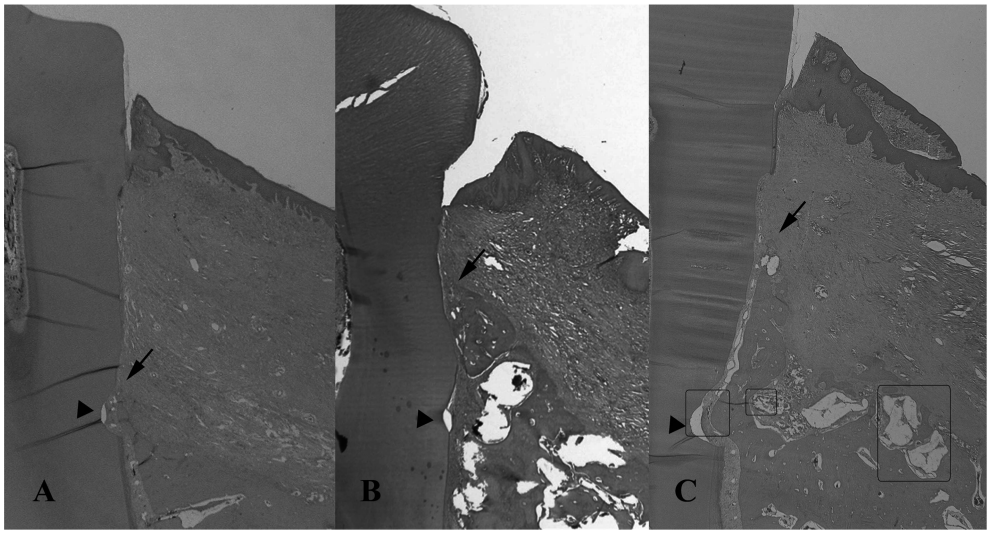

Figure 3. (A) Control group. A limited amount of bone formation is observed above the notch, (B) Histologic view of HCT group. More bone formation is observed than control. There were remnants of graft material present, (C) Histologic view of HCT/ACS group. Marked bone formation was observed above the notch along the root surface. The remaining graft materials were seen as hollow unresorbed pores (hematoxylin and eosin staining: original magnification × 20, reference notch: arrow head, highest point of new bone: arrow).

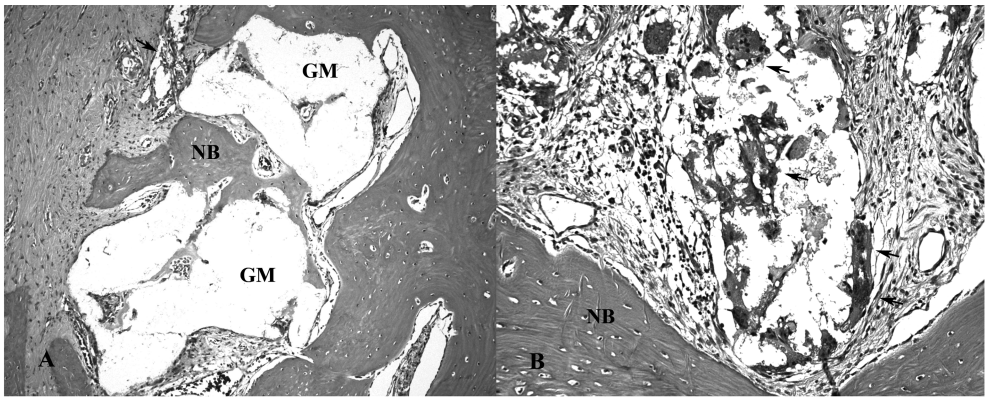

Figure 4. High magnification of HCT/ACS group: (A) At the third box on the left in Figure 3., woven bone was embedded in loose connective tissue or was observed in direct contact with the graft material. There was also new bone in the inner portion of remaining graft materials, (B) At the second box on the left in Figure 3., multi-nucleated giant cells (arrow) were observed in the remaining HCT (hematoxylin and eosin staining: original magnification, A: × 100, B: × 200, NB: new bone, GM: graft material).

Multi-nucleated giant cells were observed around the remaining graft particles (Figure 4(B)). New cementum that was thinner than the original cementum was observed along the root surface, and it extended to the level of the regenerated bone (Figure 5).

Periodontal ligament spaces were observed between the regenerated bone and the cementum, from the apical notch to the level of attachment of the coronal connective tissue (Figure 5). Periodontal ligament fibers were obliquely or perpendiculary inserted into the newly formed cementum and bone (Figure 5).

Histometric Analysis

The results of the histometric analyses are summarized in Table I. The average difference in the height of the defect was not statistically significant among the groups. In the HCT group, there was significantly more bone formation than in the control group. There was also significantly more bone formation in the HCT/ACS group than in either the control or the HCT group. Significantly more cementum regeneration was observed in the HCT/ACS than in the HCT group.

Discussion

The present study evaluated the regenerative effect of HCT in surgically prepared one-wall intrabony defects in dogs. This newly developed BCP contains an HA/β-TCP ratio of 70/30. The optimal ratio of HA/β-TCP has not yet been determined. As Nery *et al*. demonstrated that a HA/β-TCP ratio of 85/15

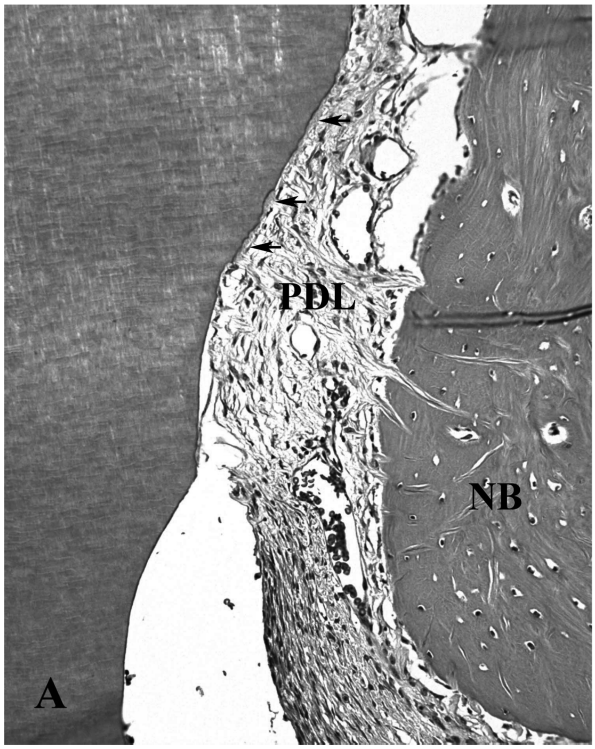

Figure 5. High magnification of first box on the left in Figure 3. Periodontal ligament fibers were obliquely or perpendicularly inserted into the newly formed cementum and bone in the notch area (hematoxylin and eosin staining: original magnification × 200, NB: new bone, PDL: periodontal ligament fiber, new cementum:arrow).

showed greater periodontal tissue attachment and bone regeneration in periodontal osseous defects,[18] BCP with an HA/β-TCP ratio of 70/30 also led to predictable bone formation in a rat calvarial defect.[32] Meanwhile, the biomaterial used in this study has an interconnected porous structure similar to that of human cancellous bone, with a porosity of 77% and a pore size of 300-500 μm under scanning electron microscopy.[22] Shetty *et al*. suggested that interconnectivity of bone substitutes is essential for bone ingrowth.[17] According to Legeros and Fleckenstein, macroporosity (diameter > 100 μm) provides a scaffold that facilitates bone-cell colonization more successfully than microporosity (diameter < 10 μm).[26,32] Considering the findings of these previous studies, it is thought that the HCT utilized in this study fulfills the physical and structural requisites for bone regeneration.

Table 1. Comparison of histometric analysis between all groups (mean ± SD in mm)

	Control	HCT	HCT/ACS
Defect height	4.31 ± 0.76	4.20 ± 0.34	4.55 ± 0.32
New bone height	0.30 ± 0.38	1.40 ± 0.72*	2.42 ± 0.34*§
New cementum height	2.78 ± 0.83	1.63 ± 0.54	2.66 ± 0.30§
Epithelial attachment	1.43 ± 0.38	1.30 ± 0.55	1.58 ± 0.63§
Connective tissue attachment	0.07 ± 0.14	0.65 ± 0.29*	0.32 ± 0.32*

* Significant statistical difference from control ($p < 0.05$)
§ Significant statistical difference from HCT ($p < 0.05$)

The biologic rationale for the choice of the one-wall intrabony defect model in dogs was as follows. The wound healing response in periodontal defects in experimental models involving dogs and monkeys appears to be comparable to that in the human.[33-34] One-wall intrabony defects are recognized to be a useful and reproducible model for the evaluation of bone substitute osteoconduction in dogs.[35] Many bone substitute candidates have already been evaluated in one-wall intrabony defects to verify their osteoconductivity and periodontal regeneration.[35-38]

The histologic and histometric results of this study indicated that the HCT resulted in greater bone formation than in the control group, a difference that was statistically significant. In the HCT group, regenerated bone was observed along the root surface and it extended in the coronal direction. This new bone was originated from mesenchymal cells in the periodontal ligament space and osteogenic precursor cells in the base of intrabony defects.[31] The HCT/ACS group showed significantly greater bone formation than the HCT and control groups. The ACS used in the HCT/ACS group has the biological property to be absorbed in about 10-14 days *in vivo*. It seemed that the ACS contributed to stabilization of the bone substitute particles and prevented the ingrowth of soft tissue into the defect at an early phase of healing. Consequently, greater bone formation was seen in the HCT that was covered with ACS.

Osteoconductive biomaterial should be resorbable in order to provide space for cell attachment, proliferation, and vascularization. A number of multi-nucleated giant cells were also detected adjacent to the remaining graft material and newly formed bone. It has been suggested that dissolution of BCP results from the phagocytosis of multi-nucleated giant cells and leads to bone replacement.[39-40] The histologic findings of this study indicated that the HCT was under active resorption, which may be associated with biologic phagocytosis of multi-nucleated giant cells, followed by bone ingrowth.

Yamada *et al*. reported that BCP might be degraded by a different mechanism, specifically by osteoclastic resorption, based on the detection of resorption lacunae on the BCP surface *in vitro*.[41] They suggested that this osteoclastic resorption contributes to osteoblastic bone formation on the surface of the β-TCP. Osteoclast-like cells have been identified on the surface of BCP in another histologic study.[42] However, we could not identify the numerous multi-nucleated giant cells on the HCT surface as true osteoclast cells and further study is required in order to explain the relationship between resorption of the HCT and osteoclastic cell activity.

It has been reported that BCP, when implanted *in vivo*,

became degraded and absorbed into the surrounding environment with the release of calcium and phosphate ions. When these released ions reached a supersaturation state in the microenvironment, bone apatite began to precipitate on the surface of the BCP.[24,26] In our histologic study, the inner surface of HCT was relatively rough going through resorption and new bone was observed in direct contact with the HCT. Another study that investigated preexisiting BCP also demonstrated similar histologic results.[42-43] Moreover, new bone ingrowth was observed at the inner portion of HCT in higher magnified histologic views of HCT/ACS group (Figure 4(A)). This means that for bone formation the HCT showed resorptive property for cell attachment, ingrowth as well as scaffolding activity.

The bone regeneration capacity of HCT in our study corresponds to the findings reported by other investigators. According to Um *et al*., the HCT used in this study induced new bone formation in a rabbit calvarial defect that was comparable to that induced by deproteinized bovine bone.[44] When used in a sinus grafting procedure, this HCT led to successful bone regeneration.[22]

New cementum was also observed in both experimental groups in the current study. The HCT/ACS group, in particular, showed significant cementum regeneration, compared with the HCT group. In addition to cementum formation, the periodontal ligament space was also well maintained between the newly formed cementum and the alveolar bone. Periodontal ligament fibers perpendicularly or obliquely inserted to the new cemenum and bone could be observed in this study. Periodontal regeneration comprises the formation of new cementum with insertion of periodontal ligament fibers, as well as alveolar bone regeneration.[1] It have been reported that root resorption could be prevented by new cementum formation.[45] Thus, it was found that this HCT also has beneficial effects in periodontal regeneration.

Conclusions

In conclusion, the results of the present study indicate that the HCT possessed osteoconductivity and showed periodontal regeneration in one-wall intrabony defects in dogs. The combination of HCT and ACS increased bone regeneration compared with HCT alone. Though the HCT showed favorable regenerative effect, there still lacks evidence on the advantage of coating HA with β-TCP. Therefore, further investigation to evaluate the influence of HA coated with β-TCP on bioactivity, including the degradation rate and cell attachment efficacy, is necessary *in vivo* and *in vitro*.

Acknowledgements

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