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Bone formation with a collagen model polypeptides/alpha-tricalcium phosphate sponge in a mandibular canine defect model

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We investigated the effect of synthesized collagen model polypeptides consisting of a proline-hydroxyproline-glycine (poly(PHG)) sequence combined with porous alpha-tricalcium phosphate (*α*-TCP) particles on bone formation in a mandibular canine defect model. The porous *α*-TCP particles were mixed with a poly(PHG) solution, and the obtained sponge was then cross-linked and analyzed by scanning electron microscopy. The optimal mixing concentration of *α*-TCP granules and the poly(PHG) solution was decided to be 0.15 g/mL by compression testing. The osteoblastic differentiation with the ALP activity of human mesenchymal stromal cells (hMSCs) cultured on the poly(PHG)/*α*-TCP was slightly greater than that of hMSCs cultured on the poly(PHG). Mandibular defects were analyzed in healthy beagles using micro-computed tomography and histological evaluation. At 2 and 4 weeks, the volume density of new bone was grater in the poly (PHG)/*α*-TCP group than in the group with only poly(PHG) ($p < 0.05$); however, there was no difference at 8 weeks ($p > 0.05$). Histological evaluation at 4 weeks after implantation revealed that the poly(PHG) had degraded and that newly formed bone was present on the surface of the *α*-TCP particles. This study demonstrates that the composite created using porous *α*-TCP particles and poly(PHG) is sufficiently adaptable for treating the mandibular canine defect model. (J Osaka Dent Univ 2023; 57: 223-231)

Key words: Bone formation; Synthesized collagen model polypeptides; Porous alpha-tricalcium phosphate

INTRODUCTION

Bone loss commonly occurs in oral and maxillofacial surgery due to infection, trauma, tumors, and cysts, as well as clefts in the alveolar bone and palate.[1-3] Bone resorption due to periodontal disease often results in tooth loss.[4] Autologous bone is widely recognized as the gold standard grafting material.[5-7] However, the amount of autogenous bone that can be harvested from an individual is limited and the procedure is relatively invasive. Therefore, a scaffold material is needed that is bioabsorbable and can eventually be replaced by autologous tissue.[8] In addition, it must have excellent handling characteristics for surgical application, as conventional granular materials tend to leak through pores and are difficult to apply to bone defects.[4, 7]

Collagen has been widely applied clinically in various forms, such as gels and sponges, because of its good biodegradability, biocompatibility, and absorbability.[4, 7-9] Collagen sponge is considered a useful scaffold matrix for several types of tissues. However, collagen from animal sources is susceptible to nonspecific cellular attachment, may be contaminated by infectious pathogens, such as prions, and has the potential for infecting humans.[10] Tanihara *et al.*[11] previously reported the chemical synthesis of a collagen model polypeptide consisting of a proline-hydroxyproline-glycine (poly(PHG)) sequence that forms a triple-helix structure. Among

the various possible calcium phosphate materials, previous studies have highlighted the potential of α-TCP particles as a bone reconstruction material because they gradually biodegrade while bone regenerates in the surrounding area.[12] In our previous study, we evaluated the effect of the combination of poly(PHG) and α-TCP particles on bone formation in a skull-deficient minipig model.[7] The material obtained by combining poly(PHG) and α-TCP particles did not induce inflammation, and complete degradation and remodeling of lamellar bone was observed. However, the solution-particle combination was difficult to apply to bone defects and tended to leak from the defect.

Several novel peptide-based biomaterial scaffolds for tissue engineering have been reported.[13] However, producing scaffolds for use in bone tissue engineering remains a major challenge. In this study, a dehydration thermal cross-linking method was used to construct poly(PHG) and porous α-TCP sponges as scaffolds for bone regeneration. We evaluated the effect of the poly(PHG)/α-TCP scaffolds on bone regeneration in surgically induced mandibular canine bone defects.

MATERIALS AND METHODS

Synthesis of poly(PHG)

Poly(PHG) was synthesized according to previously published methods.[11] Briefly, PHG was dissolved in 10 mM phosphate buffer (pH 7.4) at a concentration of 165 mM, and mixed with 33 mM of 1-hydroxybenzotriazole (Peptide Institute, Osaka, Japan) and 825 mM of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Peptide Institute). After sequential stirring for 2 hrs at 4°C and 46 hrs at 20°C, the reaction mixture was dialyzed against water for 48 hours to remove any residual reagents.

Preparation of poly(PHG)/ α-TCP sponges

The particles were prepared by pulverization of an α-TCP block with 80% of the pores showing a continuous pore structures.[14, 15] Large and small α-TCP particles were provided by Taihei Chemical Industrial, Osaka, Japan. The median sizes of the large and small particles were 580.8 μm and 136.2 μm, respectively. The large and small α-TCP particles were mixed at ratios of 50:50 mass%. Next, α-TCP particles were mixed with the aqueous poly(PHG) solution at a concentration of 10 mg/mL. The mixing concentration of α-TCP and the poly(PHG) solution required to produce the molded sponge was determined to be 0.15 g/mL. The mixture was then poured into plastic molds, and all samples were immediately frozen to −80°C and then freeze-dried for 24 hours. The freeze-dried poly(PHG)/ α-TCP constructs resembled sponge-like structures, and were subsequently cross-linked in vacuo at 140°C for 10 hours. The poly(PHG)/ α-TCP sponges were sterilized with ethylene oxide gas at 40°C. The poly (PHG) sponges without α-TCP particles as a control were also prepared in the same way.

Compression test

Cylindrical specimens (n＝3) measuring 5 mm in diameter and 7.5 mm in height were prepared in a silicon tube mold for compression tests, following the procedures out-lined in ISO 9917-1. We prepared solutions containing 0, 0.1 g and 0.15 g of α-TCP in 1 mL of 1% the poly(PHG) solution in a dry condition. The samples were tested using a universal testing machine (AGS-X; Shimadzu, Kyoto, Japan). The load cell was 500 N and the crosshead speed was 1 mm/min.[16]

Seeding of cells on the sponges

Human mesenchymal stromal cells (hMSCs) were obtained from the RIKEN Cell Bank, Tsukuba, Japan and maintained by continuous culture at 37°C in a humidified atmosphere containing 5% CO_2. The hMSCs were expanded for 7-10 days in DMEM supplemented with 10% heat-inactivated FBS, 3 ng/mL fibroblast growth factor-2, and 1% antibiotic/antimycotic in 75-mm^2 flasks. After a sufficient number of hMSCs was obtained, they were seeded at a density of 3×10^4 cells onto poly(PHG) or poly (PHG)/α-TCP placed in the wells of 24-well tissue culture plates. The cells were incubated for 3 days in a CO_2 incubator at 37°C. Subsequently, the medium was removed from the wells, and the cells

were cultured in an osteogenic differentiation medium of 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 μg/mL ascorbic acid for 7 and 14 days.

DNA content analysis

DNA content was measured on days 7 and 14. The medium was then removed, and the cells were washed twice with PBS. Afterwards, 300 μL of 0.2% Triton X-100 was added to each well, and the cells were removed by a cell scraper (Becton-Dickinson, Franklin Lakes, NJ, USA) for DNA extraction. DNA content was measured by a Quant-iTTM PicoGreen dsDNA Reagent and Kit (Invitrogen, Carlsbad, CA, USA). Fifty microliters of sample were mixed with a DNA-binding fluorescent dye solution (0.5 μL PicoGreen reagent in 100 μL 1× TE buffer), and the fluorescent intensity was measured by a microplate reader at Ex 450 nm/Em 510 nm (SpectraMax M 5; Molecular Devices, Sunnyvale, CA, USA).

Measurement of alkaline phosphatase activity (ALP)

ALP activity was measured on days 7 and 14 using LabAssayTM ALP (Wako Pure Chemical Industries, Osaka, Japan). One-hundred microliters of a working assay solution (6.7 mmol/L p-nitrophenylphosphate disodium) was added to 20 μL of the same sample used for DNA measurement, mixed thoroughly, and incubated at 37°C with 5% CO_2 for 15 min. After 80 μL of stop solution (0.2 mol/L sodium hydroxide) was added to the mixture, the absorbance was measured at 405 nm with the SpectraMax M 5. The levels of ALP activity were normalized to the amount of total DNA in the cell lysates.

Establishment of the animal model

Twelve healthy beagles 2 years of age and weighing approximately 10 kg that were obtained from Hamaguchi Animal, Osaka, Japan were used as the tibia defect model. The animals were housed in a temperature controlled environment at 24°C and were allowed food and water ad libitum. The body weight and general health of the beagles were monitored throughout the study.

Transplantation procedures

All procedures in this study were approved by the Animal Experiment Committee of Osaka Dental University and conformed to procedures described in the Guiding Principles for the Use of Laboratory Animals (Approval No. 13-03026). An initial incision was made in the skin covering an appropriate part of a mandible, and the skin and subcutaneous tissue were separated from the periosteum. A second incision was made in the periosteum of the tibia, and the periosteum was elevated and carefully dissected from the underlying mandibular defects which were 4 mm in diameter and 6 mm in depth. The defects were made using a twist drill (Astra Tech, Tokyo, Japan) with physiologic saline cooling under general anesthesia (0.5 mg/kg pentobarbital sodium) and infiltration anesthesia (1.8 ml 2% lidocaine hydrochloride and 1:80,000 epinephrine). The defects were randomly filled with one of the two treatment mixtures. A mixture of poly(PHG) and α-TCP was transplanted into the animals and poly (PHG) alone was transplanted into the control group animals. Assessments were made at 2, 4 and 8 weeks after surgery. The periosteum and skin over the defects were then sutured in two layers with 3-0 Vicryl (Ethicon, Norderstedt, Germany) and 3-0 MANI Silk (MANI, Tochigi, Japan). Six cavities were prepared and tissues were histopathologically assessed at each follow-up time in each group.

Radiographic analysis

The tibias were harvested for microradiographic examination using an SMX-130 CT micro-computed tomography (CT) apparatus (Shimadzu). Blocks of bone specimens were mounted on the turntable. The exposure conditions were 51 kV and 120 mA. The data obtained from each slice were saved at 512×512 pixels. TRI/3D BON software (Ratoc, Tokyo, Japan) was used to create a 3D reconstruction image using the volume rendering method for morphological observations. In the 3D analysis, the total volume (TV; cm^3) and bone volume (BV; cm^3)

were measured using the TRI/3D-BON software based on the obtained CT values. The volumetric density (VD) was then calculated based on the following formula: VD (%)＝BV/TV.

Histological assessment

The tibias were fixed in 10% neutral-buffered formalin (Sigma, St. Louis, MO, USA), demineralized in a solution of ethylenediaminetetraacetic acid (Sigma), dehydrated in a series of alcohol washes, and embedded in paraffin. The tibias were sectioned at 5-7 μm in the coronal plane and stained with hematoxylin and eosin (HE). Each section was observed using a BZ 9000 All-in-One Fluorescence Microscope (Keyence, Tokyo, Japan). A fluorescence filter was used to visualize the remaining poly(PHG) fragments and α-TCP particles.

Statistical analysis

The mean and standard deviation of each parameter were calculated for each group and compared by the Student's t-test using statistical software (Statcel 2; OMS Publisher, Tokorozawa, Japan). A value of $p < 0.05$ was considered significant.

RESULTS

Characteristics of the poly(PHG)/α-TCP sponge

Figure 1 shows a scanning electron microscope (SEM) image of the synthesized porous ceramic particles. The ceramic body had a continuous pore structure, with a pore diameter of approximately 5-10 μm. Figure 2 A shows the SEM image of poly (PHG)/α-TCP, and Figure 2 B shows the image of poly(PHG). The composites were composed of α-TCP particles and a 3D porous structure (with an anatomizing network).

Compression Test

The maximum compressive stress in the groups containing 0.05 g, 0.1 g and 0.15 g of α-TCP-containing solution in 1 mL of 1% the poly(PHG) solution was 0.15, 0.23 and 0.31 MPa, respectively (Figure 3).

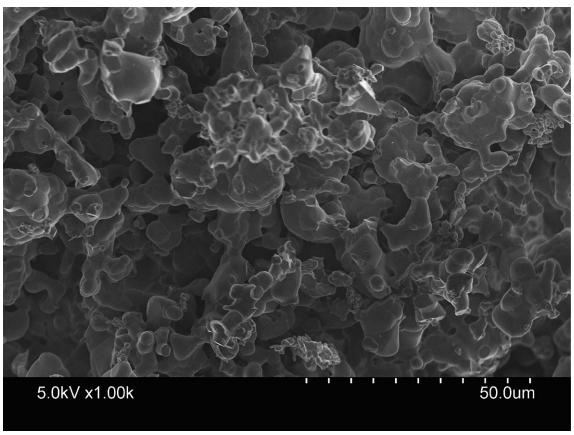

Fig. 1 Scanning electron microscope image of porous α-TCP particles.

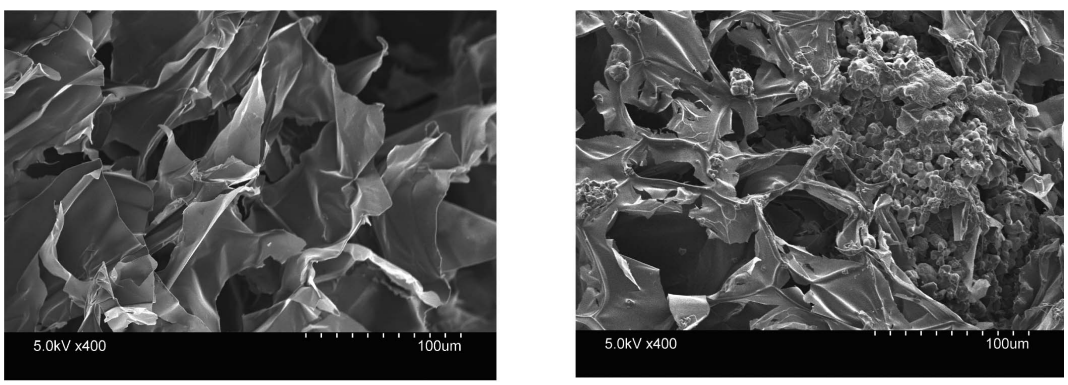

Fig. 2 Scanning electron microscope image of (A) poly(PHG) and (B) poly(PHG)/α-TCP.

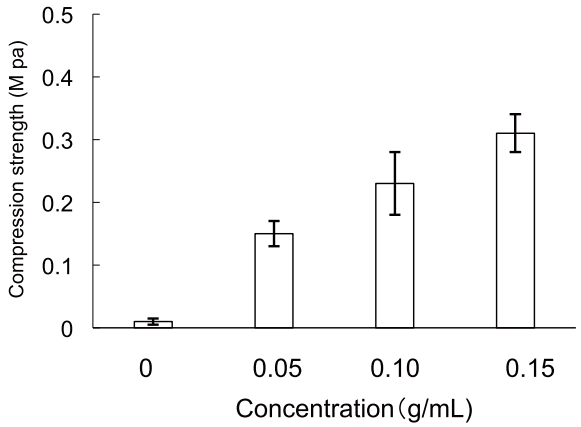

Fig. 3 Compression strength of the sponge as a function of the concentration of α-TCP particles

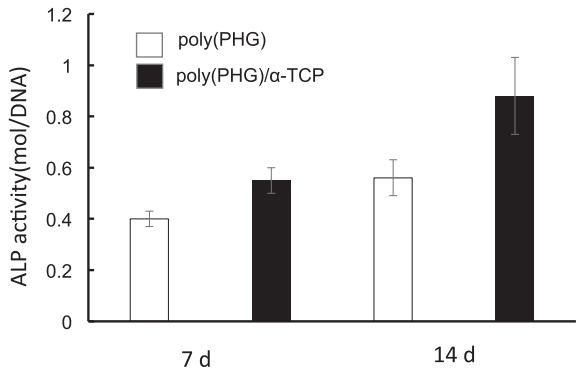

Fig. 4 ALP activity of hMSCs cultured on poly(PHG) or poly(PHG)/α-TCP for 7 and 14 days

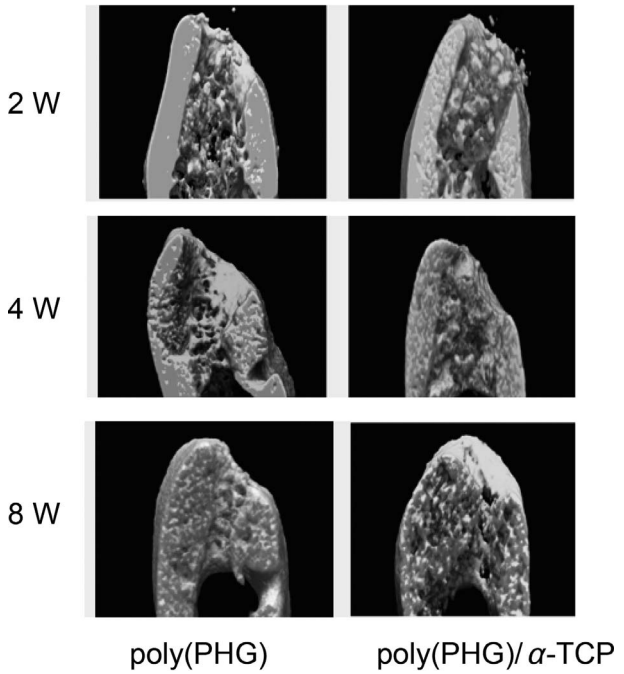

Fig. 5 Micro-computed tomography images of bone regeneration at 2, 4 and 8 weeks after surgery.

ALP activity

The osteoblast differentiation ability of hMSCs cultured on poly(PHG) alone and poly(PHG)/α-TCP was compared by using an osteoblastic differentiation marker. As a marker of early stage osteoblastic differentiation, the ALP activity of hMSCs cultured on the poly(PHG)/α-TCP was slightly higher than that of hMSCs cultured on the poly(PHG) on days 7 and 14 ($p < 0.05$) (Fig.4).

Microradiography

Representative 3D reconstructions of filling defects at 2, 4 and 8 weeks in the poly(PHG) alone (control) and the poly(PHG)/α-TCP (experimental) groups are shown in Figure 5. In the experimental group, an opaque image similar to that of the original α-TCP particles was observed at 2 weeks, and the amount of new bone increased with time, with the entire volume of each defect filled with newly formed bone beam structure at 8 weeks. In the control group, the defects were empty, showing dense formation from the edge of the defect toward the center, and thickening was observed between 4 and 8 weeks. For determination of new bone tissue using image analysis software, the average volume ratio of new bone was calculated by dividing the BV by the TV. The VD of each group at the three time points is shown in Figure 6. At 2 and 4 weeks after transplantation, the VD of the experimental group was greater than that of the control group ($p < 0.05$). However, there was no significant difference in VD between the two groups at 8 weeks after transplantation ($p > 0.05$).

Histological assessment at 2 weeks

The poly(PHG)/α-TCP and poly(PHG) used to fill the defects in the experimental and control groups, respectively, did not degrade (Fig.7). In the experimental group, the α-TCP particles were encapsulated by connective tissue and the inflammatory reactions were minimal (Fig. 7 D). Numerous os-

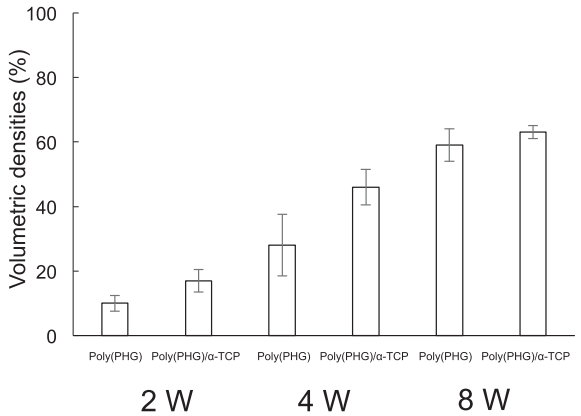

Fig. 6 Volumetric densities (%) of each group reflecting the quantity of new bone at 2, 4 and 8 weeks after surgery.

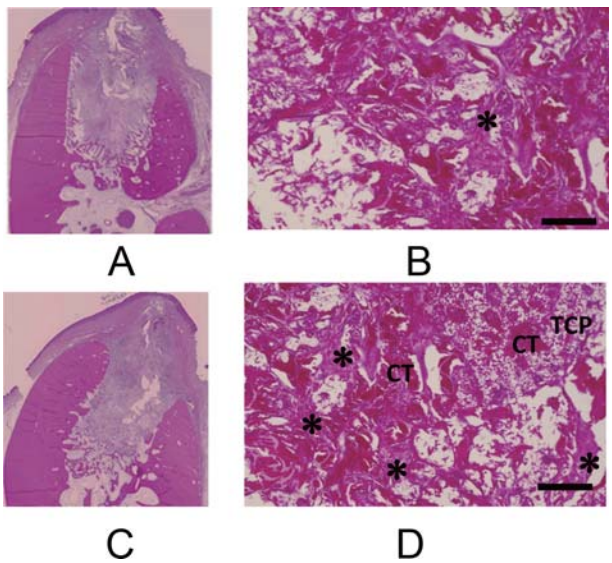

Fig. 7 Light microscopic images of hematoxylin and eosin-stained sections of bone defects at 2 weeks in (A) the control group, (B) the control group at higher magnification, (C) the experimental group, and (D) the experimental group at higher magnification (*poly(PHG), TCP: *α*-TCP particle, CT: connective tissue, Scale: 100 *μ*m).

teoblasts and new blood vessels were observed around the *α*-TCP particles. Large exophytic cells resulting from the inflammatory response were observed, but in reduced numbers compared to the control group (Fig. 7).

Histological assessment at 4 weeks

In the experimental groups (Figs. 8 B and D), *α*-TCP particles remained and reticular structures

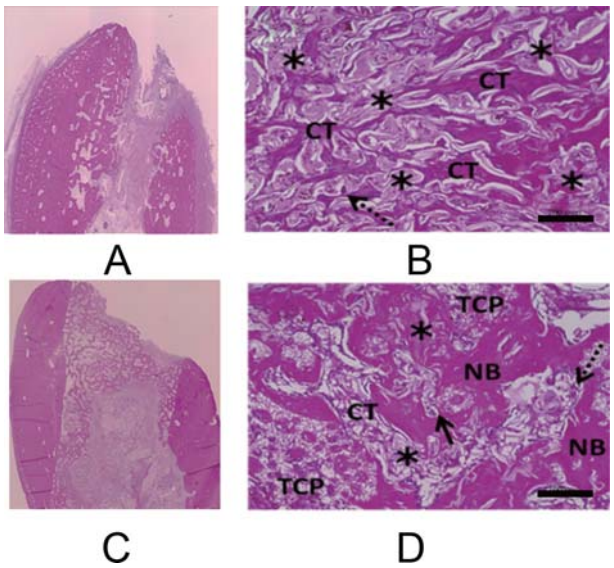

Fig. 8 Light microscopic images of hematoxylin and eosin-stained sections of bone defects at 4 weeks in (A) the control group, (B) the control group at higher magnification, (C) the experimental group and (D) the experimental group at higher magnification. (NB: New bone, Black arrow: Capillary, Black dot arrow: Large foreign cells, Scale: 100 *μ*m).

were observed inside the particles. Newly formed woven bone was deposited directly on the surface of the *α*-TCP particles and also formed from the edges of the bone defects. Furthermore, the newly formed bone was interconnected and several osteocytes were observed in an irregular pattern. In contrast, poly(PHG) was severely degraded and surrounded by connective tissue. In the control group (Fig. 8 B), many poly(PHG) fragments also remained. However, some fragments of transplanted poly(PHG) were replaced by connective tissue resembling immature bone. The number of large foreign cells in both groups was greatly reduced compared to that observed 2 weeks after surgery (Fig. 8 B). In the experimental group, the *α*-TCP particles were encapsulated by connective tissue and inflammatory reactions were minimal. Numerous osteoblasts and new blood vessels were observed around the *α*-TCP particles. Large exophytic cells resulting from the inflammatory response were observed, but in reduced numbers compared to the controls (Fig. 8 D).

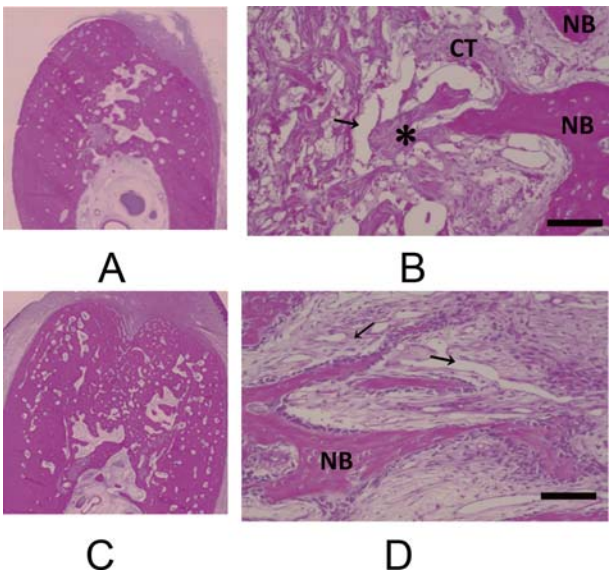

Fig. 9 Light microscopic images of hematoxylin and eosin-stained sections of bone defects at 8 weeks in (A)the control group, (B) the control group at higher magnification, (C) the experimental group, and (D) the experimental group at higher magnification (Scale: 100 μm).

Histological assessment at 8 weeks

In the experimental groups (Fig. 9 D), α-TCP particles remained and reticular structures were observed inside the particles. Newly formed interwoven bone was deposited directly on the surface of the α-TCP particles and also formed from the edges of the bone defects. Furthermore, the newly formed bone was interconnected and some osteocytes were observed in an irregular pattern. In contrast, poly(PHG) was severely degraded and surrounded by connective tissue. In the control group (Fig. 9 B), many poly(PHG) fragments also remained. However, some fragments of implanted poly(PHG) were replaced by connective tissue resembling immature bone.

DISCUSSION

Although autogenous bone is considered the best grafting material, considerable effort has been devoted to the development of resorbable bone substitutes for use in the repair of periodontal bone defects and for alveolar crest augmentation.[17, 18] In this study, porous α-TCP and poly(PHG) were combined to create a biodegradable sponge composite material for bone regeneration. The poly(PHG)/α-TCP sponge can be easily cut with scissors or a sharp knife and easily molded for use in alveolar bone augmentation. We evaluated the effect of this sponge on bone regeneration after transplantation into canine mandibular defects. The defect model in this study was intended to reflect patients coming to the dental clinic for alveolar bone defect reconstruction. Many of these patients have bone remodeling, usually after tooth extraction, and the alveolar bone defects are usually covered with hard cortical bone. In this experiment, the alveolar bone was reconstructed by creating a defect where the bone had been remodeled after tooth extraction. Compression testing showed that the compression strength of the poly(PHG)/α-TCP(0.15 g/mL)was significantly greater than that of the poly(PHG)/α-TCP(0.1, 0.05 and 0 g/mL)over the entire strain range. These results suggest that the optimal mixing concentration of α-TCP granules in the poly (PHG) solution is 0.15 g/mL.

The hMSCs are multipotent and have been differentiated into several types of mesodermal tissues, including bone, fat, and cartilage *in vitro.* Thus, hMSCs may be useful for studying the effects of bone replacement biomaterials. These cells are known to synthesize ALP. Compared to cells cultured on the poly(PHG) alone, cells cultured on the poly(PHG)/α-TCP for 7 or 14 days produced higher levels of ALP. This suggests that the activity of α-TCP in bone matrix formation is large.[19] Micro-CT has high resolution and only requires very thin tissue slices. With a larger number of slices, the spatial differences within areas of the specimen become negligible.[20] In our study, micro CT analysis showed that VD in the experimental group was greater than that of the control at 2 and 4 weeks after implantation, but not at 8 weeks. VD in the experimental group was greater at 2 weeks. This may reflect the density of the remaining α-TCP, and may not be the result of new bone formation. At 4 weeks, the density of newly formed replacement bone was greater than that of the control group. However, at 8 weeks, the defects in both groups were almost completely filled with newly formed

bone.

The processes of *in vivo* mineralization and biodegradation are complex for a composite scaffold. The balance of mineral deposition, biodegradation, and tissue regeneration changes greatly at different times after transplantation.[4, 21] With regard to the biodegradability of the poly(PHG)/*α*-TCP, the poly (PHG) sponge scaffold still remained at four weeks after implantation. Although the poly(PHG) sponge scaffold was replaced not only by soft connective tissue, but also by newly formed bone, the new bone did not show the formation of a typical bone network. In the present study, some poly(PHG) remained at 4 weeks after implantation and was replaced by a network of newly formed bone. The slow degradation rate of poly(PHG) combined with the rapid degradation of porous *α*-TCP balanced to match the rate of bone tissue regeneration. Thus, the poly(PHG)/*α*-TCP sponge showed faster and better bone induction capacities compared to the poly(PHG) sponge. Also, *α*-TCP particles that remained in the defect were in direct contact with the newly formed bone. Osteogenic progenitor cells migrated from adjacent bone edges and bottoms, attached to the surface of *α*-TCP particles, and subsequently formed new bone. These results suggest that an appropriate three-dimensional porous structure composed of poly-PHG and *α*-TCP particles played a role in providing appropriate spatial arrangement of osteogenic cells and in promoting vascular invasion.

In conclusion, this study demonstrated that a sponge created using porous *α*-TCP particles and poly(PHG), which is a chemically synthesized collagen model polypeptide, is sufficiently adaptable for treating canine mandibular bone defects.

REFERENCES

1. Matsuno T, Hashimoto Y, Adachi S, Omata K, Yoshitaka Y, Ozeki Y, Umezu Y, Tabata Y, Nakamura M, Satoh T. Preparation of injectable 3D-formed *β*-tricalcium phosphate bead/alginate composite for bone tissue engineering. *Dent Mater J* 2008; **27**: 827-834.
2. Nakano K, Kubo H, Nakajima M, Honda Y, Hashimoto Y. Bone regeneration using rat-derived dedifferentiated fat cells combined with activated platelet-rich plasma. *Materials* 2020; **13**: 1-15.
3. Yamada Y, Ueda M, Naiki T, Nagasaka T. Tissue-engineered injectable bone regeneration for osseointegrated dental implants. *Clin Oral Implants Res* 2004; **15**: 589-597.
4. Matsuno T, Nakamura T, Kuremoto K, Notazawa S, Nakahara T, Hashimoto Y, Satoh T, Shimizu Y. Development of beta-tricalcium phosphate/collagen sponge composite for bone regeneration. *Dent Mater J* 2006; **25**: 138-144.
5. Baba S, Inoue T, Hashimoto Y, Kimura D, Ueda M, Sakai K, Matsumoto N, Hiwa C, Adachi T, Hojo M. Effectiveness of scaffolds with pre-seeded mesenchymal stem cells in bone regeneration - assessment of osteogenic ability of scaffolds implanted under the periosteum of the cranial bone of rats. *Dent Mater J* 2010; **29**: 673-681.
6. Cohen M, Polley JW, Figueroa AA. Secondary (intermediate) alveolar bone grafting. *Clin Plast Surg* 1993; **20**: 691-705.
7. Sakai K, Hashimoto Y, Baba S, Nishiura A, Matsumoto N. Effects on bone regeneration when collagen model polypeptides are combined with various sizes of alpha-tricalcium phosphate particles. *Dent Mater J* 2011; **30**: 913-922.
8. Arima Y, Uemura N, Hashimoto Y, Baba S, Matsumoto N. Evaluation of bone regeneration by porous alpha-tricalcium phosphate/atelocollagen sponge composite in rat calvarial defects. *Orthodontic Waves* 2013; **72**: 23-29.
9. Yamamoto Y, Nakamura T, Shimizu Y, Matsumoto K, Takimoto Y, Kiyotani T, Sekine T, Ueda H, Liu Y, Tamura N. Intrathoracic esophageal replacement in the dog with the use of an artificial esophagus composed of a collagen sponge with a double-layered silicone tube. *J Thorac Cardiovasc Surg* 1999; **118**: 276-286.
10. Aguzzi A, Montrasio F, Kaeser PS. Prions: health scare and biological challenge. *Nature reviews Molecular cell biology* 2001; **2**: 118-126.
11. Tanihara M, Kajiwara K, Ida K, Suzuki Y, Kamitakahara M, Ogata SI. The biodegradability of poly (Pro-Hyp-Gly) synthetic polypeptide and the promotion of a dermal wound epithelialization using a poly (Pro-Hyp-Gly) sponge. *J Biomed Mater Res A* 2008; **85**: 133-139.
12. Wiltfang J, Merten HA, Schlegel KA, Schultze-Mosgau S, Kloss FR, Rupprecht S, Kessler P. Degradation characteristics of α and β tri-calcium-phosphate (TCP) in minipigs. *J Biomed Mater Res* 2002; **63**: 115-121.
13. Holmes TC. Novel peptide-based biomaterial scaffolds for tissue engineering. *Trends Biotechnol* 2002; **20**: 16-21.
14. Kitamura M, Ohtsuki C, Iwasaki H, Ogata SI, Tanihara M, Miyazaki T. The controlled resorption of porous *α*-tricalcium phosphate using a hydroxypropylcellulose coating. *J Mater Sci Mater Med* 2004; **15**: 1153-1158.
15. Kitamura M, Ohtsuki C, Ogata SI, Kamitakahara M, Tanihara M, Miyazaki T. Mesoporous calcium phosphate via post-treatment of a-TCP. *J Am Ceram Soc* 2005; **88**: 822-826.
16. Imataki R, Shinonaga Y, Nishimura T, Abe Y, Arita K. Mechanical and functional properties of a novel apatite-ionomer cement for prevention and remineralization of dental caries. *Materials* 2019; **12**: 3998.
17. Knabe C, Berger G, Gildenhaar R, Howlett CR, Markovic B, Zreiqat H. The functional expression of human bone-derived cells grown on rapidly resorbable calcium phosphate ceramics. *Biomaterials* 2004; **25**: 335-344.
18. Murugan R, Ramakrishna S. Bioresorbable composite bone paste using polysaccharide based nano hydroxyapatite. *Biomaterials* 2004; **25**: 3829-3835.

19. Yang L, Sun H-Y, Qi N-M. Novel mini *β*-TCP 3D perfusion bioreactor for proliferation and osteogenic differentiation of bone marrow mesenchymal stem cells. *Biotechnol Bioprocess Eng* 2010; **15**: 329-340.
20. Tobita K, Ohnishi I, Matsumoto T, Ohashi S, Bessho M, Kaneko M, Matsuyama J, Nakamura K. Effect of low-intensity pulsed ultrasound stimulation on callus remodelling in a gap-healing model: evaluation by bone morphometry using three-dimensional quantitative micro-CT. *J Bone Joint Surg Br* 2011; **93**: 525-530.
21. Tsumano N, Kubo H, Imataki R, Honda Y, Hashimoto Y, Nakajima M. Bone regeneration by dedifferentiated fat cells using composite sponge of alfa-tricalcium phosphate and gelatin in a rat calvarial defect model. *Applied Sciences* 2021; **11**: 11941.