ORIGINAL ARTICLE

Bone Induction by α-tricalcium Phosphate Microparticle Emulsion Containing Simvastatin

Akito TATEYAMA[1], Akihito KATO[1], Hirofumi MIYAJI[1], Erika NISHIDA[1], Yasuhiko IWASAKI[2], Syuji FUJII[3,4], Kohei KAWAMOTO[1], Kanako SHITOMI[1], Tomokazu FURIHATA[1], Kayoko MAYUMI[1], and Tsutomu SUGAYA[1]

[1]Department of Periodontology and Endodontology, Division of Oral Health Science, Faculty of Dental Medicine, Hokkaido University, Sapporo, Japan
[2]Department of Chemistry and Materials Engineering, Faculty of Chemistry, Materials and Bioengineering, Kansai University, Osaka, Japan
[3]Department of Applied Chemistry, Faculty of Engineering, Osaka Institute of Technology, Osaka, Japan
[4]Nanomaterials Microdevices Research Center, Osaka Institute of Technology, Osaka, Japan

Synopsis
To improve the degradability and operability of conventional bone graft materials, we fabricated a water-oil emulsion based on α-tricalcium phosphate (α-TCP) bone paste. Simvastatin, a lipophilic hyperlipidemia treatment agent, reportedly enhances the expression of bone morphogenetic protein-2 and subsequent bone formation. Accordingly, we assessed the bone forming effects of α-TCP bone-paste containing simvastatin in rat cranial bone defects.

Bone paste exhibited porous structure and generation of hydroxyapatite after solidification. X-ray image analysis and histological examination were carried out after implantation of bone paste into rat skull defect. The results showed that new bone was formed after implantation of bone paste containing simvastatin. In particular, bone volume in the 0.1 mg simvastatin group was significantly promoted when compared to controls (no implantation). No bone paste residue was observed in the bone defect at 4 weeks after surgery. Therefore, α-TCP bone paste containing simvastatin is degradable and beneficial for bone tissue engineering.

Key words: *α-tricalcium phosphate (TCP), bone tissue engineering, bone paste, water-oil emulsion, simvastatin*

Introduction

Significant bone loss due to periodontal disease, tumors or cysts requires the alveolar bone reconstruction. Block-shaped bone substitute materials have been used clinically to fill large bone defects [1, 2]; however, their low shapeability leads to low operability. To improve their operability, paste-like bone filling agents (bone pastes) are anticipated to be developed as bone graft materials. Calcium phosphate bone paste has been already applied for clinical use [3, 4]; however, bone paste is non-absorbable and

shows high density after solidification. Hardened bone paste is therefore thought to inhibit cell invasion and replacement into bone tissue, and resulting in long-term paste residue in the body, increased risk of infection and aberrant healing.

Alpha-tricalcium phosphate (α-TCP; $Ca_3(PO_4)_2$) has been extensively examined as a self-hardening bone substitute material that has the same composition as, but a different crystal structure from, β-TCP [5, 6]. α-TCP possesses high degradability in water and produces calcium-deficient hydroxyapatite (HA) by hydrolysis [7, 8]. Bone paste containing α-TCP would be suitable for paste-hardening via dissolution of α-TCP particles and the formation of a HA crystal network, while providing a porous structure to improve cell infiltration and biodegradation of bone cement [8, 9, 10]. Iwasaki et al. fabricated a novel bone paste using α-TCP and poly (lactic-co-glycolic acid) (PLGA) fine particles based on particle-stabilized water-oil emulsions. Fine particles and oil droplets self-aligned to form a stabilized emulsion, and then a porous HA structure was formed by the hydration reaction [11]. However, it has not yet been investigated whether emulsion-templated bone paste promotes bone formation in vivo.

Simvastatin is a lipophilic statin drug that and inhibits 3-hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol synthesis [12]. Simvastatin reportedly promotes the expression of bone morphogenetic protein-2 (BMP-2), a differentiation factor for osteoblasts, and subsequently facilitates bone formation in animal femur and skull [13, 14, 15]. Therefore, we speculate that a combination of water-oil emulsion based on α-TCP bone paste and simvastatin would induce an osteogenic effect. Accordingly, we fabricated emulsion-templated α-TCP fine particle bone paste containing simvastatin and histologically assessed its bone forming effects in rats.

Materials and Methods

1. Preparation of emulsion-templated bone paste

α-TCP was synthesized by annealing of β-TCP (BETA-TCP; Tomita Pharmaceutical Co., Ltd., Tokushima, Japan) for 5 h at 1200°C. α-TCP was crushed by ball mill (Pot mill rotator PM-001; AS ONE Corporation, Osaka, Japan) with alumina balls (1.5 mm diameter) at 450 rpm for 1 h and then passed a sieve having mesh of 25 μm (test sieve; Tokyo Screen Co., Ltd., Tokyo, Japan) to form the α-TCP fine powder. Subsequently, α-TCP powder, castor oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and distilled water were vortex-mixed for 30 s and homogenized using a probe-type ultrasonic homogenizer (Sonifier® SLPe40; Emerson Japan, Ltd., Tokyo, Japan) for 5 min to form emulsion-templated bone paste in a sample tube (Fig. 1A). Bone paste with α-TCP/oil/water blend ratio of 50/25/25 wt% was employed.

2. Characterization of the bone paste

β-TCP, α-TCP and bone paste (1 week after fabrication) were characterized using X-ray diffraction (XRD) (RINT2000; Rigaku Corporation, Tokyo, Japan). Cu Ka radiation at 40 kV and 40 mA was used. Diffractograms were obtained from 2 h = 10° to 90° at increments of 0.02° with a scanning speed of 4°/min.

The 4-week bone paste sample after fabrication was dehydrated in an ethanol series and then analyzed using a scanning electron microscope (SEM) (S-4000; Hitachi, Ltd., Tokyo, Japan) at an accelerating voltage of 10 kV after coating with a thin layer of Pt-Pd.

Bone paste with simvastatin (0 and 2.5 mg/pieces; Wako Pure Chemical Industries, Ltd.) was injected into a silicone rubber ring (2.5 mm height and 5.6 mm diameter) and then left to stand in an incubator (37°C and 95% humidity). After solidification of bone paste, blocks were provided for compression test at 1, 2, 3, 4 and 6 weeks using a universal testing machine (EZ-S; Shimadzu Corporation., Kyoto, Japan). The maximum value up to a displacement of 25% at a crosshead speed of 0.5 mm/min was taken as compressive strength (MPa).

3. Bone forming effects of bone paste in rat cranial bone

Animal experimental protocols followed the institutional animal use and care regulations of

Hokkaido University (Animal Research Committee of Hokkaido University, Approval number 13-204). Forty-five 10-week-old male Wistar rats weighing 190–210 g were given general anesthesia by intraperitoneal injection of medetomidine hydrochloride (0.15 mg/mL, Domitor; Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan), Midazolam (2 mg/mL, Dormicum; Astellas Pharma Inc., Tokyo, Japan), butorphanol tartrate (2.5 mg/mL, Vetorphale; Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and local injection of 2% lidocaine hydrochloride with 1:80,000 epinephrine (Xylocaine Cartridge for Dental Use; Dentsply Sirona K.K., Tokyo, Japan).

After exposing rat cranial bone, circular defects were created with a trephine bar (diameter 4.5 mm). Subsequently, bone defects in rats received α-TCP bone paste implants (0.1 g/defect) containing simvastatin (0, 0.01, 0.1 and 1 mg). No implantation was performed in the control group. Skin was then sutured (BioFit-D 4-0; Washiesu Medical Corporation, Tokyo, Japan) and treated with tetracycline hydrochloride (achromycin ointment; POLA PHARMA INC., Tokyo, Japan) to prevent postoperative infection. At 1 and 4 weeks post-surgery, rats were euthanized using an overdose of sodium pentobarbital (100 mg/kg, Somnopentyl; Kyoritsu Seiyaku Corporation, Tokyo, Japan). Four-week postoperative samples were assessed by X-ray images (Dexco ADX 4000 W; Dexcowin japan Co., Ltd., Hyogo, Japan), and the area of radiolucency was measured using software (ImageJ 1.41; National Institutes of Health, Bethesda, MD). Ten and thirty-five samples were collected at 1 week and 4 weeks for histological observation, respectively. Samples were fixed in 10% buffered formalin, decalcified by 10% ethylenediaminetetraacetic acid and embedded in paraffin. After thin slicing (5 μm), sections were stained with hematoxylin and eosin and observed using light microscopy. Histomorphometric measurements of newly formed bone in 4-week specimens were performed using software.

4. Statistical analysis

Means and standard deviation of each parameter were calculated for each group. Statistical differences were analyzed using Tukey's HSD test. p-values < 0.05 were considered to be statistically significant. All statistical procedures were performed using a software package (SPSS 11.0; IBM Corporation, Armonk, NY).

Result

1. Characterization of bone paste

SEM images of the emulsion based on α-TCP bone paste confirmed a porous structure; pore sizes of 50 to 100 μm were observed (Fig. 1B). XRD analysis of β-TCP powder, α-TCP powder (sintered β-TCP) and bone paste confirmed β-TCP, α-TCP and HA, respectively (Fig. 2A). Compressive strength of bone paste not including simvastatin was 0.95, 1.42, 1.47, 1.73 and 2.05 N/mm^2 at 1, 2, 3, 4 and 6 weeks, respectively. In contrast, compressive strength of bone paste including simvastatin (2.5 mg/piece) was 1.07, 1.2, 1.27, 1.52 and 1.77 N/mm^2 at 1, 2, 3, 4 and 6 weeks, respectively. Both bone pastes showed gradual increases in strength from 1 to 6 weeks (Fig. 2B).

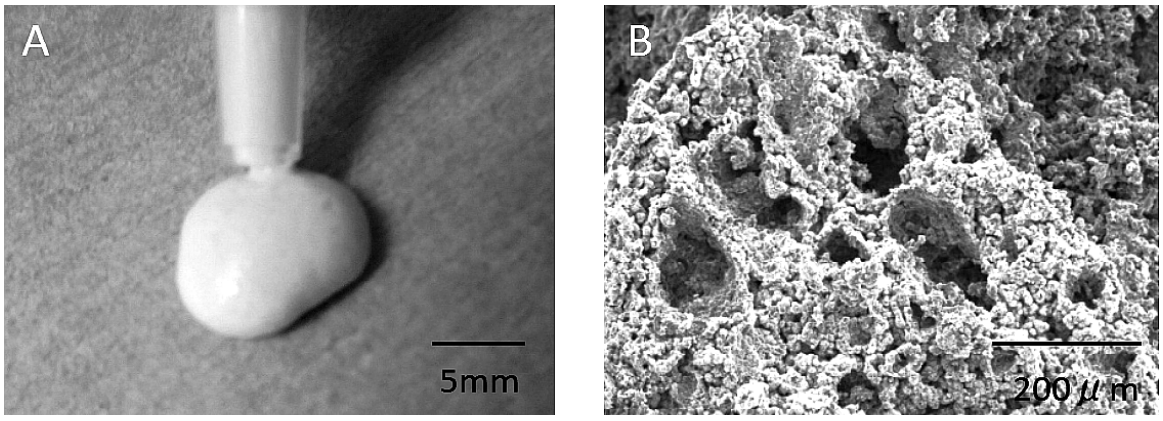

Figure 1 (A) Photograph of α-TCP bone paste. (B) SEM image of α-TCP bone paste emulsion.

No significant differences were found between pure bone paste and simvastatin containing bone paste.

2. X-ray image assessment of bone formation

In the control group, a circular defect area was clearly seen on X-ray images, while in the simvastatin-treated group, the bone defect area was markedly reduced (Fig. 3A-E). Radiolucency area related to bone defect was 13.2, 11.8, 11.5, 9.5 and 11.9 mm^2 in the control, and 0, 0.01, 0.1 and 1 mg simvastatin groups, respectively (Fig. 3F). The area in the 0.1 mg simvastatin group was significantly lower than that in controls.

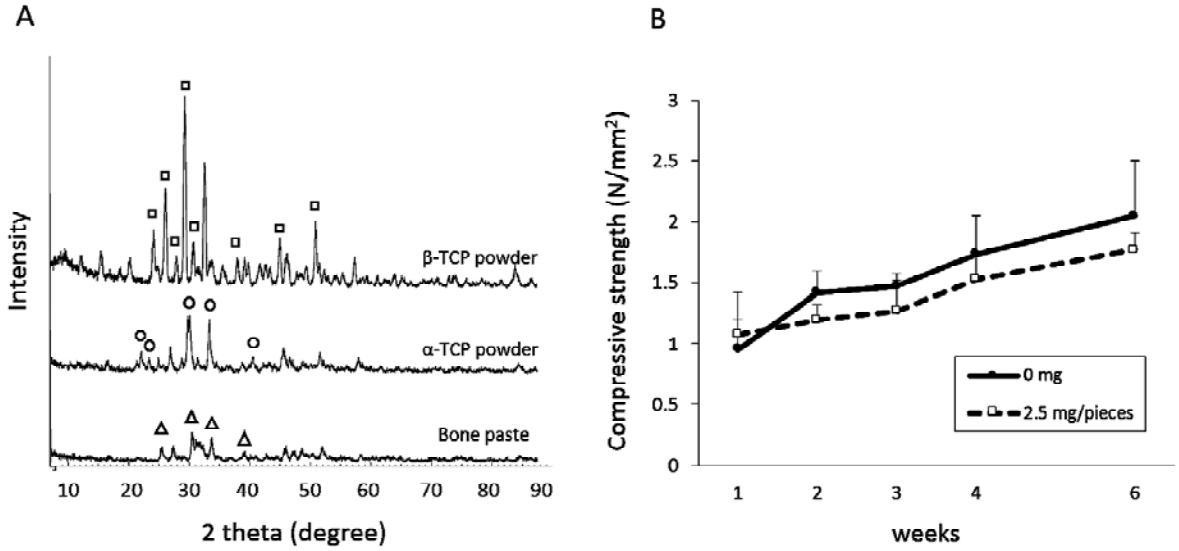

Figure 2 (A) X-ray diffraction patterns of β-tricalcium phosphate (β-TCP) powder, α-tricalcium phosphate (α-TCP) powder and bone paste emulsion. ϒ: β-TCP, ⊄: α-TCP and ∆: hydroxyapatite. (B) Compressive strength of pure bone paste and simvastatin-containing bone paste (2.5 mg/pieces).

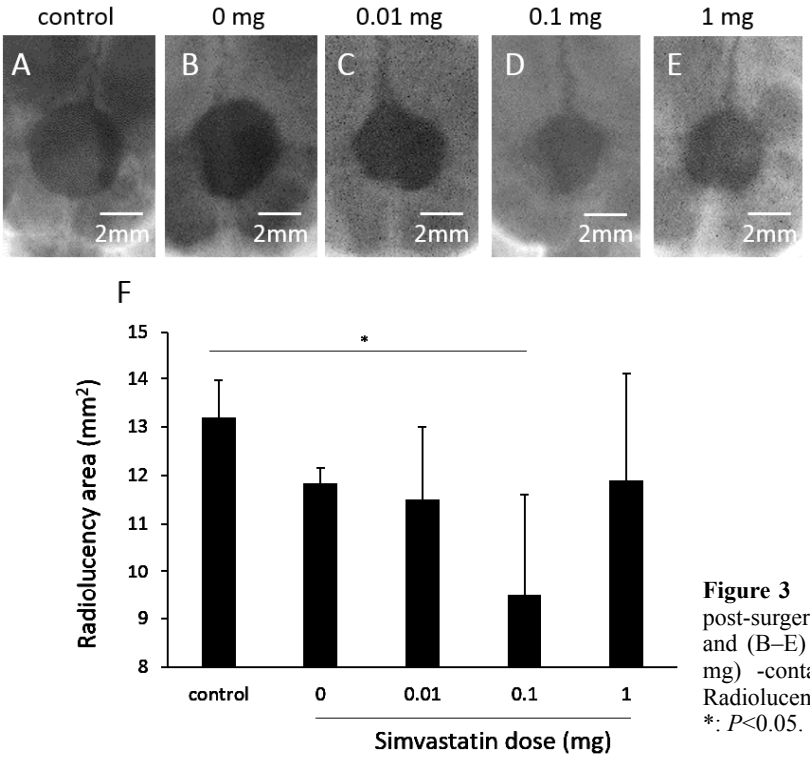

Figure 3 (A-E) X-ray images at 4 weeks post-surgery. (A) Control (no implantation) and (B–E) simvastatin (0, 0.01, 0.1 and 1 mg) -containing bone paste groups. (F) Radiolucency area at 4 weeks post-surgery. *: $P<0.05$.

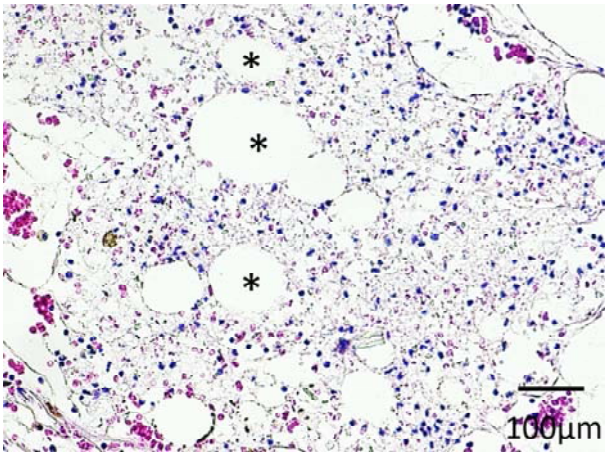

Figure 4 Histological findings at 1 week post-surgery. Oil droplets (*), inflammatory cells and erythrocytes were observed in bone paste. Staining: hematoxylin and eosin.

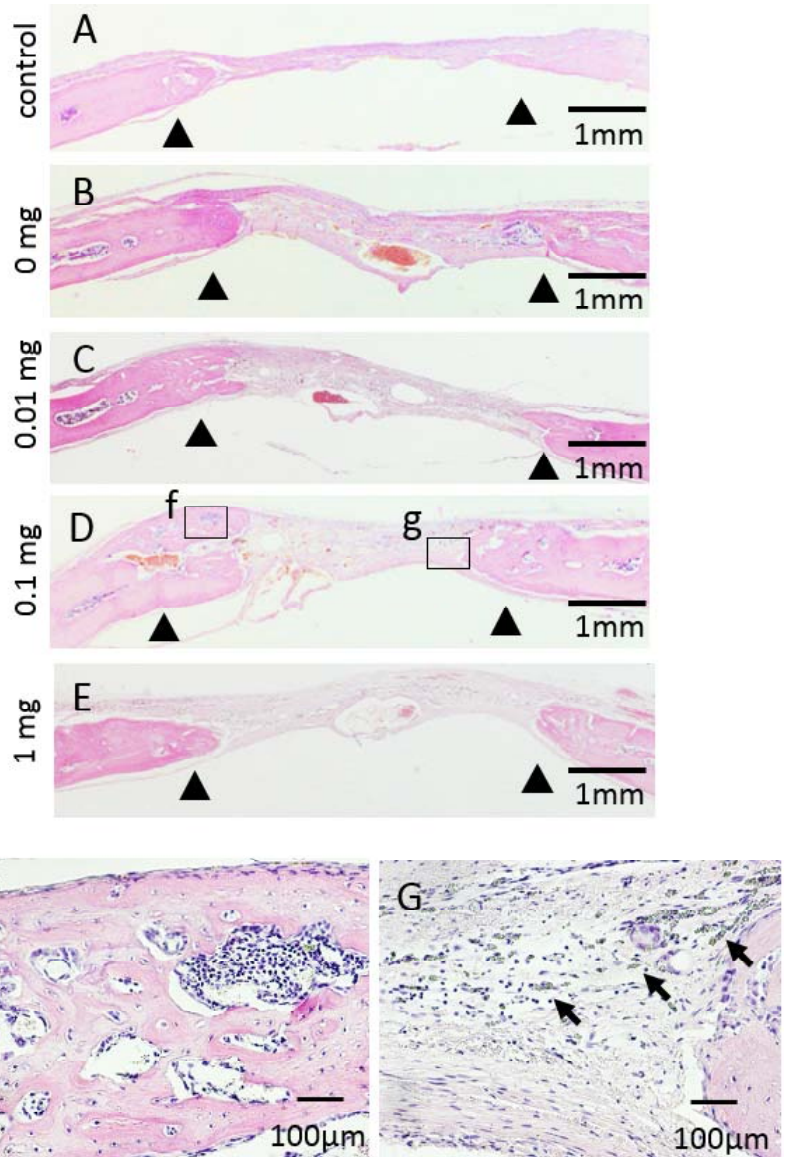

Figure 5 (A-G) Histological findings at 4 weeks post-surgery. (A) Control (no implantation) and (B–E) simvastatin (0, 0.01, 0.1 and 1 mg) -containing bone paste groups. (F, G) Higher magnification of framed area (f, g) in panel D, respectively. Arrows show bone paste residue and arrowheads show the border of the bone defect. Staining: hematoxylin and eosin.

3. Histological evaluation of bone formation

Histological images at one week post-surgery showed that bone paste residue was frequently present in the bone defect (Fig. 4). Bone paste residue showed oil droplets and ceramic-like structures containing some inflammatory cells and erythrocytes.

In control specimens (no implantation) at 4 weeks after surgery, bone healing was scarcely demonstrated. However, bone paste-treated groups showed new cranial bone formation continuous from pre-existing bone (Fig. 5A-E). In particular, application of bone paste containing 0.1 mg simvastatin increased the thickness of newly formed bone when compared to pre-existing bone (Fig. 5F). New bone included bone marrow, osteocyte- and osteoblast-like cells. Central areas of the bone defect were filled with fibrous tissue and blood vessels. Bone paste residue was sparsely found in the fibrous tissue (Fig. 5G).

New bone length was 0.3, 0.6, 0.6, 1.1 and 0.7 mm in the control, and 0, 0.01, 0.1 and 1 mg simvastatin groups, respectively. Application of bone paste tended to enhance bone healing. Bone paste containing 0.1 mg simvastatin significantly promoted bone formation when compared with the control, and 0 and 0.01 mg groups (Fig. 6).

Discussion

On SEM images, solidified bone paste exhibited pores with 50 to 100 μm diameters (Fig. 1B). α-TCP fine particles surrounded castor oil drops throughout o/w emulsion fabrication. The resulting porous structure would be beneficial for biodegradation in the body. Furthermore, interconnecting each pore enhances the ingrowth of cells and blood vessels. Iwasaki et al. fabricated an emulsion based on bone paste using α-TCP and PLGA fine particles and demonstrated the interconnected porous structure via early PLGA degradation [11]. Therefore, the bioactivity of simvastatin-containing bone paste may be increased by changing the particles in bone paste to give an interconnected structure. In addition, bone paste gradually hardened and showed HA peaks on XRD assessment (Fig. 2). Accordingly, HA via hydrolysis of α-TCP would occur in emulsion based on bone paste. In addition, the compressive strength of simvastatin containing bone paste was comparable to that of pure bone paste, suggesting that generation of HA was not inhibited by simvastatin application. The solidification of the bone paste emulsion would be beneficial for storage of HA and simvastatin in the bone defect and preservation of the regenerative field.

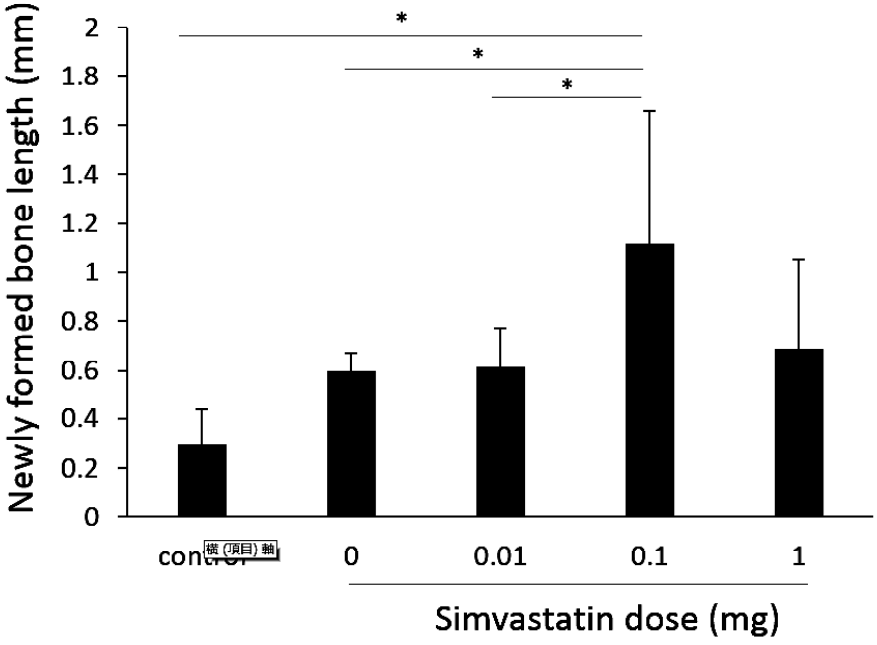

Figure 6 Newly formed bone length at 4 weeks post-surgery. *: $P<0.05$.

Histological specimens at 4 weeks confirmed that new bone induction was remarkable in the rat cranial bone defects receiving 0.1 mg simvastatin (Fig. 5 and 6). In addition, the radiolucency area in 4-week X-ray images was significantly lower in the 0.1 mg simvastatin group when compared to the control group (Fig. 3F). This suggests that application of simvastatin-containing α-TCP bone paste facilitated bone formation in cranial bone defects. Many studies have confirmed that simvastatin increases the expression of BMP-2 in bone cells [12, 16, 17]. Simvastatin is thought to activate pre-existing bone around the defect and to promote the differentiation of undifferentiated mesenchymal cells into osteoblasts. Regarding to simvastatin dose, the 0.1 mg dose group tended to show increased bone healing when compared to the 0.01 mg and 1 mg groups. Stein et al. reported that simvastatin (0.1, 0.5, 1.0, 1.5, or 2.2 mg) was implanted into rat mandibular bone. They found that application of simvastatin at a high dose (2.2 mg) caused severe swelling via inflammation and that a lower dose (0.5 mg) increased bone formation [18]. Similarly, we observed that the bone forming effects of simvastatin did not exhibit a dose-dependent relationship. The middle dose of simvastatin (0.1 mg) would be suitable for bone paste emulsion to promote bone induction in rats.

The bone paste emulsion comprised a biosafe substrate, α-TCP artificial bone, castor oil nonionic surfactant and pure water. On histological observation, 1-week specimens showed the w/o emulsion structure, with inflammatory cells and erythrocytes in bone paste residue (Fig. 4). However, residual bone paste was rarely observed in 4-week specimens. Hence, we speculate that bone paste possess good biodegradability in the body, in contrast to conventional bone cement materials. Contamination by blood clots and tissue fluid after implantation may affect the hydration of bone paste to generate low-crystallinity HA, thus accelerating in vivo degradation. Further comparative studies are needed in order to elucidate the solidification and degradation processes of the bone paste emulsion under in vitro and in vivo condition.

In this study, we fabricated an α-TCP bone paste emulsion containing simvastatin. Bone paste exhibited a porous structure, HA generation properties and time-dependent solidification. Histological examination and X-ray image analysis revealed that implantation of bone paste containing 0.1 mg simvastatin significantly promoted bone formation in rats. Based on these results, emulsion templated α-TCP and simvastatin bone paste would be beneficial for bone tissue engineering.

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Corresponding author*:*
Hirofumi Miyaji, D.D.S.,Ph.D.
Department of Periodontology and Endodontology,
Division of Oral Health Science,
Faculty of Dental Medicine,
Hokkaido University
N13, W 7, Kita-ku, Sapporo, 060-8586, Japan
Tel: +81 11 706 4266
Fax: +81 11 706 4334
E-mail: miyaji@den.hokudai.ac.jp
(Co-corresponding author)
Akito Tateyama, D.D.S.
E-mail: akitotateyama@den.hokudai.ac.jp