INTRODUCTION
Since tooth missing decreases patient's QOL, dental prosthesis, such as removable denture, bridge and dental implant, is provided to recover patient's oral functions. Loss of alveolar bone occurs after tooth extraction, which sometimes causes difficulty in subsequent prosthetic treatment. Thus, preservation and/or augmentation of alveolar ridge after tooth extraction are advantageous. Recently, various bone substitutes were developed for alveolar ridge preservation. However there are few substitutes that stimulate bone formation.

Statins are widely-used cholesterol-lowering drugs, which inhibit cholesterol biosynthesis by blocking...
lesterol biosynthesis by blocking HMG-CoA reductase, an important enzyme in mevalonate pathway. Statins include naturally existing lovastatin and chemically modified simvastatin and pravastatin, and they have been widely used for the treatment of hypercholesterolaemia. Interestingly, Mundy et al. demonstrated that simvastatin enhances BMP-2 mRNA expression in osteoblastic cells and stimulate bone formation in rodents. Following this study, the effects of statins in bone have been extensively examined both in vitro and in vivo, which confirmed positive anabolic effects of statins in bone. Statins promote differentiation of osteoblastic cells and induce osteoclast apoptosis. Furthermore, local application of simvastatin in the vicinity of bone by injection and/or with different carriers has been reported. Lee et al. applied injection style of simvastatin to rat mandibles. Wong et al. applied collagen sponge containing 0.5 mg simvastatin to parietal bone defects in rabbits and showed tiny but statistically significant stimulation of bone repair. Calvaria bone defect model has been used to examine effect of local application of simvastatin. Recently Wu et al. have demonstrated that simvastatin application with poly lactic and glycolic acid co-polymer (PLGA) to the tooth sockets increases alveolar ridge. Although PLGA is a bio-degradable polymer and used clinically as a biomaterial, this material is producing acids, which seems to be unfavorable for bone regeneration.

Tricalcium phosphate (TCP) is a biodegradable ceramics. Since β-TCP, one form of TCPs, shows favorable osteoconduction and biodegradation, β-TCP has been clinically used as a bone substitute especially for the future implant placement site. α-TCP is an isomer of β-TCP and it is more biodegradable than β-TCP. Kihara et al. have reported that α-TCP works osteoconductive and biodegradable scaffold for bone regeneration maintaining the regeneration space in rabbit parietal bone defects. Yamada et al. have further demonstrated that α-TCP works as more favorably degradable scaffold compared to β-TCP on rabbit parietal bone. These studies suggested potentiality of α-TCP as a bone substitute for the future implant placement site. The purpose of the present study is to examine whether local application of simvastatin augments the bone of the tooth-extracted site when we use α-TCP as a simvastatin carrier.

MATERIALS AND METHODS
This study was approved by the institutional committee for animal experiments (No.0080249).

Preparation of α-TCP containing simvastatin
Simvastatin was purchased from OHARA Pharmaceutical Co., Ltd. Tokyo, Japan. Porous α-TCP rods (1x1x10mm size) was kindly provided by ADVANCE Co., Ltd. Tokyo, Japan. X-ray diffraction analysis revealed that the component of the material was α-TCP and the pore size of this material was 5-8 μm. Simvastatin was dissolved in ethanol and α-TCP rods were soaked in the solution of different concentrations of simvastatin. Then, the rods were dried to volatilize ethanol. Finally, the rods containing 0.25, 0.50 and 1.0 mg simvastatin were prepared.

Fig. 1. Shape of porous α-TCP rod. The size was 10×1×1mm.
**Mandibular incisor extraction**

Forty-six male Wistar rats, 10 weeks old, 380-420g, were used. Nine days before tooth extraction, mandibular right incisors were cut three times above gingival margin every 3 days under ether anesthesia to weaken periodontal ligament. Three days after the last cutting, animals were anesthetized with an intramuscular injection of ketamine and xylazine and the right incisors were extracted.

**Application of simvastatin to the tooth sockets and experimental groups**

Immediately after tooth extraction, the α-TCP rods containing 0, 0.25, 0.50 and 1.0mg simvastatin were inserted into the tooth sockets of the animals of the experimental 4 groups: 10 rats in each group. In the animals of a control group, the tooth sockets of 6 rats were untreated. Half animals in each group were sacrificed at 4 and 8 weeks, respectively, with an injection of excess amount of the anesthetics. The right mandibles were dissected out and fixed in 10% naturalized formalin solution.

**Radiological analyses**

After excessive washing with water, firstly, the mandibles were photographed with soft X-ray (Softex SRO-M50, Sofron, Tokyo, Japan). The exposure time was 30 s at 4 kV with 4 mA on FR-IX film (Fuji Photofilm, Tokyo, Japan). Secondarily, bone mineral content (BMC) of the whole mandible was measured with dual-energy X-ray absorptiometry (DEXA) (DCS-600, Aloka, Tokyo, Japan). Thirdly, the mandibles were analyzed with micro-computed tomography (µ-CT) (InspeXio; Shimmzu Science East Corporation, Tokyo, Japan). Images of cross sections including the mesial root axis of the first molar were obtained (Fig. 2). Then, area and width of radio-opaque area in this cross section were measured (Fig. 2).

**Histological observation**

After radiological analyses, the mandibles of the rats, which were sacrificed at 8 weeks, were decalcified in 5% formic acid for 1 week at 4°C, dehydrated with ethanol and embedded in paraffin. Cross sections including the mesial root axis of the first molar, which was similar to the plane analyzed with µ-CT, were prepared and stained with hematoxin and eosin.

**Statistical Analysis**

Statistical differences from DXA and micro CT data at each time point were analyzed with One Way ANOVA and Scheffe post-hoc multiple comparison tests. Probabilities of less than 0.05 were accepted as significant.

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![Fig. 2](image_url)  
**Fig. 2.** Images of cross sections including the mesial root axis of the first molar. Area and width of radio-opaque area in this cross section were measured.
RESULTS
Throughout the experiment, neither infection nor unusual inflammatory response was observed. Soft tissues of the extracted sites evenly healed at 4 and 8 weeks in all groups. Soft X-ray photographs revealed that α-TCP rods were firmly inserted in the tooth sockets in all the animals of the experimental groups (Fig. 3). At both 4 and 8 weeks, there was a tendency that the bone around tooth-extracted site, where simvastatin was applied, was thick compared to the one in the control group (Fig. 3). This finding was the most evident in 1.0mg simvastatin group. In all experimental groups, α-TCP rods still remained in the sockets at 8 weeks. BMC was measured with dual energy X-ray absorptiometry. Corresponding with the findings in soft X-ray photos, at 4 weeks, BMC of 1.0mg was higher than the ones of the control group and 0mg group (α-TCP alone) while BMC of 0.5mg group was also higher than the control group (Fig. 4a). At 8 weeks, BMC of both 0.5mg and 1.0mg groups was higher than the one of the control group (Fig. 4b). Micro-CT analysis demonstrated that radio-opaque areas of cross sections of 0.5mg and 1.0mg groups were higher than the one of the control group.

Fig. 3. Soft X-ray photographs of the mandibles at 4 (a) and 8 weeks (b).

Fig. 4. Bone mineral contents of the whole mandibles from DXA analysis at 4 (a) and 8 weeks (b). At 4 weeks, BMC of 0.5mg and 1.0mg groups were significantly higher than that of control group. There was also significant difference between 1.0mg and 0mg group. At 8weeks, BMC of 0.5mg and 1.0mg groups were significantly higher than that of control group. (* vs the control, at P<0.05. † vs the 0mg, at P<0.05)
control group at 4 weeks (Fig. 5a). At 8 weeks radio-opaque area of cross section of 1mg group was higher than the one of the control group (Fig. 5b). At 4 and 8 weeks bone widths of the tooth-extracted sites of 0.5mg and 1mg groups were larger than the one of the control group (Fig. 6a, b).

Histological images at 8 weeks are presented in Fig. 7. In the control group the socket was mostly occupied with newly-formed bone, which connected to the socket bony wall. The bone, which surrounded the original socket, was homogenously matured bone with few lacunae (Fig. 7a). In the socket of 0mg group, in which α-TCP rod alone was applied, newly-formed bone connecting to the socket bony wall was observed (Fig. 7b). In this group newly-formed bone extended from the bony wall to α-TCP and cellular structure existed in α-TCP suggesting degradation of this material. The bone surrounding the original socket in this group contained few lacunae, which was similar to the corresponding bone in the control group. Contrarily, in 0.25, 0.5 and 1mg groups, newly-formed bone in the socket was less than the one in the control and 0mg.

![Fig. 5](image)

**Fig. 5.** Radio-opaque area in the cross section from micro CT analysis at 4 (a) and 8 weeks (b). At 4 weeks, the area of 0.5mg and 1.0mg groups were significantly higher than that of control group. At 8 weeks, there was significant difference between 1.0mg and control group. (*- vs the control, at P<0.05)

![Fig. 6](image)

**Fig. 6.** Bone width of the cross section from micro CT analysis at 4 (a) and 8 weeks (b). Bone width of 0.5mg and 1.0mg groups were significantly higher than that of control group at 4 and 8 weeks. At 4 weeks, there was also significant difference between 0.5mg and 0mg group. (*- vs control group, at P<0.05. †- vs 0mg group, at P<0.05)
Fig. 7. Histological images at 8 weeks. The control group (a), 0mg group (b), 0.25 mg group (c), 0.5mg group (d) and 1mg group (e).
groups, whereas the bone around the socket thickened outward. Notably, these thickened bone contained many lacunae, which was contrast to the control and 0mg groups (Fig. 7c, d, e).

DISCUSSION

In the present study, the effects of local simvastatin application on the alveolar ridge were examined after mandibular incisor extraction in rats. The reasons for using this animal experimental model were the following. First, using small animals like rats permits an experiment with large sample numbers. Second, large tooth sockets can be constantly and simply prepared by extracting mandibular incisors. Rat incisors continuously erupt, and the removal of occlusal contact by cutting the incisal edges every 3 days increases eruption speed and reduces the mechanical strength of the periodontal ligament. Following the procedure of a previous study, extraction of the incisors without fracturing the teeth is possible without difficulty. Finally, precise histological changes in the alveolar ridge after the incisor extraction have been already reported. However, in spite of these advantages in this experimental model, the height of alveolar ridge does not decrease significantly after the incisor extraction, probably because of the connection of the mesial part of the alveolar ridge to the alveolar ridge of the other side of the mandible subsequently mechanical stress being continuously applied to the alveolar ridge after the incisor extraction. This point is a disadvantage of the present experimental model. Kihara et al. have reported that α-TCP works well as a degradable scaffold for osteogenesis when applied to the rabbit parietal bone defects and that this material maintains the space for bone regeneration. Absorption of alloplasts in vivo has been recognized to occur through two processes, namely cell-mediated absorption by multinuclear giant cells and physical-chemical dissolution by tissue fluids. Eggli et al. suggested that absorption of TCPs is initiated via phagocytosis by multinuclear giant cells. On the other hand, Schliephake et al. suggested that their absorption begins with dissolution by tissue fluids, and is then followed by cell-mediated absorption. Thus, it is very likely that the initial absorption of TCPs is influenced not only by cell-mediated absorption but also by physical-chemical dissolution.

In the present study, radiological analyses demonstrated that α-TCP rods in all groups still remained in the sockets at 8 weeks. Although α-TCP degradation was evident in the histological image especially in 0mg group, α-TCP degradation was not extensive in the present study. However, α-TCP particles are degradable and exchange to new bone when they are applied to rabbit parietal bone defects. It is likely that body fluid flow in the present tooth socket is less than the one in the parietal bone defects resulting in less α-TCP degradation. Furthermore, surface area of the present α-TCP rod is obviously less than the one of the α-TCP particle, which probably caused less degradation of the present α-TCP rod. Regarding the α-TCP degradation, it is also clear that simvastatin inhibited the material resorption because α-TCP rods containing simvastatin were less resorbable than α-TCP alone.

Simvastatin is a common cholesterol-lowering drug used for prevention and treatment of cardiovascular diseases. Recent studies have shown that simvastatin is also capable of promoting bone formation. Since simvastatin undergoes extensive first-pass extraction in the liver after oral administration, the availability of the drug to the general circulation is low (<5%). This pharmacokinetic characteristic leads to a lower
concentration of the drug in other tissues including bone. Intermittent injections of simvastatin to mice parietal bones, once a day for five days, are required to promote local bone formation, which suggests that simvastatin concentration at the vicinity of the bone should be kept at a certain level for a certain period for stimulating local bone formation.

A carrier, which is able to release simvastatin slowly, would be effective in stimulating local bone formation. In the previous studies the following carriers have been used for local simvastatin application: methyl-cellulose, collagen, calcium sulfate and PLGA. We have also used collagen, calcium sulfate and PLGA for simvastatin carriers and applied to rat mandibular incisor sockets and/or rat parietal bone defects. In our previous experiments calcium sulfate and PLGA were suitable simvastatin carriers compared to collagen. However, degradation of calcium sulfate is fast to achieve complete bone regeneration in critical-sized parietal bone defects in rats. In addition, although PLGA works well as simvastatin carrier, this material inhibits new bone formation when it is applied to rat incisor socket. For stimulating bone formation in bone defect surrounded by bony wall like a tooth socket, a carrier, which releases simvastatin slowly and which acts as a scaffold for bone regeneration, would be ideal. Furthermore, degradable material exchangeable for newly-formed bone would be advantageous.

We have already reported that α-TCP is degradable and exchangeable for newly-formed bone when this material is applied to rabbit parietal bone defects. Since this material has porous structure, we are speculating that porous α-TCP could work as ideal simvastatin carrier. As we speculated, α-TCP containing simvastatin slowly and constantly releases simvastatin after the initial rapid release within 24 hours although this was observed in vitro incubation experiment. It is likely that the releasing mechanism at constant rate is due to mechanically trapping the hydrophobic compound in the pores of the material. Indeed, α-TCP particles containing simvastatin stimulates new bone formation exchange to new bone when applied to rat parietal bone defects. Thus, we conducted the present study. In the present study we applied α-TCP rods containing simvastatin to rat incisor socket. This combined material increased the bone width of the tooth-extracted site. Histologically, the bone around the socket thickened outward. These present results demonstrated local anabolic action of simvastatin to the bone. Notably, in the experimental groups, outside of the tooth socket there were many lacunae compared to the bone of the control group, which suggests active bone remodeling in this area. Thus, outside of the tooth socket, where simvastatin was applied, both bone formation and resorption might be active while bone formation might be more active than bone resorption. This is likely because simvastatin stimulates bone formation and it inhibits bone resorption. However, precise histomorphometric analysis with fluorescence labeling is required to confirm this speculation.

In the present study, new bone formation in the tooth socket was retarded when simvastatin was applied. Although simvastatin stimulates bone formation, this effect is in concentration-dependant manner while high concentration of simvastatin induces cellular apoptosis. Therefore, it is possible that this toxic effect of simvastatin appeared in the tooth socket. Since rat incisor socket is narrow and deep, fluid flow in the socket is less than the one in the parietal bone defect, where simvastatin stimulated new bone formation as
we have reported recently. It is reasonable to speculate that in the present study simvastatin concentration in the socket was toxic whereas simvastatin concentration outside of the socket was optimum to stimulate bone formation. The particle material would be more resorbable than the rod material because of more surface area. Since the incisor socket in the present study was narrow and deep, we could not apply the particle material to the socket. In the clinical situation the tooth socket is large enough to accept the particle material and fluid flow is more expected than the incisor socket in the present study. Thus, although we could not demonstrate the positive effect of the material on the socket healing in the present study, it is likely that the local application of simvastatin with \( \alpha \)-TCP as its carrier is clinically potential to preserve alveolar ridge after tooth extraction.

**CONCLUSION**

In the present study we examined whether local application of simvastatin augments the bone of the tooth-extracted site using \( \alpha \)-TCP as a simvastatin carrier. The present results indicate that local application of simvastatin with \( \alpha \)-TCP as its carrier would be potential to preserve alveolar ridge after tooth extraction although further studies are required.

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