Effects of Different Sizes of β-tricalcium Phosphate Particles on Bone Augmentation within a Titanium Cap in Rabbit Calvarium

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This study evaluated the effects of different sizes of β-TCP particles on bone augmentation within a titanium cap. In 20 rabbits, the calvarium was exposed and a circular groove was prepared. After marrow penetration, a standardized hemispherical titanium cap was placed in the circular groove. The cap was filled with small-sized (100-250 μm) or medium-sized (250-500 μm) β-TCP particles for the experimental site and without β-TCP for the control site. After one and three months of healing, the animals were euthanized and examined histologically. There was a statistically significant difference in the amount of mineralized bone generated between the experimental and control groups in the three-month specimens. Furthermore, the medium-sized particles showed significantly more mineralized bone than did the small-sized particles. Based on these findings, we suggested that β-TCP might be effective for bone formation and that medium-sized particles are more useful than small-sized particles in bone maturation.

Key words: β-TCP, Particle size, Bone augmentation

INTRODUCTION

Guided bone regeneration (GBR) is used to regenerate specific portions of lost bone11. Its usefulness has been shown experimentally in research studies7–8 and clinically with dental implants9–11. For placement and success of dental implants, the availability of adequate bone volume for anchorage plays a critical role. To circumvent this limitation pertaining to bone volume, a number of studies have examined the application of GBR around an implant and these studies have shown that it is a useful technique12–17. However, in several experiments, it was difficult to augment bone beyond the skeletal envelope with only a membrane18–21. Using an autogenous bone graft and graft materials is more effective than using a membrane alone11,20,21, so the use of filling materials coupled with the use of barrier membranes has recently gained new interest because this allows for a space-making effect when the membrane is not sufficiently stiff. Autogenous bone seems to be the gold standard among the numerous filling materials, but its use is limited by the necessity of additional surgical procedure, the difficulty of obtaining large amounts of donor tissue, and the risk of complications22–24. For these reasons, many attempts have been made to find a synthetic graft material that matches the features of an autogenous bone graft.

Beta-tricalcium phosphate (β-TCP) and hydroxyapatite (HA) have been used in some experimental and clinical studies25–28. β-TCP is a bone substitute material that provides osteoconductive activity and is biodegradable.

Of late, a new type of highly pure β-TCP has been manufactured using a mechanochemical method and is available for clinical use as a potent bone-grafting substitute29. In experimental studies, early bone formation was first observed around the grafted β-TCP. The grafted β-TCP then degraded gradually and was finally replaced by mature new bone. This material has osteoconductive properties, and the particle size and pore size of β-TCP influence osteoconduction. However, studies on the influence of particle size on osteoconduction have reached variant conclusions30–32.

The aim of this study, therefore, was to evaluate the effects of two different sizes of β-TCP particles on bone augmentation within a titanium cap in rabbit calvarium.

MATERIALS AND METHODS

Animals

Twenty adult male Japanese white rabbits, weighing 2.5 to 2.8 kg, were used for the study. Before the experiment, the health of the rabbits was monitored
for two weeks. The rabbits were kept in a standard
cage in an experimental animal room (24℃, 55% hu-
midity, 1 atm, 12-hour light/dark cycle) and fed a
standard laboratory diet and water. This study was
approved by the Animal Experimentation Commit-
tee of Nihon University School of Dentistry.

Titanium cap

The experimental device was a custom-made, stan-
dardized, stiff, hemispherical titanium cap with a
smooth surface (Ti > 99.5%, JIS H6400, Sankin,
Tokyo, Japan). The dimensions were 4 mm in
height, 8 mm in diameter, and 0.2 mm in thick-
ness. The cap was cleaned in 0.1 w/v% germitol
water (Maruishi Pharmaceutical Co. Ltd., Osaka,
Japan) in an ultrasonic bath to remove contamina-
tants and sterilized using ethylene oxide gas.

Graft materials

We used two particle sizes of high-purity β-TCP
(OSferion Olympus, Japan; porosity, 75%; pore size,
100-400 μm). The particle sizes were small (100-250
μm) and medium (250-500 μm).

Anesthesia and surgery

All operations were conducted under sterile con-
tions. General anesthesia was induced by injecting
pentobarbital sodium (Nembutal®, Abbot Laborato-
ries, North Chicago, IL, USA; 0.4 ml/kg) via an ear
vein and maintained by gas inhalation with halothane (Fluothane®, Takeda Chemical Industries
Ltd., Osaka, Japan; 1.5 to 2.0 vol%). The forehead
of the rabbit was shaved. An injection of approxi-
mately 1.8 ml of lidocaine HCl containing 1:80,000
epinephrine (2% Xylocaine®, Astra Japan Ltd., Fujisawa
Pharmaceutical Co. Ltd., Osaka, Japan) was used as
a local anesthetic to reduce hemorrhaging under the
skin over the calvarium.

A flap was made using a midsagittal incision
(#15 surgical blade, Feather, Osaka, Japan) and ex-
foliated from the forehead. The periosteum was in-
cised and lifted to expose the calvarium on both sides
of the midline. A circular groove was prepared on
each side of the midline using a trephine drill (Bone
trephine 131001, Technica, Tokyo, Japan) with an
inner diameter of 8 mm, under profuse irrigation
with sterile saline. The circular groove did not pene-
trate the calvarium. This groove ensured a tight
seal between the edge of the titanium cap and the
surface of the skull. The depth of the groove was
standardized by drilling into the bone until the cut-
ting edge of the trephine was just below the bone
surface at a depth not exceeding 0.5 mm. Nine small
holes were drilled with a No. 2 round bur to induce
bleeding from the bone marrow space within the cir-
cle (Fig. 1). Two standardized titanium caps were
pressed into the circular grooves on each side of the
midline (Fig. 2). Prior to placement, the experimen-
tal caps were either filled with β-TCP as the exper-
imental site or without β-TCP as the control site.
Experimental sites were further divided into two
groups and filled with either small- or medium-sized
β-TCP particles.

The periosteum was replaced, covering as much
of the titanium cap as possible, and was sutured with
absorbent suture (5-0 Opepolix® II, Azwell, Osaka,
Japan). Each reflected flap was repositioned to cover
the titanium cap and sutured with interrupted su-
tures (4-0 silk Mani® suture, Mani Co. Ltd., Tochigi,
Japan). As the periosteum was not elastic, it was
impossible to cover the top of the cap (approximately
4 mm above the bone surface). Postoperatively, the
rabbits received 25,000,000 units of penicillin G
(Sigma-Aldrich, St. Louis, MO, USA) in a volume of
0.1 ml/kg, administered as a single intramuscular
injection. The rabbits were divided equally into two
groups and allowed to heal for either one month or
three months.

Fig. 1 A cutaneous flap was demarcated.

Fig. 2 A titanium cap was placed in the bone with the
margin of the cap fitted neatly in the groove.
Specimen preparation
After healing for either one month or three months the animals were euthanized with an overdose of pentobarbital. The calvarium bone with the titanium caps was dissected out, fixed in 10% neutral buffered formalin, dehydrated, and embedded in polyester resin (Rigolac-2004, Rigolac-70F, Nisshin EM Co. Ltd., Tokyo, Japan). One sagittal non-decalcified ground section (approximately 200 μm thick) from the central part of the titanium cap and through the entire cap — with generated tissues within each cap — was prepared with a slow-speed diamond saw (Micro cutter, MC-201, Maruto, Tokyo, Japan). The sections were mounted on acrylic glass slabs, ground and polished to a final thickness of 50 μm, stained with basic fuchsin and methylene blue, and examined under a light microscope (Olympus AH-2, Tokyo, Japan).

Histological analysis
Histological examination, photography, and morphometric assessment of the sections were performed under a light microscope equipped with a morphometric system connected to a personal computer (PC). In the histological examination, the upper and lower halves of the cap are divided by a red line, and each part was observed by high magnification. For the central section obtained from each specimen, the histomorphometric data were recorded by a computerized image analysis program (Adobe Photoshop®, 7.0J, Adobe Systems, Tokyo, Japan) on Windows PC. Slides were digitized with a solid-state 35 mm slide scanner and a CCD linear photodiode array with interface to the PC. Measurements were then extracted from the digital images with an interactive image processing software package.

The percentage area of newly generated tissue consisting of mineralized bone and marrow spaces in each histological central section was calculated relative to the area bounded by the hemispherical shape of the titanium cap and the parent bone; this latter volume was taken to be 100%. The areas occupied by newly generated tissue were computer-analyzed by pixel counting. Furthermore, the cross-sectional areas of generated mineralized bone were determined and expressed as percentages of the total tissue area generated within each space. All specimens were examined with respect to the inside of the cap to preclude any error margin between samples (Fig. 3).

Statistical analysis
Means and standard deviations were calculated for the percentage areas of newly generated tissue and mineralized bone under the titanium caps after one and three months (n=5). The Mann-Whitney U-test was used to analyze differences in the percentage areas of newly generated tissue and mineralized bone after one and three months. The Wilcoxon signed-rank-test was employed to analyze the differences between the experimental and control caps. Differences with P values <0.05 were considered significant.

RESULTS

Clinical observations
In all of the animals, the surgical sites healed uneventfully with no signs of infection or exposure of the titanium cap. No signs of inflammation or other adverse reactions were found when the cutaneous layer above the surgical sites was dissected and removed.

Histological observations
1) Low magnification of a section of the titanium cap
The percentages of newly generated tissue and mineralized bone in each sagittal histological section were evaluated in the 20 central-section specimens. Various amounts of newly generated tissue and mineralized bone had formed underneath the cap with or without β-TCP (Figs. 4a, b, c and 5a, b, c). In the one-month specimens, mineralized bone in the experimental site reached approximately 2/3 the height of the cap (Figs. 4b, c), while newly generated tissue in the control site reached only 1/2 the height of the cap (Fig. 4a). β-TCP particles remained in the specimens regardless of the particle size; however, fewer β-TCP particles were found in the three-month specimens than in the one-month specimens (Figs. 4b, c and 5b, c).

2) High magnification of a section of the titanium cap with small-sized particles in the one-month speci-
Fig. 4 Sagittal histological section of the titanium cap in a one-month specimen (original magnification ×3).
(a) Control. Newly generated tissue reaches to approximately 1/2 the height of the cap.
(b) Small-sized particles. Newly generated tissue reaches to approximately 2/3 the height of the cap.
(c) Medium-sized particles. Newly generated tissue in the experimental site reaches to approximately 2/3 the height of the cap.

Fig. 5 Sagittal histological section of the titanium cap in a three-month specimen (original magnification ×3).
(a) Control. Although newly generated tissue reaches to approximately 2/3 the height of the cap, the space is still not filled.
(b) Small-sized particles. Newly generated tissue has formed surrounding β-TCP. β-TCP particles remain in the upper part of the cap.
(c) Medium-sized particles. Newly generated bone has formed surrounding β-TCP. Amount of remaining β-TCP particles is decreased.
Phagocytosis by osteoclast-like cell was observed in the upper part of the cap, where osteoclast-like cell included the β-TCP particles (Fig. 6e). Many lacunae, osteoblast-like cells, and canaliculi were found in the lower part of the cap (Fig. 6f).

3) High magnification of a section of the titanium cap with medium-sized particles in the one-month specimens
Osteoclast-like cell attached to a β-TCP particle was noted in the upper part of the cap (Fig. 7e). Many lacunae, canaliculi, osteoblast-like cells, and osteoids were observed in the lower part of the cap (Fig. 7f).

4) High magnification of a section of the titanium cap with small-sized particles in the three-month specimens
Many remaining β-TCP particles were observed in the upper part of the cap. A small amount of newly generated tissue invaded the interparticulate space between β-TCP particles (Fig. 8c). Vascular channels contained vessels (Fig. 8f). Osteocytes, Haversian canal, osteoblast-like cells, and osteoids could also be observed in the lower part of the cap (Fig. 8d, f).

5) High magnification of a section of the titanium cap with medium-sized particles in the three-month specimens
A Haversian canal surrounded by lamellar bone and bone marrow were noted in the upper part of the cap (Fig. 9c). High magnification showed marrow tissue and fat cells. Osteoblast-like cells and osteoids could also be observed (Fig. 9e). A Haversian canal surrounded by lamellar bone and bone marrow were likewise observed in the lower part of the cap (Fig. 9d). Osteoblast-like cells and osteoids were also observed (Fig. 9f).

Newly generated tissue and mineralized bone formation
Between the small-sized and medium-sized particles in the one-month specimens, a statistically significance difference was observed regarding the relative amount of newly generated tissue. However, there were no statistically significant differences in the amount of newly generated tissue between the experimental and control groups for each β-TCP particle size in the one- and three-month specimens (Table 1).

Regarding mineralized bone formation in the newly regenerated tissue, there was a statistically significant difference between the control and experimental groups in the three-month specimens. Furthermore, there was a statistically significant difference in the relative amount of mineralized bone between the small-sized and medium-sized particles. However, in the one-month specimens, there were no
Fig. 7  Medium-sized particles in a one-month specimen.
(a) Upper part of the cap.
(b) Lower part of the cap.
(c)-(d) Higher magnification of framed area in (a) (b) respectively (original magnification×60).
(e) Higher magnification of framed area in (c) Osteoclast-like cell attached to β-TCP particle (▽) (original magnification×200).
(f) Higher magnification of framed area in (d) Lacuna, canaliculi (↓), and osteoblast-like cells (*) (original magnification×200).

Fig. 8  Small-sized particles in a three-month specimen.
(a) Upper part of the cap.
(b) Lower part of the cap.
(c) Higher magnification of framed area in (a) (original magnification×60).
(d) Higher magnification of framed area in (b) Haversian canal (▽) are observed (original magnification×60).
(e) Higher magnification of framed area in (c) Vascular channels containing vessels (◇) (original magnification ×200).
(f) Higher magnification of framed area in (d) Osteoblast-like cells (*) are observed (original magnification×200).
Fig. 9  Medium-sized particles in a three-month specimen.
(a) Upper part of the cap.
(b) Lower part of the cap.
(c) Higher magnification of framed area in (a) Haversian canal (☆) surrounded by lamellar bone (original magnification ×60).
(d) Higher magnification of framed area in (b) Haversian canal (☆) surrounded by lamellar bone (original magnification ×60).
(e) Higher magnification of framed area in (c) Bone marrow, and osteoblast-like cells (⋆) (original magnification ×200).
(f) Higher magnification of framed area in (d) Bone marrow, and osteoblast-like cells (⋆) (original magnification ×200).

Table 1  Percentage areas of newly generated tissue under the titanium cap

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<th>1-month (n=5)</th>
<th>3-month (n=5)</th>
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<td>Small particles</td>
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<tr>
<td>Experiment</td>
<td>39.1±3.3</td>
<td>70.1±8.4</td>
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<td>Control</td>
<td>44.7±9.6</td>
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<tr>
<td>Medium particles</td>
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<td></td>
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<tr>
<td>Experiment</td>
<td>47.7±6.6</td>
<td>78.9±2.3</td>
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<tr>
<td>Control</td>
<td>53.1±10.0</td>
<td>79.8±5.3</td>
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Values are mean±SD (%).
* Mann-Whitney U-test, P<0.05.

Table 2  Percentage areas of mineralized bone in the newly generated tissue under the titanium cap

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<th>1-month (n=5)</th>
<th>3-month (n=5)</th>
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<td>Small particles</td>
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<tr>
<td>Experiment</td>
<td>32.3±5.1</td>
<td>34.2±1.8</td>
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<tr>
<td>Control</td>
<td>33.7±11.1</td>
<td>26.6±2.5</td>
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<tr>
<td>Medium particles</td>
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<td></td>
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<tr>
<td>Experiment</td>
<td>30.3±7.2</td>
<td>44.4±4.8</td>
</tr>
<tr>
<td>Control</td>
<td>31.9±4.5</td>
<td>27.9±1.5</td>
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Values are mean±SD (%).
* Wilcoxon signed-rank test, P<0.05.
** Mann-Whitney U-test, P<0.05.
significant differences in the relative amount of mineralized bone generated between the control and experimental groups. Furthermore, there were also no significant differences based on particle size (Table 2).

DISCUSSION

The results of this study demonstrated that \( \beta \)-TCP was more effective than a mere titanium cap to augment the generation of bone beyond the skeletal envelope and into areas where there was previously no bone present. Successful outcomes with GBR require the fulfillment of certain biological principles, namely, wound stabilization, exclusion of competing tissues, and space-making ability.\(^{12,31-37}\) For all the specimens in this study, newly generated tissue and mineralized bone were noted under the space created by the titanium cap with or without \( \beta \)-TCP. Further, we did not observe inflammatory cell infiltration or abundant fibrous connective tissue ingrowth in either the one- or three-month specimens. Hence, as shown in this study, the barrier material must be sufficiently occlusive to prevent any invasion of fibrous connective tissue into the area to be regenerated with new bone. Insufficient peripheral stability or sealing of the wound area would therefore not pose any problem in our model, which used stiff, standardized, titanium caps.

In the histological examination, mineralized bone in the one-month specimens had already reached approximately 2/3 the height of the cap in the experimental group. On the other hand, mineralized bone in the control reached only 1/2 the height of the cap. Thus, \( \beta \)-TCP might be effective for initial bone formation. However, there were no statistically significant differences in the amount of newly generated tissue between the experimental and control groups for either particle size of \( \beta \)-TCP in the one-and three-month specimens.

As \( \beta \)-TCP was expected to be osteoconductive, it acted as a scaffold in this study. This was done in the light of some reports that have examined the osteoconductive properties of calcium phosphate. Bowers et al.\(^{27}\) reported bone and osteoid formation around graft particles and stated that \( \beta \)-TCP appeared to serve as a nidus for new bone formation. Wada et al.\(^{38}\) reported that osteoblasts were present on the surfaces of \( \beta \)-TCP particles, and our study also showed osteoblasts and osteoid formation around \( \beta \)-TCP particles.

Studies examining the influence of particle size in various graft materials on osteoconduction have reached variant conclusions. Mellonig\(^{39}\) recommended 250-to 750-\( \mu \)m-sized particles for the treatment of intrabone defects. Zaner et al.\(^{40}\), on the other hand, recommended 300-to 500-\( \mu \)m-sized particles of various graft materials to treat periodontal defects. However, no statistically significant differences were observed for the bone fill rates conducted with demineralized freeze-dried bone allograft (DFDBA) using particle sizes of 250-500 or 850-1,000 \( \mu \)m in a clinical study.\(^{30}\) To date, no consensus has been reached regarding the optimal particle size to use for graft materials.

In the one-month experimental group, osteoblast-like cells and bone formation were observed regardless of \( \beta \)-TCP particle size. Remaining \( \beta \)-TCP particles and phagocytosis of \( \beta \)-TCP by osteoclast-like cells were also observed. In the three-month specimens, the amount of mineralized bone was increased, a Haversian canal and osteocytes were observed, and enclosed lamellar bone was seen. Moreover, specimens with medium-sized particles showed marrow tissue and mineralized bone. However, the upper part of the specimens with small-sized particles showed remaining \( \beta \)-TCP and a small amount of cell infiltration. This is because \( \beta \)-TCP must first be absorbed by osteoclast-like cells. Then, an osteoblast-like cell will appear, the bone matures, and the osteoid formed.

The morphology of a graft material is thought to contribute to its osteoconductive capacity. This is because particle size and shape are thought to influence the resorption and replacement phenomena, while interparticulate space is thought to influence the infiltration of vascular cellular elements and bone formation. Our study showed a significant difference in the amount of mineralized bone between the experimental and control sites in the three-month specimens. The amount of mineralized bone differed depending on the presence or absence of \( \beta \)-TCP. The presence of \( \beta \)-TCP brought about an increase in the amount of mineralized bone because \( \beta \)-TCP discharges Ca and P ions when it is absorbed by a surrounding cell.\(^{40}\) In this manner, the dissolution of \( \beta \)-TCP may provide the Ca and P ions needed for mineralized bone formation.\(^{41}\)

In the one-month specimens, there was a statistically significant difference in the relative amount of newly generated tissue between the small-sized and medium-sized particles. Thus, interparticulate space might have a significant effect on the infiltration of vascular cellular elements in the early stage. Indeed, in the three-month specimens, there was also a significant difference in the amount of mineralized bone between the medium-and small-sized particles, where a greater proportion of the small-sized particles remained. It was possible that when small-sized particles became densely packed in the cap, it was done at the expense of interparticulate space. As a result, with small-sized particles there was insufficient interparticulate space to allow the migration and ingrowth of cells, blood vessels, and bone.\(^{40}\) With the migration and ingrowth processes hindered, the increase of newly generated tissue in the early stage...
was also impeded. With diminished increase in the newly generated tissue, the amount of mineralized bone likewise decreased. The findings of this study therefore suggested that particles with dimensions less than 100 μm seemed to possess less mineralization potential versus medium-sized particles which could permit sufficient interparticulate space.

CONCLUSION

In this study, we examined the effects of using different sizes of β-TCP particles for bone augmentation within a titanium cap in the rabbit calvarium. Within the limitations of the study resulting from the use of only 20 specimens, β-TCP was found to be effective at increasing the amount of newly generated tissue and mineralized bone. Further, it was found that medium-sized β-TCP particles were more useful than the small-sized particles for promoting the formation of mineralized bone. It appeared that suitable interparticulate spaces between the β-TCP particles were necessary to augment the formation of bony tissues, but further investigations are needed to examine the effects using larger particles and for longer time periods.

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