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# Effects of the enamel matrix derivative and $\beta$ -tricalcium phosphate on bone augmentation within a titanium cap in rabbit calvarium

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Abstract: In vitro studies suggest that enamel matrix derivative (EMD) affects the early stages of osteogenic maturation by stimulating bone cell proliferation. In the present study, we evaluated the effects of EMD and  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) on bone augmentation within a titanium cap in rabbit calvaria, using 14 adult male Japanese white rabbits. The calvarium was exposed, a circular groove prepared, the marrow penetrated, and a standard hemispherical titanium cap placed in the groove. The cap was filled with a mixture of  $\beta$ -TCP and EMD at the experimental site, and was filled with  $\beta$ -TCP alone at the control site. At 1 and 3 months after cap implantation, animals were euthanized, and histological sections prepared. The sections were stained with basic fuchsin and methylene blue, and were examined using light microscopy. At 1 month, EMD tended to increase the amount of bone, but there was no significant difference in the amount of new tissue and mineralized bone between the experimental and control sites. The present findings indicate that the present mixture of EMD and  $\beta$ -TCP does not accelerate bone formation, compared with  $\beta$ -TCP alone. (J. Oral Sci. 47, 209-217, 2005)

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#### Introduction

In recent years, guided bone regeneration (GBR) has been used successfully to treat bone defects around osseointegrated implants and to augment severely resorbed jawbones before implant placement (1-7). A barrier membrane is placed over the bone defect to create an isolated space between the bone and the membrane. This space can only be populated by osteoprogenitor cells from the adjacent bone tissue, allowing bone to regenerate within the defect. One of the most frequent reasons for failure of bone regeneration using the membrane technique is the collapse of the membrane toward the surface of the bone as a result of the pressure from overlying soft tissues (3-4). Clinicians and researchers have investigated methods of preventing such collapse, by placing various different graft materials under the membrane.

Beta-tricalcium phosphate ( $\beta$ -TCP) and hydroxyapatite have been used in many experimental and clinical studies (8-11).  $\beta$ -TCP is a bone substitute that is osteoconductive and biodegradable. A new high-purity  $\beta$ -TCP has been manufactured using a mechano-chemical method, and is a potent bone-graft substitute that is available for clinical use (12). However, this new  $\beta$ -TCP is not osteoinductive, although it is highly osteoconductive.

Histological examination of human biopsy specimens (13) and histological evaluations of the effects of enamel

matrix protein derivative (EMD) on periodontal defects in monkeys (14,15) indicate that EMD promotes formation of new periodontal attachments, including regeneration of alveolar bone. *In vitro* studies suggest that EMD affects the early stages of osteogenic maturation by stimulating bone cell proliferation (16-18). Takayama et al. postulated that the effects of EMD are mediated by bone morphogenetic protein (BMP)-like molecules present within EMD. Recent findings regarding the *in vivo* role of EMD in bone regeneration and bone augmentation suggest that combined application of EMD and GBR can significantly affect bone healing (19,20).

In the present study, we performed a detailed evaluation of the effects of EMD and  $\beta$ -TCP on bone augmentation within a titanium cap in rabbit calvaria.

# **Materials and Methods**

# Animals

We used 14 adult male Japanese white rabbits, weighing 2.5 to 2.8 kg each. Before the experiment, the health of the rabbits was monitored for 2 weeks. The rabbits were kept in a standard cage in an experimental animal room (24°C, 55% humidity, 1 atm, 12-h light/dark cycle), and were fed a standard laboratory diet and water. This study was approved by the Animal Experimentation Committee of Nihon University School of Dentistry, Japan.

# Anesthesia and surgery

All operations were conducted under sterile conditions. General anesthesia was induced by injecting pentobarbital sodium (Nembutal®, Abbot Laboratories, North Chicago, IL, USA; 0.4 ml/kg) via an ear vein, and was maintained by halothane inhalation (Fluothane®, Takeda Chemical Industries, Osaka, Japan; vol%, 1.5 to 2.0%). The forehead of each rabbit was shaved and disinfected with 70% ethanol. Under local anesthesia with lidocaine-HCl (2% Xylocaine<sup>®</sup>, Astra Japan, Fujisawa Pharmaceutical, Osaka, Japan) containing epinephrine (dilution, 1: 80,000), a cutaneous flap was created by making a linear incision that was raised laterally. The periosteum was incised and lifted to expose the calvarial bone on both sides of the midline. Two large holes were drilled on each side of the sagittal suture using a trephine bur (diameter, 4 mm), and 9 smaller holes were drilled around each center hole using a No. 2 round bar, to induce bleeding from the marrow space. Care was taken to irrigate the wounds thoroughly and avoid involvement of the sagittal and coronal sutures during drilling. A stiff, standardized titanium cap (21,22) was fixed to each calvarium. One titanium cap (experimental site) was filled with  $\beta$ -TCP (OSferion Olympus, Japan; porosity, 75%; pore size, 500-1,000 µm) mixed with EMD (Emdogain® Gel, Biora AB, Malmö, Sweden). The other cap (control site) was filled with  $\beta$ -TCP alone. The fixed caps were covered carefully by replacing the periosteum and cutaneous flaps, which were then sutured with absorbable sutures (5-0 Opepolix<sup>®</sup> II, Azwell, Osaka, Japan). Each reflected flap was repositioned to cover the titanium cap, and was sutured with interrupted sutures (4-0 silk Mani<sup>®</sup> suture, Mani, Tochigi, Japan). Because the periosteum is 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 into 2 equal groups that were allowed to heal for 1 or 3 months, respectively.

# Specimen preparation

After healing for 1 or 3 months, the animals were euthanized with an overdose of pentobarbital. The calvarium bone with the titanium caps was dissected, fixed in 10% neutral buffered formalin, dehydrated, and embedded in polyester resin (Rigolac-2004, Rigolac-70F, Nisshin EM, Tokyo, Japan). We prepared 1 sagittal nondecalcified ground section (approximate thickness, 200  $\mu$ m) from the central part of the titanium cap, encompassing the entire cap and tissues that were generated within the cap, using 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  $\mu$ m, stained with basic fuchsin and methylene blue, and examined under a light microscope (Olympus AH-2, Tokyo, Japan).

#### Histological analysis

We performed histological examination, photography, and morphometric assessment of the sections, using a light microscope equipped with a morphometric system connected to a personal computer. The histomorphometric data for the central section obtained from each specimen were recorded using a computerized image analysis system (Adobe Photoshop<sup>®</sup>, 7.0 J, Adobe Systems, Tokyo, Japan). Images taken at 2.5 × magnification were digitized using a solid-state 35-mm slide scanner and a CCD linear photodiode array interfaced to the computer. The measurements were extracted from the digital images using an interactive image processing software package.

For each histological central section, we calculated the percentage area of newly generated tissue that consisted of mineralized bone and marrow space, relative to the area bounded by the hemispherical shape of the titanium cap and parent bone; the latter volume was designated as 100%. We determined the cross-sectional area of generated mineralized bone, expressed as percentage of the total area of tissue generated within each space (Fig. 1).

#### Statistical analysis

Means and standard deviations were calculated for the percentage areas of newly generated tissue and mineralized bone under the titanium caps after 1 and 3 months of healing. The Mann-Whitney *U*-test was used to analyze differences in the percentage areas of newly generated tissue and mineralized bone between the 2 healing-duration groups (1 and 3 months). The Wilcoxon signed-rank test was used to analyze differences between the 2 caps (experimental and control). Differences with P < 0.05 were considered significant.

# Results

#### Clinical observations

In all rabbits, the surgical sites healed uneventfully with no signs of infection or exposure of the titanium cap. When the cutaneous layer above the surgical sites was dissected and removed, there were no signs of inflammation or other adverse reactions.

#### Histological observations

# Low magnification of sections of the titanium cap

We evaluated the percentage of newly generated tissue and mineralized bone in sagittal histological sections of 14 central specimens. Various amounts of newly generated tissue and mineralized bone had formed underneath both



Fig. 1 Schematic of a sagittal histological section through the center of a titanium cap, illustrating the measurements performed. a = area within the titanium cap; b = area of newly generated tissue except  $\beta$ -TCP, expressed as a percentage of a; c = area of newly generated mineralized bone, expressed as a percentage of b; d = baseline equaling the cross-sectional contour of the original parent bone.

the experimental and control caps. The newly generated tissue consisted of slender mineralized bone of various sizes and large marrow spaces in the parent bone (Figs. 2 and 3). At both experimental and control sites,  $\beta$ -TCP particles were detected in the sections. The number of  $\beta$ -TCP particles was greater after 1-month healing than after 3-month healing (Figs. 2 and 3). In the 1-month experimental and control specimens, more than the half of the original  $\beta$ -TCP remained in the cap, and some mineralized bone surrounded the  $\beta$ -TCP (Fig. 2). In the 3-month experimental and control specimens, mineralized bone surrounded the  $\beta$ -TCP (Fig. 3).

#### High magnification of experimental sections at 1 month

The sections contained new mineralized bone surrounded by  $\beta$ -TCP particles and Haversian canals, which were surrounded by lamellar bone. High magnification showed osteoblast-like cells and osteoids (Fig. 4).





Control site



- Fig. 2 Sagittal histological section of the titanium cap in a 1month specimen.
  - (a) Newly generated tissue reaches to approximately 2/3 the height of the cap in experimental site.
  - (b) Newly generated tissue reaches to approximately 2/3 the height of the cap in control site.

# High magnification of control sections at 1 month

Osteoclast-like cells attached to  $\beta$ -TCP particles were observed in the upper part of the cap. Osteoblast-like cells and osteoids were observed in the lower part of the cap (Fig. 5).

# High magnification of experimental sections at 3 months

The sections contained new mineralized bone surrounded by  $\beta$ -TCP particles. The parent bone had fused with the new mineralized bone. Osteocytes, osteoblast-like cells, and osteoids were also observed (Fig. 6).

# High magnification of control sections at 3 months

The sections contained new mineralized bone surrounded by  $\beta$ -TCP particles. The parent bone had fused with the new mineralized bone. Osteoblast-like cells and osteoids were also observed (Fig. 7).

Newly generated tissue and mineralized bone



Control site



- Fig. 3 Sagittal histological section of the titanium cap in a 3month specimen.
  - (a) Newly generated tissue surrounds the  $\beta$ -TCP in experimental site.
  - (b) Newly generated tissue surrounds the  $\beta$ -TCP in control site.

# formation

After 1 month of healing, newly generated tissue occupied  $46.6 \pm 12.6\%$  of the titanium caps at the control sites and  $49.1 \pm 7.6\%$  at the experimental sites; the difference was not significant (P = 0.345). After 3 months of healing, newly generated tissue occupied  $69.3 \pm 5.1\%$  of the titanium caps at the control sites and  $68.4 \pm 3.3\%$  at the experimental sites; the difference was not significant (P = 0.753).

After 1 month of healing, the percentage of mineralized bone in the newly generated tissue was  $36.8 \pm 10.3\%$  at the control sites and  $42.2 \pm 13.1\%$  at the experimental sites; the difference was not significant (P = 0.075). After 3 months of healing, the percentage of mineralized bone in the newly generated tissue was  $41.2 \pm 10.6\%$  at the control sites and  $43.3 \pm 6.8\%$  at the experimental sites; the difference was not significant (P = 0.917).

# Discussion

The present results indicate that GBR using  $\beta$ -TCP with or without EMD produces bone augmentation within a titanium cap in rabbit calvaria. Successful GBR requires wound stabilization, exclusion of competing tissues, and adequate space (3,23-25). The barrier materials must be sufficiently occlusive to prevent invasion of fibrous connective tissue into the area of new bone regeneration. In all present specimens, newly generated tissue and mineralized bone were observed under the space created by the titanium cap. Therefore, in the present model, which uses stiff, standardized titanium caps, there is sufficient peripheral stability and sealing of the wound area.

 $\beta$ -TCP would have to be osteoconductive in order to be effective as a scaffold. There have been studies of the osteoconductive properties of calcium phosphate. Bowers et al. (10) reported formation of bone and osteoids around graft particles, and reported that  $\beta$ -TCP appears to serve as a nidus for new bone formation. Wada et al. (26) observed osteoblasts on the surfaces of  $\beta$ -TCP particles, similar to the present observation of formation of osteoblasts and osteoids around  $\beta$ -TCP particles. However,  $\beta$ -TCP is not osteoinductive because it does not contain the proteins necessary to induce bone formation.

Recent in vitro studies suggest that EMD can enhance bone formation by stimulating proliferation and differentiation of bone-forming cells (16-18). Ohyama et al. (17) reported that EMD converted C2C12 cells into osteoblast or chondroblast lineages. Takayama et al. (18) reported that EMD promoted the osteogenic differentiation of pluripotent mesenchymal cells, and reported that this effect was mediated by BMP-like molecules present in the EMD. Similar results have been obtained in animal studies



- Fig. 4 Experimental site in a 1-month specimen.
  - (a) Upper part of the cap.
  - (b) Lower part of the cap.
  - (c) Higher magnification of framed area in (a). Harversian canals (arrows) are visible.
  - (d) Higher magnification of framed area in (c). Osteocytes, osteoblast-like cells (arrow-heads) are visible.
  - (e) Higher magnification of framed area in (b). Haversian canal (arrow) surrounded by lamellar bone.
  - (f) Higher magnification of framed area in (e). Bone marrow, and osteoblast-like cells (arrowheads) are visible.



- Fig. 5 Control site in a 1-month specimen.
  - (a) Upper part of the cap.
  - (b) Lower part of the cap.
  - (c) (e) Higher magnification of framed area in (a) (b) respectively.
  - (d) Higher magnification of framed area in (c). An osteoclast-like cell is attached to  $\beta$ -TCP particle (arrow).
  - (f) Higher magnification of framed area in (e). Osteoblast-like cells (arrowheads) are visible.



- Fig. 6 Experimental site in a 3-month specimen.
  - (a) Upper part of the cap.
  - (b) Lower part of the cap.
  - (c) (e) Higher magnification of framed area in (a) (b) respectively.
  - (d) Higher magnification of framed area in (c). Lacunae (arrows), osteoblast-like cells (arrowheads) are visible.
  - (f) Higher magnification of framed area in (e). Lacunae (arrows) and canaliculi are visible.



- Fig. 7 Control site in a 3-month specimen.
  - (a) Upper part of the cap.
  - (b) Lower part of the cap.
  - (c) (e)Higher magnification of framed area in (a) (b) respectively.
  - (d) Higher magnification of framed area in (c). New mineralized bone is surrounded by  $\beta$ -TCP particles.
  - (f) Higher magnification of framed area in (e). Lacunae and canaliculi are visible.

(27,28). Therefore, in the present study, we used a combination of EMD (which can stimulate bone formation) and  $\beta$ -TCP, because we expected that EMD would have an osteoinductive effect.

However, the present results indicate that EMD had little effect on bone formation, although it appears to have promoted initial bone formation. After 1 month of healing, the percentage of mineralized bone in the newly generated tissue was  $36.8 \pm 10.3\%$  at the control sites and  $42.2 \pm 13.1\%$  at the experimental sites. In addition, light microscopy showed that the mineralized bone was more mature at the experimental sites than at the control sites. Moreover, at the experimental sites, many osteoblast-like cells were observed in the upper part of the cap.

By contrast, in the 3-month specimens, there was no significant difference in the relative amount of generated mineralized bone between the control and experimental sites. In 2 of the 7 specimens from the 3-month group, the amount of newly generated tissue and mineralized bone formation was slightly but significantly greater at the control site than at the experimental site (Fig. 8). These results suggest that BMP-like molecules in the EMD had a weak osteoinductive effect. EMD did not promote osteoblast activity, but it promoted the osteogenic differentiation of pluripotent mesenchymal cells.

Previous studies of use of a combination of EMD and

graft material have been aimed at preventing the decrease in the regeneration space that can occur with EMD alone. In the present study, instead of focusing on the space, we examined bone augmentation within a titanium cap in rabbit calvaria.

It is difficult to augment bone beyond the skeletal envelope using only a membrane (29,30). The use of a membrane with autogenous bone grafts and graft materials is more effective than using a membrane alone (31,32). Consequently, the use of filling materials with a barrier membrane has recently attracted new interest, because

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

	1-month	3-months
Experimental	$49.1\pm7.6$	$68.4 \pm 3.3$
Control	$46.6 \pm 12.6$	69.3 = 5.1
		Values are mean + SD (%3).

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

	1-month	3-menths
Experimental	$42.2 \pm 13.1$	$43.3\pm 6.8$
Control	$36.8 \pm 10.3$	$41.2 \pm 10.6$

Values are mean  $\pm$  SD (%).



Fig. 8 Specimen with small amount of mineralized bone formation.

- (a)Upper part of the cap.
- (b) Lower part of the cap.
- (c) Higher magnification of framed area in (a). There is no newly generated tissue between the  $\beta$ -TCP particles.
- (d) Higher magnification of framed area in (b). Newly generated tissue surrounds the  $\beta$ -TCP.

such methods allow for a space-making effect when the membrane is not sufficiently stiff.

In the present study, a decrease in the space was not a problem, because we used stiff, standardized titanium caps. Consequently, we were able to examine the effects of EMD on bone formation. We found no significant difference in promotion of new bone formation between  $\beta$ -TCP alone and the combination of EMD and  $\beta$ -TCP, implying that EMD is not an additional stimulus for bone formation. This brings into question the effectiveness of the use of EMD proteins to generate new bone in clinical situations.

In conclusion, the present findings indicate that GBR of the rabbit calvarium using a titanium cap produces significant bone augmentation. The combined application of EMD and  $\beta$ -TCP did not increase bone formation, compared with use of  $\beta$ -TCP alone.

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