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# Effect of a chitosan sponge impregnated with platelet-derived growth factor on bone augmentation beyond the skeletal envelope in rat calvaria

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**Abstract:** We evaluated the ability of platelet-derived growth factor (PDGF) to promote bone augmentation beyond the skeletal envelope in rat calvaria. The calvariae of 14 rats were exposed, and two plastic caps—one with 0.03% PDGF and a chitosan sponge and one with a chitosan sponge alone—were placed. Microcomputed tomography and histologic sections were used to determine the amount of bone augmentation within the plastic caps. Bone volume was calculated using measurement software. Bone volume and amount of bone augmentation were significantly greater in the PDGF group than in the control group. In conclusion, a chitosan sponge containing 0.03% PDGF enhanced bone formation beyond the skeletal envelope in rat calvaria. (J Oral Sci 56, 23-28, 2014)

Keywords: PDGF; bone regeneration; rat; chitosan; micro-CT.

## Introduction

Guided bone augmentation (GBA) is the process of creating new bone by guiding bone cells to an area beyond the original outer or inner skeletal envelope. We previously studied the effects of various regenerative factors and biomaterials on GBA in rat calvaria but found it difficult to accurately measure the amount and height of newly generated bone in all specimens (1-3).

Platelet-derived growth factor (PDGF) is a potent mitogen and chemotactic factor for mesenchymal cells and has a role in proliferation (4), chemotaxis (5-8), and matrix apposition (9). In addition to its action on osteoblasts, it is stored within the bone matrix. PDGF also regulates maturation and remodeling of newly formed blood vessels (10).

The effects of PDGF have been studied in animal models of cranial and alveolar-ridge defects (11-19). However, these studies yielded conflicting results regarding promotion of new bone formation. In those studies, PDGF was applied at various concentrations using different carrier systems. In a previous study, we reported that PDGF delivered via a collagen carrier enhanced bone formation in rat calvaria (20).

The use of chitosan (poly-N-acetyl glucosaminoglycan) in bone regeneration has attracted considerable interest. A bioresorbable chitosan sponge loaded with various growth factors was used to induce bone forma-

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tion. This biodegradable and nontoxic natural biopolymer has several biomedical applications: it enhances bone formation *in vitro* (21) and *in vivo* (22,23). In addition to these biomedical applications, chitosan regulates release of bioactive agents, including growth factors (24).

In the present study, we evaluated the effects of various carriers on GBA in rat calvaria within plastic caps.

## Materials and Methods

### Animals

Seven male Fischer rats (age, 12 weeks; 200–250 g) were included in the study. The animals were kept in plastic cages in an animal experimentation room (22°C, 55% humidity, 12/12 h light/dark cycle) and had *ad libitum* access to food and water. This study was approved by the Animal Experimentation Committee of Nihon University School of Dentistry, Japan (AP11D005).

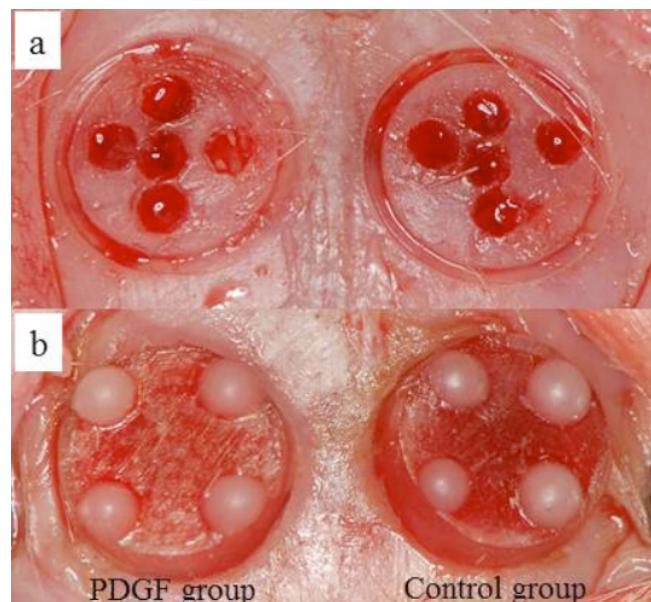
### Preparation of PDGF-impregnated chitosan sponges

Chitosan sponges (HemCon Dental Dressing; Hakuho Co., Tokyo, Japan) were used as scaffolds for freeze-dried recombinant rat PDGF-BB (R&D Systems, Inc. Minneapolis, MN, USA). Sponges impregnated with 0.03% PDGF and sponges without PDGF were prepared similarly, except for the use of aseptic saline solution. Aseptic saline solution (20 µL), with or without PDGF, and a chitosan sponge were implanted into each bone defect.

### Experimental design

The animals were anesthetized by intraperitoneal injection of 0.5 mL lidocaine (Xylocaine, 1:8 dilution; Astra Zeneca, Osaka, Japan). The dorsal cranium was shaved and prepared aseptically for surgery. A 20-mm-long incision was made in each scalp along the sagittal suture. A circular groove was made on each side of the midline using a trephine drill (inner diameter, 5 mm) under profuse irrigation with sterile saline. Five small holes were drilled with a #2 round bur to induce bleeding within each circle. During the creation of the groove and holes, care was taken to avoid dura penetration (Fig. 1a).

Cylindrical plastic caps (standardized columnar shape; height, 1.5 mm; diameter, 4.4 mm) were placed on both sides of the midline, and composite resin landmarks were fixed on the plastic caps. The midsagittal suture was not included in the bone defects, to avoid contributing to bone healing and to limit the risk of damage to the superior sagittal sinus. During the surgical procedure, care was taken to avoid damaging the dura mater or puncturing the superior sagittal sinus. Before placement, each plastic cap was filled with a chitosan sponge (diameter, 4 mm;



**Fig. 1** a: A circular groove (5 mm) was cut with a trephine, and five small holes were drilled using a round bur. b: The plastic cap was pre-filled with either 0.03% PDGF in a chitosan sponge (for the experimental site) or with saline alone in a chitosan sponge (for the control site). A plastic cap was fixed to each groove.

height, 2 mm) impregnated with either 0.03% PDGF (experimental site) or saline (control site; Fig. 1b).

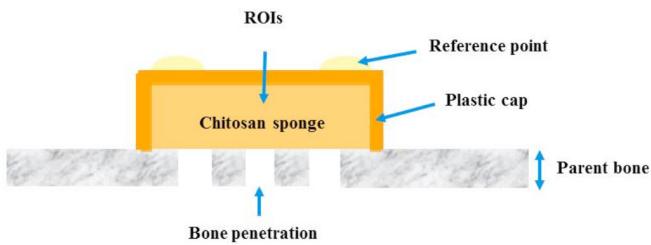
### Imaging

Microcomputed tomography (micro-CT) was performed with a device (R\_mCT; Rigaku, Tokyo, Japan) equipped with a microfocus X-ray tube (L9181S; focal length, 7 µm; Hamamatsu Photonics, Hamamatsu, Japan). The X-ray source and image intensifier were connected by a basal plate, and a direct-drive motor enabled I-arm rotation on the vertical plane.

The rats were anesthetized with sodium pentobarbital and placed on the stage, and images of regions of interest (ROIs) were captured. Micro-CT imaging was repeated from 1 to 12 weeks after surgery.

### Micro-CT analysis

The exposure parameters were 90 kV and 100 µA. The images were reconstructed on a personal computer using specially designed i-View software (Kitasenju Radist Dental Clinic, i-View Image Center, Tokyo, Japan). The relative amounts of cortical bone and soft tissue were measured by micro-CT. We also measured bone volume (BV) within the plastic caps using voxel images and BV measurement software (Kitasenju Radist Dental Clinic, i-View Image Center), which calculated gray values and numbers of voxels with corresponding gray values in



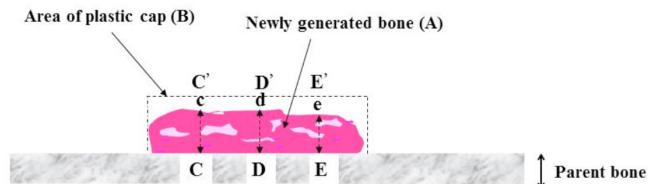
**Fig. 2** Schematic of the plastic cap, the area visualized using micro-CT, and the measurements taken.

ROIs (Fig. 2). Histograms showing the X-ray absorption rate on the *x* axis and CT voxel numbers on the *y* axis were created for the CT imaging fields. Histograms of X-ray absorption rates showed peaks for hard and soft tissues, and the threshold was set at the value representing the trough between these peaks. The number of voxels for X-ray absorption rates exceeding the threshold was counted. Finally, BV was calculated, and the number of voxels was multiplied by the voxel volume. BV was measured on the first postoperative day in the ROIs and again each week under the same conditions. Then, the increase in BV (considered to represent new bone) was calculated by subtracting the BV on day 1 from each subsequent value.

#### Histomorphometric analysis

The rats were killed with an overdose of pentobarbital at 12 weeks after the last micro-CT scan. The calvarial bone with the plastic cap was resected, fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, and processed into 5- $\mu\text{m}$ -thick sections for hematoxylin and eosin staining. One sagittal decalcified ground section from the center of each plastic cap was prepared with a microtome. We assessed the histologic and morphometric characteristics of the section under a light microscope that was equipped with a morphometric system and connected to a personal computer. Histomorphometric data for the central section from each specimen were recorded using a computerized image-analysis system. Images taken at  $\times 40$  magnification were digitized using a solid-state 35-mm slide scanner and a charge-coupled device linear photodiode array interfaced with the computer. Measurements were extracted from the digital images using an interactive image-processing software package.

For each central histologic section, we calculated the percentages of areas of mineralized bone relative to the area bounded by the plastic cap and parent bone (designated as 100%). We determined the cross-sectional area of newly generated bone, which was expressed as a percentage of the height and total area of a represen-



**Fig. 3** Measurement of histologic sections. Newly generated bone was calculated using the formula  $A/A + B \times 100(\%)$ . The height of newly generated bone was calculated using the formula  $(Cc/CC' + Dd/DD' + Ee/EE')/3 \times 100(\%)$ .

tative histologic section selected and compared to the appropriate CT image, taking care to match specific homologous anatomical features in the bony anatomy (Fig. 3). The histologic sections were evaluated by a single examiner who was blinded to the details of the study.

#### Statistical analysis

Means and standard deviations were calculated for BV, height, and percentages of areas of newly generated bone under the plastic caps at 12 weeks. The Wilcoxon rank test was used to evaluate differences between the experimental and control sites. In all analyses, a *P* value of  $<0.05$  was considered to indicate statistical significance.

## Results

Healing progressed uneventfully, without complications, in all animals.

#### CT images

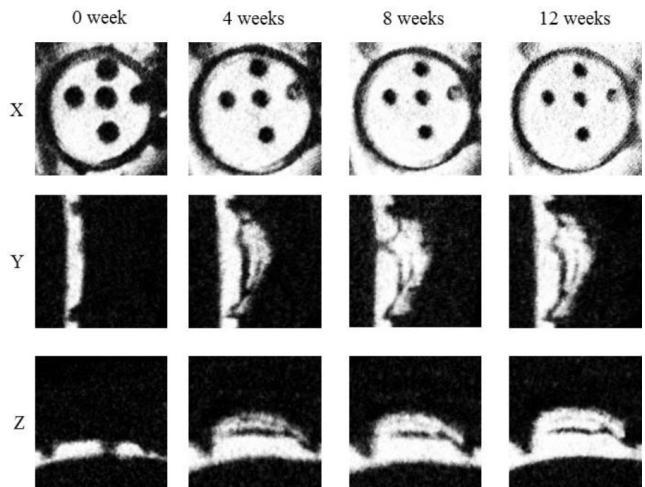
Micro-CT images showed a gradual increase in radiopacity over time in the experimental and control groups. At 12 weeks, the thin layer of radiopacity reached the top of the plastic cap in the 0.03% PDGF group and halfway up the plastic cap in the control group (Figs. 4 and 5).

BV increased in a time-dependent manner in the PDGF and control groups. The increase in BV in the PDGF group was significantly greater (by 1.8- to 2.1-fold) than that in the control group at 4 weeks (Fig. 6).

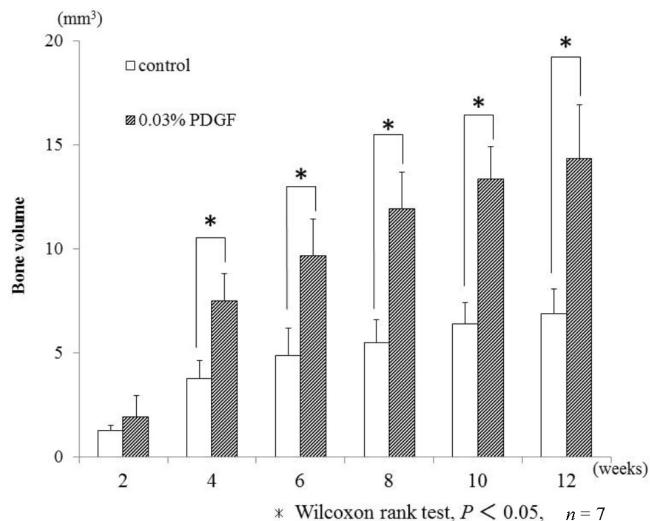
#### Histologic observation and histomorphometric analysis

Lamellar bone in the lower part of the ROIs and adjacent parent bone were thicker in the PDGF group than in the control group. The newly generated bone reached approximately to the top of the plastic cap in the PDGF group specimens (Fig. 7).

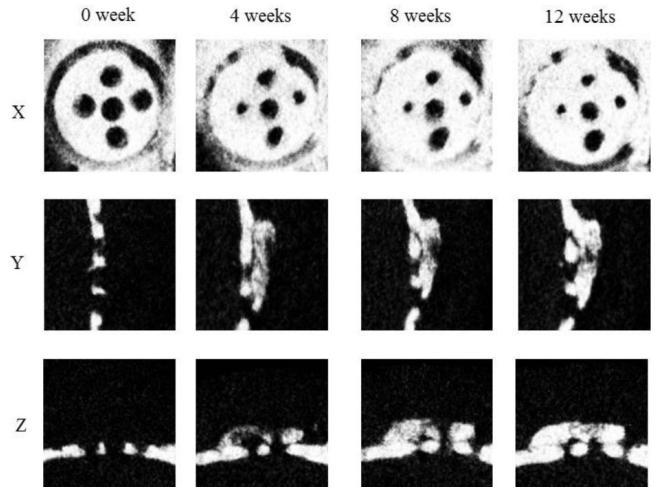
Histomorphometric analysis revealed that the height of newly generated bone was significantly greater in the PDGF group (Table 1).



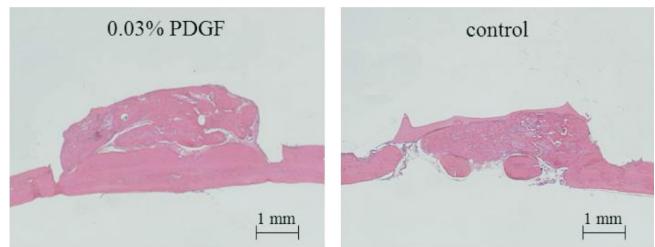
**Fig. 4** X-Y-Z images of the plastic cap in the 0.03% PDGF group.



**Fig. 6** Bone volumes (vertical axis) in the PDGF and control groups at 2, 4, 6, 8, 10, and 12 weeks (horizontal axis).



**Fig. 5** X-Y-Z images of the plastic cap in the control group.



**Fig. 7** Histologic sections from the PDGF and control groups at week 12.

**Table 1** Histomorphometric analysis at 12 weeks

	Amount of newly generated bone (%)	Height of newly generated bone (%)
0.03% PDGF	$61.5 \pm 7.9^*$	$87.5 \pm 8.8^*$
control	$30.3 \pm 4.2$	$44.2 \pm 4.5$

$n = 7$ , \* $P < 0.05$  vs control, Wilcoxon rank test

## Discussion

We evaluated the effects of PDGF on GBA beyond the skeletal envelope in rat calvaria. The skeletal envelope in the calvaria was designed according to our previously reported surgical procedure (3). The advantages of our model include lack of spontaneous regeneration and use of uniform plastic caps to ensure standardization of defect size. However, we found that, within an occluded space, augmentation of mature bone beyond the skeletal envelope was difficult in a variety of experimental conditions (1-3).

Micro-CT imaging showed significantly more new BV in the PDGF group at 4-12 weeks. The amount of bone regeneration within the plastic cap was significantly greater in the PDGF group than in the control group, which was confirmed by histologic analysis. Lee et al.

(19) evaluated bone regeneration using a dome-shaped, 3-mm-tall, molded poly/tricalcium phosphate (TCP) membrane containing PDGF-BB in rabbits and found that new bone filled the space at 4 weeks. Schwarz et al. (17) found that biphasic calcium phosphate with PDGF supported the initial stages of GBA in chronic lateral ridge defects. Simion et al. (16) evaluated the outcome of vertical ridge augmentation in a standardized dog model by combining purified recombinant human (rh)PDGF-BB with a deproteinized block of bovine bone. This combination resulted in the regeneration of significant amounts of new bone without the use of a membrane. Al-Hazmi et al. (19) reported that BV and height were significantly greater in a group treated with xenografts and rhPDGF-BB than in a control group.

Previous animal studies reported application of

>1 µg/mL PDGF-BB to treated sites (10). A study in humans showed that 0.3 mg/mL PDGF-BB significantly improved radiographic bone formation in advanced periodontal osseous defects (25). Our results showed that 0.03% (0.3 mg/mL) PDGF produced significant bone formation within the plastic caps. The mode and duration of delivery are important considerations in the use of growth factors for bone augmentation. In our previous study (20), as compared with 0.01% PDGF with collagen carrier, 0.03% PDGF with collagen carrier resulted in better bone formation and was thus used in the present study. However, the ideal carrier for rhPDGF-BB remains unknown. We used a chitosan carrier in the present study. The delivery of rhPDGF-BB via a chitosan sponge induced bony filling of large rat calvarial defects (14). Similarly, Nash et al. (12) reported that rhPDGF-BB delivered in a collagen or beta-TCP collagen matrix stimulated healing in long bones in rabbits (15). Vikjaer et al. (13) reported that a single dose of rhPDGF-BB stimulated bone formation in critical calvarial defects in rabbits. In addition, rhPDGF-BB increases restoration of bone lost due to advanced periodontal disease in humans. Change in BV did not significantly differ between the present study, which used a chitosan sponge, and a previous study, which used a collagen sponge. This suggests that bone augmentation is similar with chitosan and collagen sponges.

In the present study, a greater amount of mature bone formed in defects treated with PDGF than in control defects. In addition, lamellar bone was thicker in the PDGF group than in the control group. These results are consistent with those of Vikjaer et al. (13) and Schwarz et al. (17). Because PDGF is involved in the maturation and remodeling of newly formed blood vessels, angiogenic and vasculogenic cells may be important initial responders to application of this mitogenic factor. However, this hypothesis needs to be tested in future studies. Our data indicate that the quantity of mineralized tissue decreased with distance from the parent bone and that no such tissue formed within the plastic caps. In a histologic analysis, Simion et al. (16) observed a lack of bone formation in the center of a bovine block, indicating that bone regenerates from parent bone. BV differed significantly between the PDGF and control groups at 4 weeks in the present study, although we anticipated that PDGF would have a more rapid effect. Due to differences in experimental design, animal model, vehicle carrier system, and PDGF concentration, it may not be appropriate to compare the findings of Simion et al. (16) with the results of the present study.

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