Abstract: The present study was designed to compare the bone augmentation ability of absorbable collagen sponge (ACS) with that of hydroxyapatite/collagen composite (HAP/Col) using a rat calvaria defect model. Bone defects were created artificially on the surface of the calvariae of 10-week-old male Fisher rats, and then cylindrical plastic caps filled with ACS or HAP/Col were placed on the defects. This area was designated as the region of interest (ROI) and new bone formation was observed at 0, 4, 8, and 12 weeks after surgery using micro-CT. Histological examinations were performed using sections obtained from 12-week-old rats. Prominent new bone formation was observed in the HAP/Col group relative to the ACS group; onset of new bone augmentation was evident from 4 weeks after surgery in the HAP/Col group and from 8 weeks in the ACS group. Histological examination revealed that the entire area of the cap was filled with newly formed bone intermingled with the HAP/Col composite. Bone mineral density in the HAP/Col group was double that in the ACS group. These results indicate that the use of HAP/Col contributes significantly to new bone augmentation.

Keywords: absorbable collagen sponge; hydroxyapatite/collagen composite; calvaria; bone augmentation.

Introduction
Basic periodontal treatments are indispensable for preventing the progression of periodontitis. However, once periodontal disease has progressed to a severe state, alveolar bone loss may occur, leading to tooth loss. Even in this situation, the affected tooth will be retained in the oral cavity if the alveolar bone defect is repaired. Although the most reliable repair can be achieved by grafting of autologous bone obtained from the iliac crest (1), this approach has several limitations (2-4). Recently, various artificial materials have been developed for use in bone augmentation (5,6). Collagen-based materials have been used successfully because of their high biocompatibility (7). Among them, resorbable collagen plugs (RCPs) are widely used for repair of tooth extraction sockets. Hydroxyapatite (HAP) is one of the most important constituents of natural bone, and composite materials consisting of collagen and HAP have been...
The usefulness of guided bone augmentation (GBA) for generation of new bone beyond the original skeletal envelope has been examined using the rat calvaria defect model (9). The aim of the present study was to compare the new bone augmentation ability of absorbable collagen sponge (ACS) with that of HAP/Col composite using the rat calvaria model.

**Materials and Methods**

**Animals**

Ten-week-old male F344/Jcl rats (CLEA Japan, Tokyo, Japan) weighing 200-220 g were used. The animals were kept at constant temperature (22°C) and humidity (55%) under a 12/12-h light/dark cycle. This study was approved by the Animal Experimentation Committee of Nihon University School of Dentistry, Japan (AP16D002).

**Materials**

Absorbable collagen sponge (ACS, Teruplug) and multiporous HAP/Col composite (HAP/Col, Refit) were obtained from Olympus Terumo Biomaterials (Tokyo, Japan) and Pentax Hoya (Tokyo, Japan), respectively.

**Experimental design**

General anesthesia was induced in the rats by isoflurane inhalation followed by intraperitoneal injection of a mixture of 0.15 mg/kg dexmedetomidine hydrochloride, 2.0 mg/kg midazolam, and 2.5 mg/kg butorphanol tartrate. The dorsal area of the cranium was shaved and prepared aseptically for surgery. A 20-mm-long incision was made in the scalp along the sagittal suture. Circular grooves (5 mm inner diameter) were created on each side of the midline using a trephine bur under profuse irrigation with sterile saline. The surfaces of the bone were drilled with a #2 round burr, and five small circles were generated to induce bleeding (Fig. 1a). The defects were covered with cylindrical plastic caps as described previously (10). The caps were filled with ACS or HAP/Col, and then fixed on the grooves. The material-filled regions were designated as regions of interest (ROIs) (Fig. 1b) and analyzed using the following methods.

**Micro computed tomography (CT) analysis**

Images were reconstructed on a personal computer using i-View software (J. morita MFG Corp., Kyoto, Japan). Relative CT values for cortical bone and soft tissue were measured by micro-CT (R-mCT2 system; Rigaku, Tokyo, Japan). Bone volume (BV) and bone mineral density (BMD) were measured using specialist software (Kitasenju Radist Dental Clinic, Tokyo, Japan). For BMD measurements, gray scale values and the numbers of voxels corresponding to them were calculated in the ROIs.

**Histological analyses**

The rats were perfused with 4% paraformaldehyde at 12 weeks after surgery. The calvarial bone with the bone defects was resected and fixed in 4% paraformaldehyde. The bone was then decalcified with 10% EDTA solution (Wako Pure Chemical, Osaka, Japan), embedded in paraffin wax, and cut into 4-μm-thick sections for hematoxylin/eosin (H&E) and alkaline phosphatase (ALP) staining. For tartrate-resistant acid phosphatase (TRAP) staining, the specimens were immersed in tartaric acid and acid phosphatase solution (Wako).

**Statistical analysis**

Data are presented as mean ± standard deviation (SD). The Mann-Whitney- U rank test was used for statistical analysis. P value of <0.05 were considered statistically significant.
 Results

 CT images

CT images from each experimental group were compared at 0, 4, 8, and 12 weeks after surgery. In the collagen group, bone outgrowth started at 8 weeks and increased slightly until 12 weeks after surgery. In the HAP/Col group, on the other hand, bone outgrowth started at 4 weeks and increased gradually thereafter. At 12 weeks, the whole cap area was filled with newly formed bone (NB) (Fig. 2).

Histological analysis

Histological sections were prepared at 12 weeks and compared after H&E staining (Fig. 3). In the collagen group, a relatively small amount of NB was observed above the thin bridging bone area. Consistent with the CT image, the cap area was filled with NB intermingled with the grafted material (GM) in the HAP/Col group. In both groups, no inflammatory reaction was detected. TRAP and ALP staining revealed that the numbers of osteoclasts (OCs) and osteoblasts (OBs) were not significantly different in either of the groups.

CT analysis

BV (Fig. 4, left panel) and BMD (Fig. 4, right panel) were then measured. In the HAP/Col group, both BV and BMD increased with time, and by 12 weeks had reached values approximately double those in the collagen group. To further confirm the increased bone formation in the HAP/Col group, the area and height of NB were measured in HE-stained specimens (Fig. 5). These values in the HAP/Col group were 1.67- and 1.75-fold higher than those in the ACS group, respectively. The CT images were further analyzed by measurement of BMD. NB was recognizable by its green color (Fig. 6). The analysis confirmed that the green area was significantly wider in the HAP/Col group than in the collagen group (Fig. 6). Taken together, these results suggested that application of HAP/Col increased the outgrowth of NB much more prominently than did collagen.
Discussion

In the present study, the bone augmentation ability of HAP/Col was compared with that of ACS. The results clearly indicated that application of HAP/Col induced prominent bone augmentation in comparison with ACS. NB formation started earlier in the HAP/Col group (4 weeks in the ACS group) and the NB had filled the entire cap area by 12 weeks. Histological analysis revealed that the filled area was composed of NB intermingled with the HAP/Col. The main constituents of natural bone are collagen and hydroxyapatite. Collagen has telopeptide on its N- and C-terminal ends, and these protrusions make the collagen polymerize, resulting in the formation of insoluble collagen (11). Collagen lacking these protrusions is referred to as atelocollagen and can be obtained by enzymatic or alkaline treatment. It has significantly lower immunogenicity and can be used relatively safely for biological treatment (11). The combined use of ACS with platelet-derived growth factor (PDGF) has been shown to augment NB formation effectively (12). The absence of severe immune cell infiltration in both groups might have been attributable to these properties.

Hydroxyapatite, a component of calcium phosphate ceramics, is widely used for bone regeneration and can be synthesized by mixing calcium and phosphate precursors (13). Hydroxyapatite has properties similar to those of natural bone, but it is amorphous and needs to be crystalized for clinical application. The hydroxyapatite used in this study was crystallized by the low temperature method and was expected to be incorporated into the newly formed bone efficiently (14,15). In fact, the implanted HAP/Col was replaced by NB in most areas. Moreover, the NB was histologically evident until 12 weeks after surgery. On the other hand, the implanted ACS was not successfully replaced by NB, and the implanted ACS had
disappeared by 12 weeks, indicating that ACS is more resorbable than HAP/Col. BMD was also compared between the ACS and HAP/Col groups; at 2 weeks it was 2.7-fold higher in the latter than in the former, and remained 1.67-fold higher at 12 weeks after surgery. Although the BMD also increased in the ACS group, the rate of increase was more rapid in the HAP/Col group. Isolated areas created by the plastic caps were utilized to compare the degree of vertical bone augmentation. It was expected that significant NB augmentation would have been attributable to infiltration of greater numbers of osteoblasts and osteoclasts around these isolated spaces. Contrary to expectation, however, the numbers of these cells contributing to bone remodeling were equivalent in both groups. As the numbers of these cells were counted in histological specimens at 12 weeks, the possibility that they had accumulated at a much earlier time point could not be excluded.

All the results indicated that HAP/Col promotes faster bone augmentation than ACS. As the BMD of NB was also much higher in the HAP/Col group, this material appears to be reliable and safe for clinical application.

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Conflict of interest
None of the authors have any conflict of interest to declare.

References