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Biodegradation property of beta tri-calcium phosphate-collagen composite in accorded with bone formation: a comparative study with Bio-Oss Collagen® in a rat critical size defect model

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Short title: Biodegradability of b-TCP-collagen composite

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Abstract

PURPOSE: The objective of this study was to compare osteoconductivity and biodegradation properties of an in-house fabricated beta-tricalcium phosphate (b-TCP)-collagen composite with those of Bio-Oss Collagen® using a rat calvarial critical-size defect model.

MATERIALS AND METHODS: b-TCP–collagen composite material was fabricated by mixing b-TCP granules having a particle size of 0.15–0.8 mm and 75% porosity, with bovine dermis-derived soluble collagen sponge. The dry weight ratio of b-TCP granules-to-collagen ratios was 4:1. Bio-Oss collagen or the b-TCP–collagen composite was used to fill a 5.0-mm diameter calvarial defect in rats. The defects were evaluated by histological and histomorphological analyses of decalcified histological sections with hematoxylin and eosin staining 6 and 10 weeks, respectively, after surgery.

RESULTS: The defect implanted with the b-TCP composite contained immature bone structures with dense connective tissue in contrast to the abundant fibrous tissue, but no trabecular structure was observed within the defect implanted with Bio-Oss-collagen, at 6 weeks postoperatively. Eventually, the defect filled with the b-TCP composite was covered with dense, continuous, mature bone tissue with complete replacement of the graft material. However, in defects filled with Bio-Oss-collagen, only dense connective tissue, containing limited amounts of immature trabecular bone and abundant remnant Bio-Oss particles, was observed. Histomorphological analysis revealed that the b-TCP composite caused greater tissue augmentation with a larger volume of bone tissue observed in the defect and greater bioabsorption of remnant material than Bio-Oss-collagen.

CONCLUSION: These results indicated that the b-TCP composite has greater osteoconductivity with greater reduction of residue than Bio-Oss-collagen; these properties of the b-TCP–collagen composite yielded mature bone formation.

Key words: alloplast, bio-absorption, bone regeneration, bone substitute, socket preservation
Introduction

Development of alveolar augmentation procedures such as sinus elevation and alveolar ridge augmentation, is still of great concern in implant dentistry. Advancement of the capability of bone substitute would enhance clinical outcomes of bone augmentation. Ideally, the requirements for bone substitute are wide-ranging. An intraosseous defect with a narrow and complex geometry requires flexibility of the bone substitute to allow dense packing. Substantial mechanical strength of the bone substitute would help to maintain the volume of space for bone regeneration, by resisting the pressure of the mucosal/gingival flap in alveolar reconstruction or Schneider’s membrane in sinus augmentation. Furthermore, replacement of the bone substitute by de novo bone tissue without a reduction in the augmentation volume is favorable for osseointegration of the subsequent implant placement. However, these properties have a paradoxical relationship and to date, no bone substitute has ever fulfilled all of these properties.

Architectural elaboration is one of several approaches for enhancement of the clinical efficacy of a bone substitute. Block form gives a bone substitute mechanical strength to retain the volume of tissue augmentation against external forces. The application becomes restricted only with a flattened ridge but not to the bone cavities such as intra-osseous defects and maxillary sinus. Moreover, there are concerns regarding cellular entry, angiogenesis, and tissue ingrowth within a block bone substitute. When using a porous structure that allows tissue ingrowth, there is a reduction in the mechanical strength of the block material, without changes in flexibility. In contrast, using a particle form bone substitute allows packing of a bone cavity and loading of the substitute onto the alveolar ridge, regardless of the size, dimensions, surface morphology, or geometry of the recipient site. However, the mass of loaded particles tends to be susceptible to the effect of external compressive force. This requires a great deal of effort to prevent collapse of the site grafted with bone substitute particles. Hence, other ingenuity in addition to architectural designing is necessary for further development of bone substitute.
Fabrication of a composite material with a combination of inorganic particles and organic components such as purified collagen or biodegradable polymers was another promising strategy for the development of the ideal bone substitute\textsuperscript{19}. Collagen-based composite materials have been extensively explored in the literature\textsuperscript{6,25,28}. Collagen is the most abundant protein in animals, and its purified derivatives exhibit bio-absorbable properties and excellent biocompatibility. Collagen has excellent flexibility which permits a large variety of physical forms, such as a sponge, membrane, and hydrogel\textsuperscript{21}. Moreover, as a drug delivery vehicle for exogenous growth factors, it allows a favorable sustained release, along with simple diffusion and matrix degradation, thus enabling a prolonged supply of the impregnated agent into the local tissue. On the other hand, a collagen matrix needs to be combined with a solid bone substitute such as inorganic particles to reinforce the inherently weak mechanical strength of the collagen matrix. Among many trials, a composite material, consisting of bovine bone mineral particles and a purified procaine collagen matrix sponge (Bio-Oss collagen\textsuperscript{®}) in a ratio of 9:1, has been proven to exhibit favorable osteoconductivity, space-making properties and clinical efficacy. However, bovine bone mineral particles have low bio-absorbability, thus remaining in new bone tissue.

Beta-tricalcium phosphate (b-TCP) is one of the representative calcium phosphate-based alloplastic materials that exhibit biodegradation properties, defined as the replacement of implanted material by newly formed organs. The material has been widely applied in orthopedic and alveolar reconstruction surgery. It biodegrades relatively slowly, which is generally recognized to be in harmony with bone modeling\textsuperscript{9,33}. This is based upon a high water solubility, which enables dissolution in tissue fluid, and absorption by osteoclasts \textit{in vivo}\textsuperscript{4,9,18,33}. Beta-TCP also has substantial physical strength; it provides a three-dimensional scaffold for bone regeneration against the pressure of tissue shrinkage\textsuperscript{3}. Moreover, b-TCP has the potential to function as a source of calcium and phosphate ions for the local tissue during the degradation process, which possibly
results in stimulation of osteoblastic function and promotion of bone formation. In the light of these physicochemical and biological characteristics of b-TCP, we hypothesized that the b-TCP–collagen composite material would rival or surpass Bio-Oss collagen in osteoconductivity and space-making property, and would show nearly complete replacement by bone tissue during the healing period, because of its excellent biodegradation property in contrast with the poor absorption property of Bio-Oss collagen. The purpose of this study was to evaluate the osteoconductive and biodegradable characteristics of b-TCP–collagen composite material in comparison with Bio-Oss collagen a rat calvarial critical-size defect model in comparison with Bio-Oss collagen.

Materials and Methods

Preparation of the composite material

The b-TCP–collagen composite material was prepared by Olympus Terumo Biomaterials Corp. (Tokyo, Japan). The b-TCP granules were prepared by mechanochemical synthesis. Briefly, a mixed slurry was prepared by wet-mixing calcium hydrogen phosphate and calcium carbonate with a calcium/phosphate molar ratio of 1.5. Subsequently, the mixed slurry was ground into a powder by friction. This then resulted in the formation of calcium-deficient hydroxyapatite by mechanochemical reaction. After drying and modeling with deflocculant, a b-TCP block was synthesized by sintering the modeled material at 1050 °C. The b-TCP had a calcium/phosphate molar ratio of 1.5 with 75% porosity. The block was crushed into a powder, and then sieved to obtain granules measuring 0.15–0.8 mm in diameter.

The collagen component of the b-TCP–collagen composite material was prepared according to methodology previously described in the literature. Briefly, original collagen fibers were obtained from bovine dermal connective tissue. Atelocollagen, which was first subjected to protease (pepsin) treatment to remove teropeptide, was arranged into either fibrous collagen by neutralization with phosphate-buffered saline at 37 °C, or into heat-denatured collagen by high-temperature heat
treatment at 60 °C. Subsequently, the 0.15–0.8-mm b-TCP granules were blended into the collagen mixture, during which the heat-denatured collagen was mixed with the fibrous collagen at a volume ratio of 1:9. After lyophilization, the b-TCP–collagen composite was cross-linked into a spongy form by heat dehydration at 110 °C for 6 h.

The dry weight ratio of b-TCP granules-to-collagen was 4:1. The appearance of the composite was that of a typical collagen sponge. This indicated that a collapse of the structural integrity of collagen sponge due to addition of b-TCP granules had been almost completely avoided (Figure 1A). A soft X-ray image (65KVp, 8.0 mA in power; Takara Medical DX-68, Takara Medical Inc., Fukuoka, Japan) and a micro-CT-based volume rendering image (μCT 40, Scanco Medical AG, Bassersdorf, Switzerland) using a contrast threshold obtained by imaging a coin, suggested that the inherent mineral density of the composite with the 4:1 ratio was relatively low (Figures 1B and C). The composite with the 4:1 ratio was apparently flexible and elastic, in contrast with the relatively delicate texture of the commercial bovine bone-collagen composite (Bio-Oss collagen®, Geistlich, Wolhusen, Switzerland). For this reason, the composite with the 4:1 ratio was expected to have superior operability and physical properties for defect filling. Therefore, the composite with the 4:1 ratio was employed in this study. The composite and Bio-Oss collagen were prepared in the shape of discs which were 5.0-mm in diameter and 2.0-mm in thickness.

The commercial bovine bone-collagen composite were purchased for experiment. The composite material comprised of granules of Bio-Oss® (Geistlich) spongiosa with the addition of 10% highly purified porcine collagen type I. The collagen acts, according to the manufacturer, as a cohesive for the granules. Bio-oss particles were manufactured by boiling with sodium hydrate for deproteinization and sintering at 600°C in furnace.

Animal surgery
Fourteen-week-old male Sprague-Dawley rats were anesthetized by inhalation of 1.2% isoflurane. The parietal region was shaved and scrubbed with 10% povidone iodine solution, and the cranium was carefully exposed after skin and periosteal incision. The flat surfaces of the cranium were selected for critical bone defects. Two circular, bicortical cranial bone defects of 5.0 mm diameter were created across the sagittal suture between the coronal and lambdoid sutures using a trephine bur (Figure 2A). This size of defect was regarded as the critical-size defect for rat’s calvaria in literature\(^1\)\(^{23}\), which was known as the defect size resulting in nonunion without filling with certain bone graft material\(^24\). Care was taken to avoid injury to the dura mater and other deep tissues. Subsequently, the b-TCP–collagen composite was placed in one defect, and the Bio-Oss collagen in the other in each rat (Figure 2A). To verify the validity of the size of the defect, the defect was created in another rat, which did not receive any material implantation. The defects were covered with an absorbable collagenous barrier membrane (BioMend®, Zimmer, Inc., IN, USA) prior to placing skin sutures.

This study was conducted at the laboratory of Hamri Co., Ltd. (Ibaraki, Japan). The animals were maintained according to the National Institutes of Health Guide for the Care and Use of Laboratory Animal. The animal experiment protocols were approved by the laboratory Animal Care Committee of Hamri Co., Ltd. (Ibaraki, Japan).

**Histological specimen preparation and histological and histomorphometrical analysis**

Animals were sacrificed at 6 and 10 weeks (each of \(n = 4\)) postoperatively. All the cranial bones were removed and fixed for 1 week in 10% neutral buffered formalin, and then decalcified in 10% ethylenediamine tetraacetic acid for 10 days. Decalcified specimens were dehydrated in ascending grades of ethanol and embedded in paraffin wax. Embedded samples were sectioned (3.5-μm serial slices) using a microtome in the coronal direction of the artificial defect and the cranial bone (Figure 2B). Sections were stained with hematoxylin and eosin. Histological observation and photography were performed for sections of the middle portion of each cavity, using a light microscope (BX51;
Olympus, Tokyo, Japan) and a digital still camera (DP72; Olympus, Tokyo, Japan). The histological images were color coded and histomorphologically measured according to the structures observed within the defect, including new bone (green), original bone (red), new non-osseous tissue (light blue), and remnant b-TCP or Bio-Oss particles (blue).

These measurements were recorded as a percentage of the total defect area. The defect area subjected to analysis was sectioned using lines connecting the edges of the defect margin (Figure 2C) using an image analyzer (ImageJ; NIH, Bethesda, ML, USA). The image analyzer was used according to the definitions stated below, for various areas in the defect.

*New tissue area:* the area comprising tissues other than generated bone (light blue).

*New bone area:* the area of newly formed bone (green).

*Remnant defect area:* the area devoid of any tissue or material (no color).

*Remnant material area:* the area comprising remnant-implanted material. (blue)

**Statistical analyses**

Statistical analysis was performed using a commercial computer program (SPSS, Standard Version, SPSS Japan, Tokyo, Japan). Bonferroni multiple comparison and Student’s t-test were used after the repeated two-way analysis of variance (Two-way ANOVA) to compare the defects filled with b-TCP–collagen composite and those filled with Bio-Oss collagen. This was also compared for the same material between healing periods, with regard to the percentage of new tissue, new bone, remnant defect, and remnant material area. Statistical significance was set at $P < 0.05$.

**Results**

**Histological observation of defect healing**

After 6 weeks of healing, only limited formation of a fibrous-like tissue, without any observable
bone tissue, was seen in the defect that had received no material implantation (Figure 3A). The defect implanted with Bio-Oss collagen demonstrated newly formed trabeculae with relatively high maturity, mainly near the edge of the defect. Dense connective tissue formation was observed around the voids, containing a bone-like structure without osteocytes, suggesting that this was remnant Bio-Oss collagen (Figure 3B). In contrast, the defect implanted with the b-TCP composite demonstrated a marked amount of thick and dense trabecular bone within relatively dense connective tissue, not only at the edge, but also in the center of the defect (Figure 3C). In addition, bone formation on the parietal side was highly observed. The voids with surrounding connective tissue were observed in the ventral part of the defect, which faced the dura mater.

After 10 weeks, the defect implanted with Bio-Oss collagen showed trabecular formation to some extent on the remnant Bio-Oss material, but did not show complete closure of the defect with newly formed bone (Figure 3D). The thickness of the tissue within the defect implanted with the Bio-Oss collagen was apparently less than that of the original cortical bone. In contrast, thick, dense and compacted bone tissue almost completely closed the defect implanted with the b-TCP composite. The thickness of the newly formed bone was equivalent to that of the original bone. A minimal amount of small remnant composite particles was observed in the ventral region of the defect (Figure 3E). Contiguous bone-bridge at the parietal side of cortical surface, to close the bone defect was observed not only in the defect filled with b-TCP composite, but also in the defect filled with Bio-Oss collagen.

High magnification images at 10 weeks postoperatively demonstrated that the defect implanted with Bio-Oss collagen contained immature trabecular bone (Figure 3F, asterisks) within a dense fibrous network with vascular canals (Figure 3F, triangles). Bio-Oss residuals remained visible (Figure 3F, arrowheads) throughout the region. In contrast, the b-TCP composite had mainly been replaced by mature bone tissue (Figure 3G, asterisks), and had the appearance of cortical bone tissue.
Histomorphological evaluation of tissue augmentation in the defects

Two-way ANOVA indicated that there was no significant interaction between the healing time and implanted materials in terms of the percentage of new tissue area and residual material area (Figure 4A-D). However, there was a significant difference between the defects implanted with the b-TCP-collagen composite (TCP in Figure 4) and the Bio-Oss collagen (BO in Figure 4) in terms of the percentage of new tissue area at week 6, new bone area at week 10, remnant defect area at week 10 and residual material area at weeks 6 and 10. There was a significant difference between the healing times in the percentage of new tissue area, new bone area and residual material area of the defect implanted with the b-TCP-collagen composite, and in the percentage of residual material area of the defect implanted with the Bio-Oss collagen.

The defect implanted with the b-TCP-collagen composite was greater in terms of the percentage of new tissue area at 6-weeks postoperatively than the defect implanted with the Bio-Oss collagen at this time-point (Figure 4A). Although the percentage of new tissue area reduced, when measured at 6-10 weeks (Figure 4A), implantation of the b-TCP-collagen composite yielded a substantial increase in new bone area during the healing period, in contrast to the defect implanted with the Bio-Oss collagen; the new bone area in the former defect was over three times greater than that in the latter defect (Figure 4B).

The percentage of remnant defect area remained less than 40% in the defect implanted with the b-TCP composite, whereas this value remained 40% or more in the defect implanted with the Bio-Oss collagen; this was 1.8 times greater than that in the defect implanted with the b-TCP composite (Figure 4C).

The percentage of residual material area was reduced with the healing period both in the defect
implanted with the b-TCP composite and in the defect implanted with the Bio-Oss collagen. This value was consistently 2 times lower in the defect implanted with the b-TCP-collagen composite than that implanted with the Bio-Oss collagen. The value changed from 10% to 3% in the defect implanted with the b-TCP composite, and from 19% to 10% in the defect implanted with the Bio-Oss collagen (Figure 4D).

Discussion

In light of clinical benefits, bone substitutes must have mechanical property and osteoconductivity to support the bone healing process, with prevention of collapse in the augmentation region. In this study, the osteoconductivity and biodegradation property of a collagen-based composite material were evaluated using a rat calvarial critical-size defect model. The defect passed through the calvarial bone, which forced the implant material to undergo intracranial pressure from the side of the dura mater (approximately 10 mmHg in rats). This pressure could simulate the mucosal tissue shrinkage and compressive pressure of Schneider’s membrane. In addition, the calvarial bone consisted mainly of cortical bone with minimal amounts of bone marrow cells. This provided a limited source of cells of osteogenic lineage, when compared with other models with different bone sites such as the femur, where periosteal, endosteal, or bone stromal cells and their interactions are involved in healing. In fact, bone formation was predominant on the parietal side both in the defects filled with b-TCP composite and Bio-Oss collagen; this suggested that only osteoprogenitor cells from periosteal mostly contributed to bone healing in this defect model. This experimental model could therefore test the mechanical stability and the osteoconductivity of the implant material in a simulated extraction socket, sinus cavity, and/or large alveolar defect.

The histological observations of the defect without material implantation after 6 weeks of healing indicated that the bone defects of 5.0-mm diameter used in this study was indeed the critical size for bone regeneration in rat calvaria. Within this experimental bone defect model, the
b-TCP-collagen composite yielded substantial new bone formation over time, without collapse of the implanted area. The critical size defect healed almost completely not only through bone elongation from the bony walls of the defect, but also through trabecular formation at the center of the defect. The trabecular bone had a thick, compacted, matured, and continuous structure, similar to that of the original cortical bone structure. In contrast, newly formed tissue in the defect implanted with the Bio-Oss-collagen consisted mainly of dense connective tissue. The contents of this defect included only immature trabecular bone surrounding the remnant material, and the thickness of this new growth was apparently less than that of the original cortical bone.

Histomorphometrical analysis supported the histological observation and revealed a substantial increase in new bone area in the defect implanted with the b-TCP composite. The newly formed tissue formation that occurred during the early healing phase and the new bone formation that occurred during the late healing phase were greater than those in the defect implanted with the Bio-Oss collagen. Progressive tissue generation by the b-TCP-collagen composite was also supported by a consistent reduction in the percentage of remnant defect area as compared with that of the Bio-Oss collagen. These results suggested that the b-TCP composite material exhibited excellent osteoconductivity and mechanical stability. These properties of the b-TCP-collagen composite may be sufficient to overcome the limitations of the available osteogenic cellular supplements, as well as to resist the compressive forces from the surrounding tissues, such as a mucosal flap or Schneider’s membrane, for prevention of collapse of a grafted region.

The bioabsorption properties, which is lacking on bovine bone mineral, of b-TCP would contribute to greater osteoconductivity of the b-TCP composite than that of the Bio-Oss collagen. Beta-TCP in the composite should release calcium ions into the surrounding environment upon dissolution. These ions could modulate osteoblastic viability, motility, proliferation, and differentiation through activation of calcium-sensing receptors, enhancement of calcium influx into cells, and subsequent
intracellular calcium signaling pathways\textsuperscript{2, 34}. A basic chemical study evidenced that b-TCP has an inherent bioabsorbable property determined by the solubility product constant. Furthermore, b-TCP is markedly more soluble than synthetic hydroxyapatite\textsuperscript{13, 33}. In addition, the increased porosity of b-TCP granules resulted in accelerated dissolution in liquids under circulating conditions due to an increase in the granular surface area. The porosity of the b-TCP granules used in the present study (75\%) was relatively higher than that of commercially available b-TCP granules used in previous in vivo and human studies\textsuperscript{10, 11, 16, 35}. This implies that the b-TCP granules were progressively dissolved, even within the collagen sponge and indicates that the b-TCP–collagen composite material has the potential to promote bone formation by enhancing osteoblastic function through calcium supplementation, in contrast to the Bio-Oss collagen.

The implanted b-TCP composite was eventually replaced with mature living bone tissue containing vascular structures with progressive disappearance of the implanted composite. These observations indicated that the b-TCP-collagen composite exhibited bio-absorption properties that aid bone modeling and remodeling. Histomorphometrical analysis demonstrated that the percentage of residual material surface area was consistently lower in the defect implanted with the b-TCP composite material than in the defect implanted with the Bio-Oss collagen at both weeks 6 and 10. The value in the defect implanted with the b-TCP composite was reduced to less than 5\% at week 10, as compared to approximately 10\% in the defect implanted with the Bio-Oss composite. Although substantial reduction of the percentage of residual material within the healing period was seen in the defect implanted with both types of composite material, the b-TCP particles in the composite material that was replaced by new bone completely disappeared. In contrast, the newly formed dense connective tissue in the defect implanted with the Bio-Oss collagen contained apparent residual particles of the Bio-Oss material, thus demonstrating that only the collagen component had been replaced by dense connective tissue and that the Bio-Oss particles were merely surrounded but not absorbed by the newly formed tissue.
The physicochemical properties of b-TCP and the enzymatic characteristics of collagen components may underlie the biodegradation of the b-TCP composite. Collagen components in the composite used in this study included insoluble fibrous collagens and water-soluble heat-denatured collagens. Heat-denatured collagen underwent a dissolution reaction with tissue fluids. Meanwhile, fibrous collagen were decomposed by collagenase, which is secreted by many types of cells, including neutrophils, macrophages, endothelial cells, fibroblasts, and osteoblasts. Hence, the degradation of two different types of collagen fibers provided the space for cellular invasion and subsequent tissue growth within the composite, without causing its structure to collapse. Moreover, bioabsorption of b-TCP was considered to occur via 2 processes: chemical dissolution within tissue fluids and cellular absorption by multinucleated giant cells, such as osteoclasts. The b-TCP components in the composite underwent initial dissolution within the tissue fluid. After trabecular deposition on the material, gradual replacement by bone tissue occurred via incorporation into the bone remodeling cycle. Theoretically, these inherent properties facilitated the biodegradation process of the composite material in coordination with bone modeling and remodeling, without reducing the niche for bone regeneration.

Collagen is the most useful matrix for the fabrication of a composite comprising biomaterial, and inorganic materials. Many types of collagen-based inorganic composite materials have been reported in the literature. There are many inherent advantages to the use of a collagen matrix in in combined with inorganic materials as a bone substitute. A collagen matrix has an excellent excipient property that facilitates compressing or trimming to adapt to a defect’s shape. Moreover, incorporation of granular material into a collagen matrix is likely to retain the granules at the grafting region, rather than direct loading of only granules at the region. A spongy form of collagen matrix may help to retain blood clots from the host bone; these clots contain autogenous proteins and regenerating bone cells.
Moreover, the favorable sustained-release action of the collagen matrix would be advantageous for localized delivery of liquid exogenous growth agents, such as recombinant bone morphogenetic protein and organic as well as inorganic chemical compounds. In this study, a water-soluble calcium phosphate compound was employed as a composite material within a collagen matrix. Evidence suggests that an excessive local concentration of calcium ions induces osteoblastic cell death and dysfunction as a result of distorted cellular calcium metabolism. The collagen component of the composite may moderate the release of calcium ions from b-TCP granules into the defect region. This would encourage osteogenic cell growth and contribute to substantial bone healing, as was observed in this study. Accurate quantification of the calcium ions released from the composite would be an interesting topic for future research.

This study demonstrated that the osteoconductivity of b-TCP-collagen composites with reduction of residue are superior to those of the clinically well-known collagen composite material, Bio-Oss collagen®. This material promises to yield a favorable outcome when used in bone augmentation procedures for site development prior to implant placement.

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Figure 1
Figure 2

B  Soft X-ray images indicating upper side view of sample

C  Indexed color image

Longitudinal histological section with HE staining
Figure 4
Figure Legends

Figure 1. (A) Photographs, (B) soft X-ray images, and (C) micro-CT image, taken using a coin as calibration threshold, of beta-tricalcium phosphate (b-TCP)-collagen composite material. (D) b-TCP–collagen composite or Bio-Oss collagen are pinched with forceps exerting equal force.

Figure 2. (A) Top-view image of two rat calvarial critical-size defects across the sagittal suture, and scheme showing positioning of the implanted material in the bone defect. (B) Upper side view soft X-ray image of a sample (left) and a histological section stained with hematoxylin and eosin (right), showing preparation of the histological sections in the sagittal direction of the artificial defect and the cranial bone. (C) Color coding example for histomorphological measurements based on the structures in the defect, including new bone (green), original bone (red), new non-osseous tissue (light blue), and remnant b-TCP particles (blue).

Figure 3. Representative histological sections stained with hematoxylin and eosin at 6 and 10 weeks postoperatively. Lower magnification images (2.0 × magnification) showing the entire bone defect implanted with or without Bio-Oss collagen or b-TCP–collagen composite (from A to E), and higher magnification images (20 × magnification) showing the center of the bone defect implanted with Bio-Oss collagen or b-TCP–collagen composite or (F and G). Bars indicate 2.0 mm in A-E and 0.5 mm in F and G.

Figure 4. Histograms showing 6 and 10-week histomorphological results for the percentage of new tissue area, new bone area, remnant defect area, and residual material area in the defect implanted with Bio-Oss collagen (BO) and in that implanted with b-TCP composite (TCP). Data are shown as mean ± SD (n = 4). *P < 0.05, significant difference between the materials or between healing periods for each material (Bonferroni multiple comparison or Student’s t-test).