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ORIGINAL ARTICLE

Biomaterials

Angiogenesis and new bone formation in novel unidirectional porous beta-tricalcium phosphate: a histological study

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Abstract

Affinos® (Kuraray Co., Ltd., Tokyo, Japan) is a beta-tricalcium phosphate (TCP) artificial bone comprising a novel unidirectional porous structure with 57% porosity. This study examined angiogenesis and bone formation over time with unidirectional porous beta-TCP (UDPTCP). Ten Japanese White rabbits were used in this study. A 5 × 8-mm rectangular area of periosteum was resected, followed by preparation of a cortical bone defect using a high-speed bur. UDPTCP was embedded in the defect in the direction of the pores, parallel to the axis of the tibia. Tissue samples were harvested at 2 weeks ($n = 3$) and 6 weeks ($n = 7$) after implantation. Just before euthanasia, the vasculature of the lower limb was perfused with saline from the femoral artery and filled with MICROFIL® (Flow Tech, Inc., Carver, MA) to create a vascular cast. The tibia was cut longitudinally at the center of the material. Decalcified sagittal sections treated with hematoxylin and eosin staining, undecalcified sagittal sections treated with Villanueva-Goldner staining, and axial unstained sections were used for histological evaluation. The lengths of the largest vessels and newly formed bone at the material border were measured in a sagittal section. Both lengths were significantly larger at 6 weeks than at 2 weeks. In the axial sections at 2 weeks, newly formed vessels filled with blue dye grew along the pores of the UDPTCP. Mature bone tissue with a lamellar structure was observed at 6 weeks. Our histological findings demonstrated that angiogenesis and bone formation occur over time in UDPTCP.

Keywords Beta-tricalcium phosphate artificial bone · Angiogenesis · New bone formation

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Introduction

Artificial bone is commonly used as graft material for orthopedic surgery in Japan because of the small number of facilities dedicated to bone banking and the commercial unavailability of allograft materials [1]. Our group previously reported the basic experiments and clinical results of hydroxyapatite with a novel unidirectional pore structure [2, 3].

Because beta-tricalcium phosphate (beta-TCP) is easily absorbed and has high osteoconductive capacity, it is gradually degraded and ultimately replaced by mature new bone [4, 5].

Affinos® (Kuraray Co., Ltd., Tokyo, Japan), which was codeveloped with the Department of Orthopedic Surgery, Faculty of Medicine, University of Tsukuba, is a beta-TCP artificial bone with 57% porosity that consists of a novel unidirectional porous structure in which intercommunicating holes of 25–300 μm are arranged in one direction [6]. This

structure makes Affinos® (Kuraray Co., Ltd.) a good candidate for graft materials in orthopedic surgery; therefore, further exploration is warranted. Affinos® (Kuraray Co., Ltd.) allows rapid penetration of tissue into the material; its strength resists the compression stress along the orientation of the unidirectional pores, and it is easy to handle during specific usage [7].

Adequate vascularity is indispensable for the promotion of appropriate bone development, regeneration, and remodeling [8, 9]. However, angiogenesis and bone formation that occur in this material over time are unclear. This study examined angiogenesis and bone formation over time in unidirectional porous beta-TCP (UDPTCP).

Materials and methods

Implant materials

UDPTCP blocks were obtained from Kuraray Co., Ltd. (Tokyo, Japan). Its oval pores with diameters of 30–250 µm penetrated the material. The initial compression strength of UDPTCP was approximately > 9 MPa parallel to the direction of the pores and > 1 MPa perpendicular to the pores. High interconnectivity of the material was confirmed by the rapid suction of blood [7].

Animal materials

All protocols involving animals were approved by the institutional review board for animal testing at Tsukuba University. Institutional guidelines for the care and use of laboratory animals were observed.

Ten Japanese White rabbits were used in this study. General anesthesia was induced by an intramuscular injection of a mixture of ketamine (50 mg/kg body weight) and xylazine (14 mg/kg body weight). A 5 × 8-mm rectangular area of periosteum was resected, and a cortical bone defect in a 4.5- × 7-mm rectangular shape was prepared using a high-speed bur. UDPTCP was embedded in the defect in the direction of the pores, which was parallel to the axis of the tibia (Fig. 1). Tissue samples were harvested at 2 weeks ($n = 3$) and 6 weeks ($n = 7$) after implantation. Just before euthanasia, the vasculature of the lower limbs of the rabbits were perfused with saline from the femoral artery and filled with Microfil® (Flow Tech, Inc., Carver, MA) to create a vascular cast [10]. The femoral artery was cannulated with a 24-gauge venula needle and perfused with heparinized saline. The femoral vascular bundle was clamped proximally, and the femoral vein was prepared as an outlet by either cutting or cannulation with a venula needle. Perfusion was continued until the effluent from the femoral vein was clear and did not contain blood. Contrast medium (MV-120 [blue]

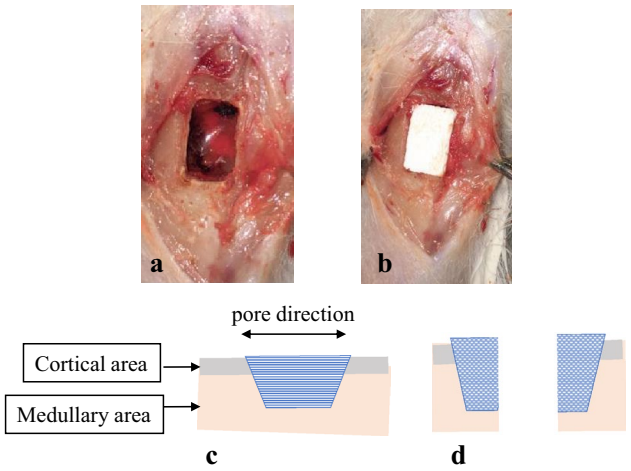

Fig. 1 Unidirectional porous beta-tricalcium phosphate was embedded in the defect in the direction of the pores, parallel to the axis of the tibia (**a**, **b**). Schematic representation of the sagittal (**c**) and axial sections (**d**)

Microfil®; Flow Tech, Inc.) was then injected in the femoral artery (Fig. 2). Microfil® (Flow Tech, Inc.) is a liquid silicone rubber formulation that cures within approximately 60 min after the addition of stannous octoate and ethyl silicate. It can be injected in vascularized structures to form a solid cast [11]. Perfusion pressure during the Microfil® (Flow Tech, Inc.) injection was monitored and maintained between 90 and 140 mmHg. After an injection of 20–30 mL of Microfil® (Flow Tech, Inc.) and confirmation that the effluent consisted of 100% Microfil® (Flow Tech, Inc.) solution, the femoral vascular bundle was clamped distal to the injection and outlet sites. After allowing the Microfil® (Flow Tech, Inc.) to cure (approximately 90 min), the right tibia was harvested following euthanasia, which was performed by administering an overdose of pentobarbital through the auricular vein during the saline infusion.

Histological analysis

The tibias were fixed in a neutral buffered 10% formalin solution. Then, they were cut longitudinally at the center of the UDPTCP insert into approximately equal halves. Half of the tibias were used for preparing decalcified slices for hematoxylin and eosin staining at 6 weeks and observed histologically. The other half of the tibias were used for preparing undecalcified sections as previously described [12]. Briefly, the bone specimen was embedded in methyl methacrylate, sectioned near the cut surface using a diamond saw, and ground to a thickness of 30 µm. The obtained sagittal section was subjected to Villanueva Goldner staining at 2 weeks and 6 weeks. Additionally, an axial section was cut 3 mm from the proximal end of the UDPTCP; this section was not subjected to staining at 2 weeks and 6 weeks. The

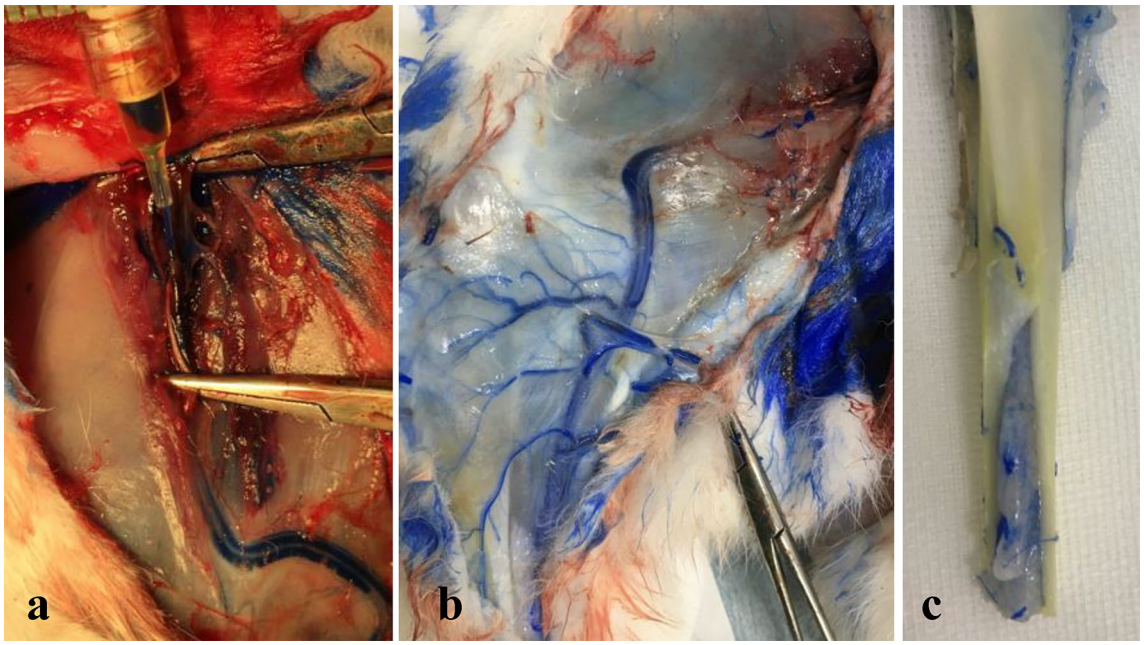

Fig. 2 The femoral artery was cannulated with a 24-gauge venula needle and perfused with Microfil® (Flow Tech, Inc.) (**a**). The blue silicone rubber was allowed to polymerize at room temperature (**b**, **c**)

lengths of the largest vessels and newly formed bone at the material border were measured in the sagittal section. We randomly selected five regions of interest of 200 µm on one side to measure the largest diameter of the blood vessels in the axial section. The average of five specimens was calculated as the diameter of blood vessels and compared between the two groups. All data are presented as mean ± standard deviation. Statistical analysis was performed using the Student's *t* test. $P < 0.05$ indicated a statistically significant difference.

Results

The UDPTCP blocks were stably inserted in the tibial bone defect in all animals. No breakages in the material were visualized at the time of implantation or specimen collection. All animals were maintained in good physical condition after implantation. Weight loss and infections at the implantation site were not observed at the time of specimen collection.

In the decalcified sagittal sections subjected to hematoxylin and eosin staining, new bone formation was observed in UDPTCP added to the inner wall, and multinucleated giant cells in the resorptive lacunae were observed in some parts (Fig. 3).

Newly formed bone was observed as a green area in the sagittal sections after Villanueva-Goldner staining. Capillaries that newly formed with the UDPTCP material were observed as blue areas of Microfil® (Flow Tech, Inc.) (Fig. 4). In the axial sections that were not subjected to staining, new tissues gradually formed regular structures within the pores, and newly formed vessels filled with blue dye grew along the pores of the UDPTCP at 2 weeks. Mature bone tissue with a lamellar structure was observed at 6 weeks (Fig. 5).

The average length of the largest area of newly formed bone at the material border was 794.7 ± 156.4 µm at 2 weeks and 3481.3 ± 27.9 µm at 6 weeks. The average length of the largest vessels at the material border was 1351.3 ± 674.1 µm at 2 weeks and 3051.6 ± 176.4 µm at 6 weeks. Both lengths were significantly larger at 6 weeks than at 2 weeks ($p = 0.001$ and $p = 0.045$, respectively). In the axial sections, the average diameter of the vessels was 39.4 ± 11.8 µm at 2 weeks and 50.0 ± 24.8 µm at 6 weeks, indicating no significant change ($p = 0.31$) (Fig. 6).

Discussion

In this study, we visualized blood vessels in artificial bone with Microfil® (Flow Tech, Inc.). The new blood vessels elongated in the unidirectional pores of the materials over time, eventually reaching the center of the material at 6 weeks.

Chazono et al. reported that active new bone formation occurred when osteoblastic lining cells were in direct contact with β-TCP in decalcified specimens of rabbits [5]. Collagen fibers and blood vessels became entangled with pores

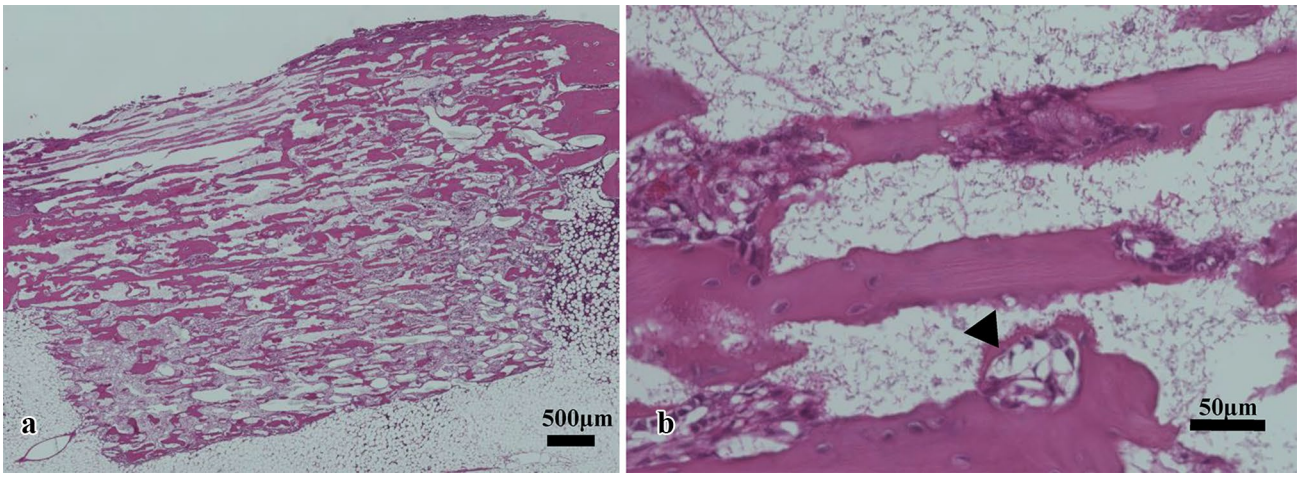

Fig. 3 Decalcified sagittal sections subjected to hematoxylin and eosin staining performed 6 weeks after transplantation (**a** ×12.5 magnification; **b** ×200 magnification). Multinucleated giant cells in the resorptive lacunae were observed at higher magnification (black triangle)

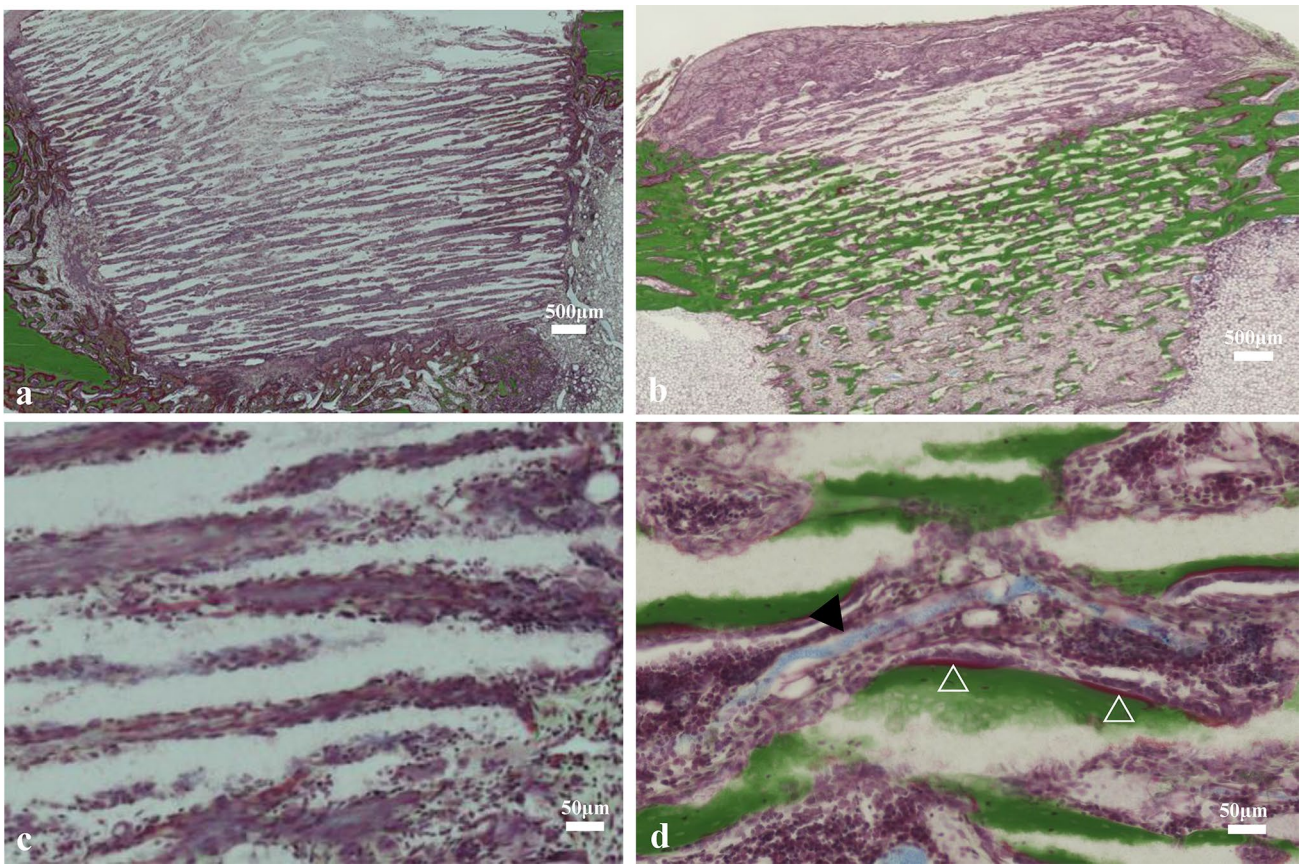

Fig. 4 Undecalcified sagittal sections subjected to Villanueva Goldner stain. Histological findings at 2 weeks (**a**, **c**) and 6 weeks (**b**, **d**) after implantation. Sagittal sections at low (×12.5) magnification and high (×100) magnification are displayed in the upper (**a**, **b**) and lower (**c**, **d**) images. Newly formed bone is observed as a green area at the edge of the material at 2 weeks. White triangle indicates osteoids. Black triangle indicates newly formed vessels, with the UDPTCP material observed as blue areas of Microfil® (Flow Tech, Inc.)

during the bone replacement process, and they also induced bone formation before the material began absorption [13].

Lafage-Proust reported that there is accumulating evidence that the bone vasculature is a crucial partner in the

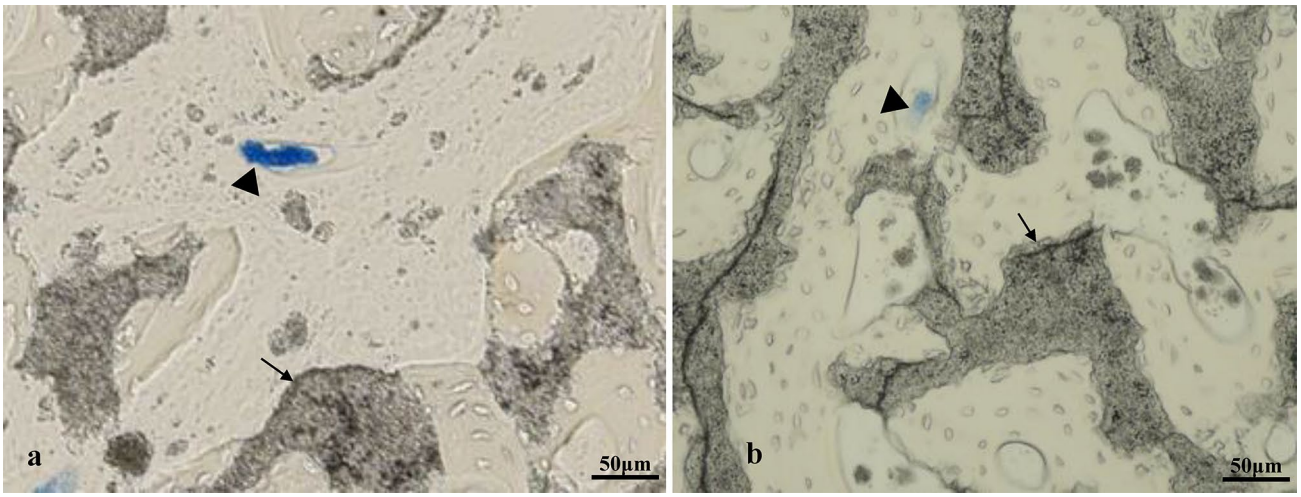

Fig. 5 Undecalcified axial sections unstained (×100 magnification) at 2 weeks (**a**) and 6 weeks (**b**). Newly formed vessels filled with blue dye grew (black triangle) along the pores of the unidirectional porous beta-tricalcium phosphate (black arrows). Mature bone tissue with a lamellar structure was observed at 6 weeks

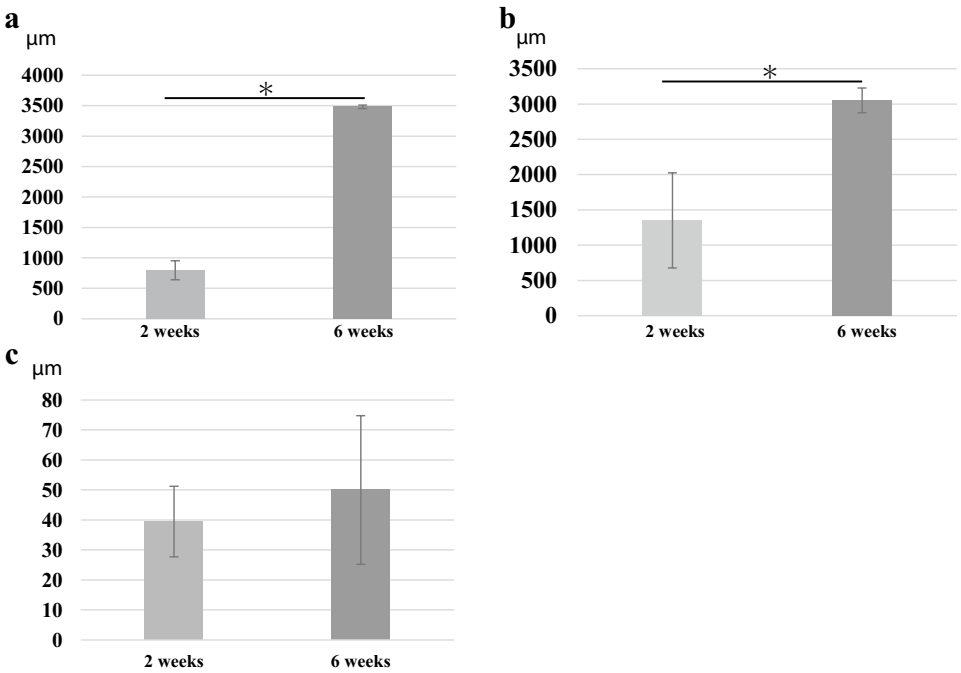

Fig. 6 The length of the largest area of newly formed bone (**a**) and the length of the largest vessels (**b**) at the material border increased significantly between 2 and 6 weeks after implantation. The average diameter of the vessels was 39.4 ± 11.8 μm at 2 weeks and 50.0 ± 24.8 μm at 6 weeks, without significant change ($p = 0.31$) (**c**)

bone remodeling process and has a role in resorption/formation coupling [14].

There is a coupling mechanism that enhances osteogenesis as osteoblasts up-regulate vascular endothelial-derived growth factor (VEGF) and promotes angiogenesis [9]. Collin-Osdoby reported that basic fibroblast growth factor regulates multiple aspects of in vivo osteoclast-mediated bone resorption by promoting localized recruitment, formation, and differentiation [15]. Osteoclast progenitors promote bone vascularization and osteogenesis [16]. During both modes of osteogenesis, angiogenesis occurs in association with the production of VEGF by the activities of either hypertrophic chondrocytes or differentiating mesenchymal cells [8].

In UDPTCP, the frost pillar shape leads to a capillary phenomenon, and the liquid component is a structure that is easy to create [7]. Tissue invasion in artificial bone is considered an important factor in bone replacement. Our experimental results suggested that the unique pore structure of UDPTCP contributes to material remodeling.

Limitations

There were some limitations to this study. First, we used a single cortical bone defect model, and the defect was not of critical size. Bone formation in larger beta-TCP implants would be needed to repair larger bone defects encountered in the clinical setting. Second, certain properties such as osteoclast and osteoblast cells were not analyzed by immunostaining. Osteoclasts and osteoblasts were indicated and characterized by tartrate-resistant acid phosphatase staining and alkaline phosphatase staining, respectively [17, 18]. Therefore, more detailed studies are needed to determine the optimal number of osteoclasts and osteoblasts needed to investigate their contribution to a coupling mechanism.

Conclusions

Our histological findings demonstrated that angiogenesis and new bone formation occur over time in UDPTCP, which is a useful material for bone substitution and repairing bone defects in fractures.

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Compliance with ethical standard

Conflict of interest The authors declare that they have no conflict of interests.

Ethics approval and consent to participate All protocols involving animals were approved by the institutional review board for animal testing at Tsukuba University.

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