

## Effect of $\beta$ -tricalcium phosphate and porous hydroxyapatite bone substitutes on bone regeneration in alveolar bone defects around dental implants

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**Although artificial materials are preferred over biological bone substitutes because there is no risk of infection, clinical outcomes are less favorable. We histologically investigated the influence on new bone formation of two types of artificial bone substitutes used to fill in experimental bone defects around implants. Nine dogs were used as the experimental animals, the bilateral mandibular, premolars were extracted, and, after a 3-month observation of osseous healing, 4 bone defects with a diameter of 5.0 mm and a depth of 10 mm were prepared in the alveolar bone of the extracted regions in each animal using a trephine bur. Implants were placed in the distal regions of the bone defects, and the animals were divided into 4 groups : Bone defects around the implants were filled with porous hydroxyapatite (HA), autologous bone,  $\beta$ -tricalcium phosphate ( $\beta$ -TCP), or not filled (controls). The wounds were completely closed, and samples were collected 2, 4 and 8 weeks after the implant treatment.**

**Bone mass continuously increased in the  $\beta$ -TCP group, whereas no increase was noted over 8 weeks in the HA group. No significant difference was observed between the  $\beta$ -TCP and control groups. New bone formation was observed earlier in the  $\beta$ -TCP group than in the HA group. Since HA is not absorbed by osteoclasts, the entrance of cells, such as osteoblasts, into the filled material may have been delayed in the HA group, resulting in the delayed formation of new bone, for which a combination with inclusion of autologous bone may be desirable. (J Osaka Dent Univ 2015 ; 49(1) : 69–84)**

**Key words : Implant ; Autologous bone ;  $\beta$ -TCP ; HA**

### INTRODUCTION

Recent dental implant treatments has been dominated by prosthetics, which attaches great importance to function and esthetics ; however, bone mass and width as well as favorable bone quality in the implant placement region of alveolar bone may not be sufficient during surgery. Fresh autologous bone grafting is generally done to overcome this problem. However, when collecting autologous bone fragments, there is the stress of surgery on the healthy region, and only a limited amount of bone can be collected.<sup>1</sup> To minimize surgical stress, the use of various bone substitutes has been investigated. These include allogeneic freeze-dried bone, decalcified free-

ze-dried bone, xenogeneic bovine dried bone (Bi-OssR, Nu-OssR), and the artificial materials, beta-tricalcium phosphate ( $\beta$ -TCP), bioactive glass, and hydroxyapatite (HA).

Although the clinical outcome for allogeneic and xenogeneic bone is superior to artificial materials, their application to patients has been hindered by concerns that the bone may contain unknown pathogens due to its biological origin. Therefore, improvements are urgently needed in the clinical outcomes of artificial materials that have little possibility of infection by unknown pathogens. We selected  $\beta$ -TCP and HA as bone substitutes because they have generally been applied clinically as stable bone substitutes in the fields of orthopedics and dentistry.<sup>2-4</sup> Implants were

placed in alveolar bone defects prepared on the assumption of bone dehiscence, the experimental bone defects around the implants were filled with the two artificial bone substitutes, and their influence on new bone formation and osseointegration were histologically investigated.

## MATERIALS AND METHODS

### Experimental animals

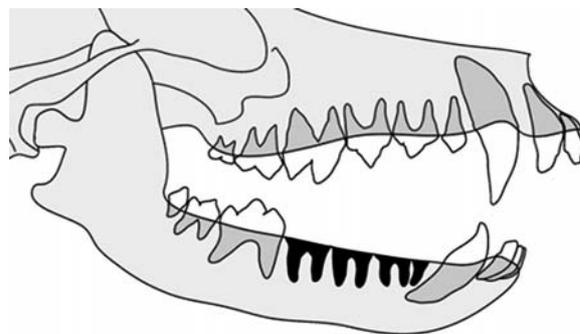
Nine adult female beagles weighing 10–12 kg were used. These animals were maintained with sufficient filtered water and food pellets in a thermostatic chamber in the animal facility of the Inagawa Research Center, Institute of Dental Research, Osaka Dental University. They were kept for some time before the initiation of experiments to confirm their health by observing general and oral conditions. Animals were handled following the Osaka Dental University Regulations on Animal Experimentation (Approval number : 13–03011).

### Materials

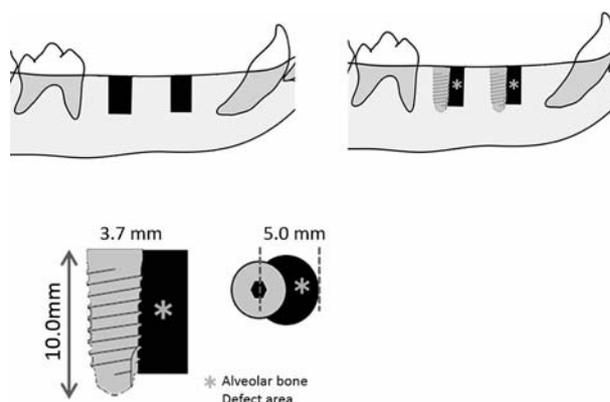
We used implants (3.7 mm in diameter 10 mm long (BrainBase Corporation, Tokyo, Japan) coated with HA. Neobone<sup>®</sup> (MMT, Osaka, Japan) was used for HA, and OSferion (Olympus<sup>®</sup>, Tokyo, Japan) was used for  $\beta$ -TCP. The diameters of the granules in both materials were 250–1,000  $\mu$ m.

### Experimental procedures

Prior to implant placement, ketamine hydrochloride (Nomopain<sup>®</sup> injection for animals, Meiji Seika, Tokyo, Japan) was intramuscularly injected into the beagles at 10 mg/kg body weight, followed by an injection of pentobarbital sodium (Nembutal<sup>®</sup>, Dainippon Sumitomo Pharma, Tokyo, Japan) into the cephalic vein at 20 mg/kg body weight. The bilateral mandibular premolars were extracted under general anesthesia. After confirming callus formation in the extraction sockets at 3 months, cavities for implant placement were prepared at two sites in the alveolar ridge of the mandibular premolar region on each side (4 sites in total) under general and local infiltration anesthesia, as described above. Bone defects were prepared on the mesial side of the cavities using a trephine bur 5.0 mm



**Fig. 1** Preparation of the animals. The bilateral mandibular premolars were extracted, and callus formation in the extraction sockets was confirmed by dental radiography 3 months later.



**Fig. 2** Implant placement. A bone defect 5.0 mm in diameter and 10 mm long was made in the alveolar bone using a trephine bur under saline irrigation at two sites on each side (4 sites in total) in each animal. Implants were placed in the distal region of the bone defect, prosthetic bone materials were placed around it, and the region was completely closed with sutures.

in diameter and 10 mm long on the assumption of bone dehiscence, and an implant was placed in each cavity (Figs. 1–2).

Four experimental groups were established : bone defects on the mesial side of the implants were filled with blood clots only (control group), crushed autologous bone only (autologous bone group), HA granules (HA group), and  $\beta$ -TCP ( $\beta$ -TCP group). Bone fragments produced during preparation of the bone defects were used for crushed autologous bone. The surgical wounds were completely closed with silk thread. After this treatment, 300 mg of lincomycin hydrochloride (Lincocin<sup>®</sup> Injection, Pfizer, Tokyo, Japan) was intramuscularly administered and 15 mg/kg body weight of clarithromycin (Claris<sup>®</sup> Dry Syrup for

children, Taisho Pharmaceutical, Tokyo, Japan) was orally administered for 3 days to prevent infection. The animals were fed a soft diet, and the sutures were removed one week after implant placement.

### Observation period and sample collection

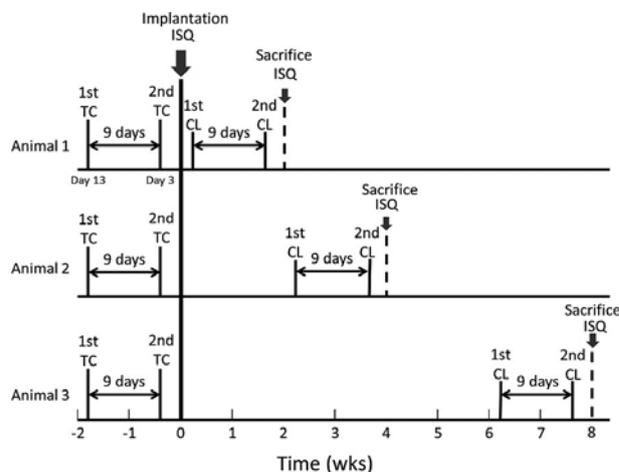
All experimental groups were divided for observations 2, 4 and 8 weeks after implant placement. Tetracycline (TC) (Sigma-Aldrich Japan, Tokyo, Japan) and Calcein (CL) (Wako Pure Chemicals, Osaka, Japan) were used as bone-labeling materials. The labels were dissolved with saline and subcutaneously administered at 20 and 10 mg/kg body weight, respectively. After completion of the observation period, the animals were euthanized with an intramuscular ketamine hydrochloride injection at 10 mg/kg body weight, followed by intravenous overdoses of 5,000 units of an anticoagulant (Novo-Heparin® Injection 1000, Mochida Pharmaceutical, Tokyo, Japan) and pentobarbital sodium. The animals were fixed by the perfusion of 70% ethanol through the bilateral common carotid arteries, and the mandible of the experimental region was excised with the surrounding healthy bone en bloc. Specimens were fixed by immersion in 70% ethanol, trimmed using a diamond cutter, and undecalcified sections were prepared and observed.

### Preparation of undecalcified ground sections (Villanueva bone stain)

Staining solution was prepared by mixing Villanueva bone stain powder with 100 mL of 70% ethanol. The specimens were immersed in this solution and embedded. After dehydration with ethanol, the samples were directly immersed and embedded in MMA. The samples were polymerized, and after confirming appropriate hardness, the center of the implant was cross-sectioned in the mesio-distal direction, and undecalcified ground sections were prepared. The bone-defect regions were observed under natural, polarized, and fluorescent lights using an incident-light microscope (RX-51 ; Olympus).

### Bone-labeling schedule

TC was administered 13 days before implant placement and re-administered 10 days later. CL was also



**Fig. 3** Bone labeling schedule for the animals sacrificed at 2, 4 and 8 weeks.

TC : Tetracycline CL : Calcein.

administered 13 days before euthanasia and again 3 days before. This schedule is shown in Fig. 3.

### Implant stability quotient

The implant stability quotient (ISQ) was measured at the time of implant placement and mandibular excision in all groups using an Osstell Mentor (Sasaki, Tokyo, Japan). ISQ was measured at 4 sites : the occlusal, mesial, distal, and buccal surfaces.

### New bone morphometry and lamellar bone : Measurement of the fibrous bone ratio

A Histometry RT digitizer and software, CSS-840 Cancerous Bone Measurement Version (System Supply, Ina, Nagano, Japan), were used in the bone morphometry system. Six visual fields centered on the central region of the implant were observed under a microscope at 200 times magnification with a 10 x eyepiece, 20 x objective lens, and 1.0 intermediate magnification.

### Counting of osteoblasts and osteoclasts

Only osteoblasts involved in lamellar bone formation arranged in one row near the lamellar bone were counted. All osteoclasts within the measurement range were counted.

### Statistical analysis

The experimental groups were compared using the Mann-Whitney U-test with the significance level set at 5%.

## RESULTS

### Implant stability quotient (ISQ) (Fig. 4)

The ISQ value at the time of implant placement was 52–54 in all groups. It decreased after 2 weeks, but increased again at 4 and 8 weeks in all groups.

### Histological findings

#### Control group (Fig. 5)

Although no new bone had formed after 2 weeks, abundant fibrous tissue was noted around the implants. Even though osteoid formation from existing bone was partially observed on the bone surface, the arrangement of the osteoblasts was rough (Fig. 6). After 4 weeks, new bone formation reached the implants. However, under polarized light it was found to mostly be fibrous bone (Fig. 7). Although the ratios of lamellar and fibrous bone were similar around implants at 8 weeks when observed under polarized light, no new bone had formed on the implant surface, which was covered by fibrous tissue (Fig. 8).

#### Autologous bone group (Fig. 9)

Osteoid formation from the grafted bone was detected

from 2 weeks, and new bone that reached the implants was partially observed (Fig. 10). Grafted bone was partially absorbed at 4 weeks, and new bone consisting of a mixture of fibrous and lamellar bone was observed under polarized light (Fig. 11). Lamellar bone was also present on the implant surface after 8 weeks. Although the grafted bone was mostly absorbed, it partially remained even at 8 weeks and was covered by new bone (Fig. 12).

#### $\beta$ -TCP group (Fig. 13)

Osteoid formation was noted on granules around existing bone at 2 weeks; however, only fibrous tissue was present around the implants (Fig. 14). At 4 weeks, osteoid and fibrous bone had formed on granules around the implants, but not on the implant surface. Observations under fluorescent light revealed that many osteoclasts were present on the surface of granules (Fig. 15). New bone had also formed on the implant surface at 8 weeks. Observations under polarized light revealed that this new bone was mostly fibrous bone; however, although one layer of lamellar bone was also detected, the quantity was very small (Fig. 16). There were fewer granules at 8 weeks than at 4 weeks.

#### HA group (Fig. 17)

Although formation of new bone was observed from the bottom of the bone defect cavities toward the implant surfaces from week 4, no new bone or osteoid was present in the central region of the bone defects after 2 or 4 weeks. Even though new blood vessels and osteoclasts were observed in HA granules under high magnification, no osteoblasts were present (Figs. 18–19). The number of new blood vessels and osteoclasts decreased farther from the bone surface. At 8 weeks, osteoid and fibrous bone were present on the implant surfaces and upper and lower regions of the bone defects; however, no new bone had formed in the central regions of the bone defects. Observations under polarized light revealed that lamellar bone and fibrous bone were mixed in the new bone. Osteoclasts, new blood vessels, and osteoblasts were also present in the central region of the bone defects (Fig. 20).

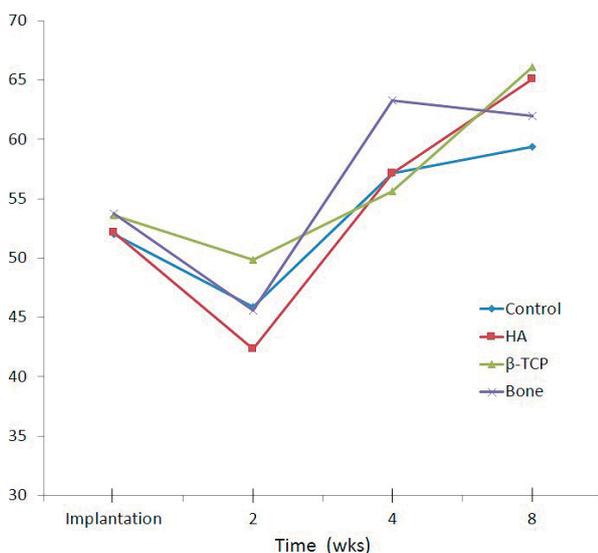
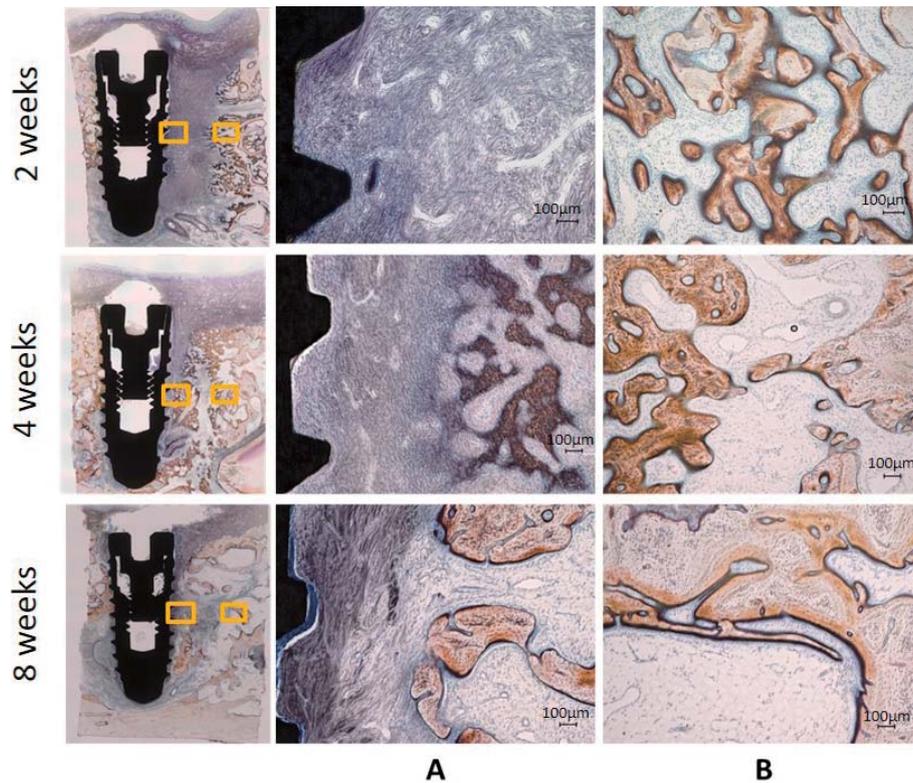
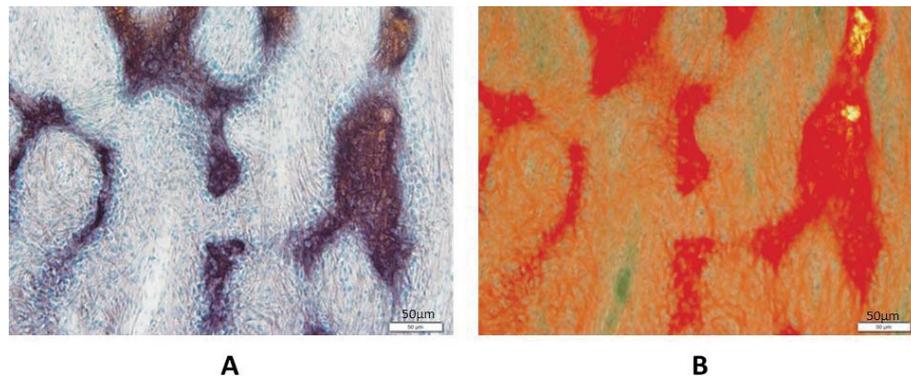


Fig. 4 Changes in the implant stability quotient (ISQ) over time.



**Fig. 5** Villanueva bone stain with the controls on the implant side (A), and the bone side (B).

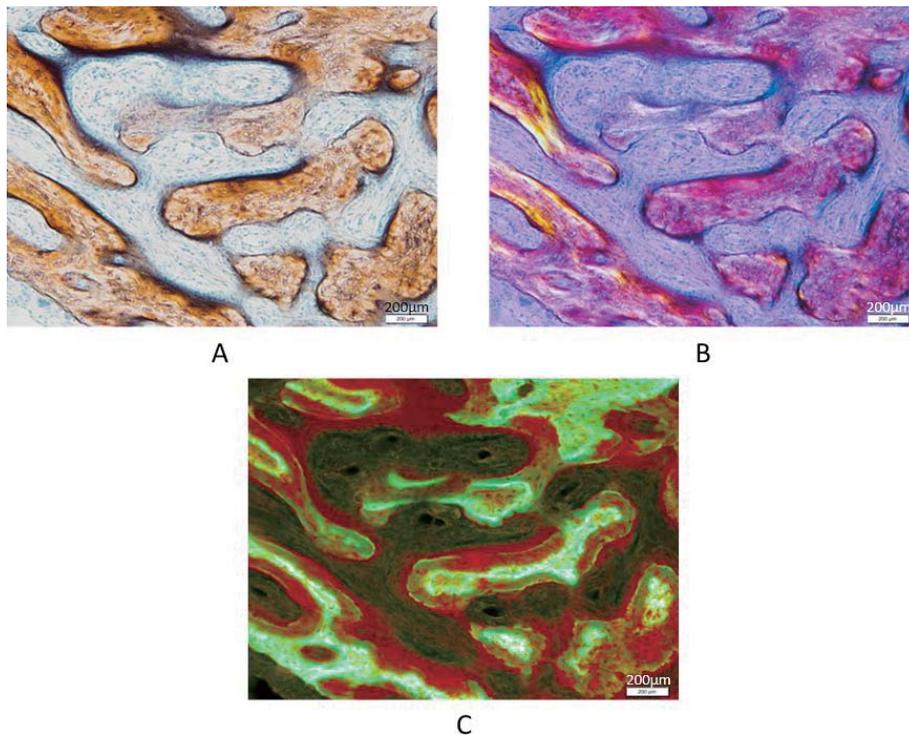


**Fig. 6** Histology of bone defects at 2 weeks in the controls under natural light (A), and fluorescent light (B).

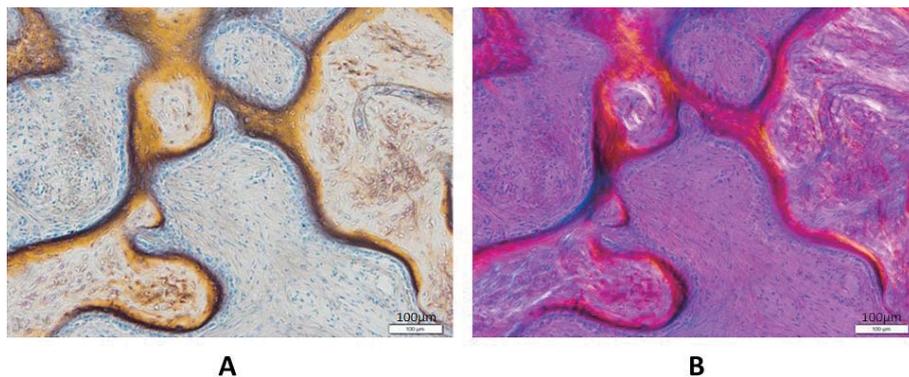
**Osteoid and new bone areas**

Although the osteoid area (Fig. 21) decreased with time from 2 weeks in the autologous bone group, it increased with time in the controls. In the  $\beta$ -TCP and HA groups, although the osteoid area increased from 2 to 4 weeks, no increase was noted from 4 to 8 weeks. A comparison among the experimental groups show-

ed that the area was significantly wider in the autologous bone group than in the other groups after 2 weeks. A significant difference was only observed between the autologous bone and HA groups at 4 weeks, and no significant difference was noted among any group at 8 weeks. The new bone area (Fig. 22) increased with time in all groups. At 2



**Fig. 7** Histology of bone defects at 4 weeks in the controls under natural light (A), polarized light (B), and fluorescent light (C).



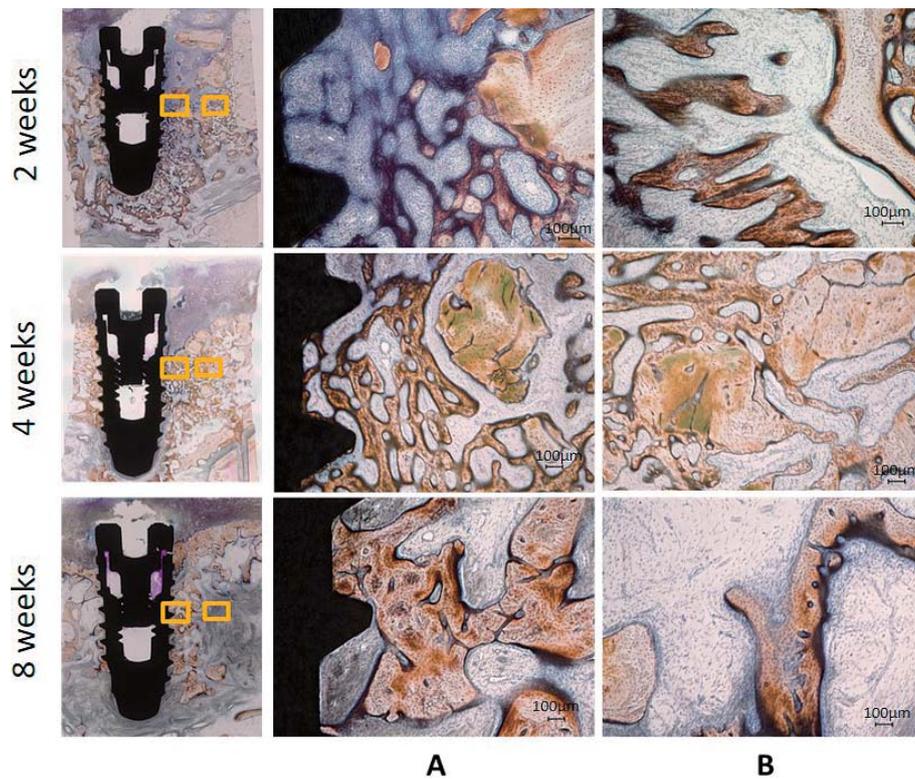
**Fig. 8** Histology of bone defects at 8 weeks in the controls under natural light (A), and fluorescent light (B).

weeks, this area was significantly wider in the autologous bone group than in the other groups. At 4 weeks, a significant differences were observed in the new bone area among the autologous bone group, controls, and the HA group. However, no significant difference was noted with the  $\beta$ -TCP group. At 8 weeks, the new bone area in the autologous bone group was significantly different from that in the con-

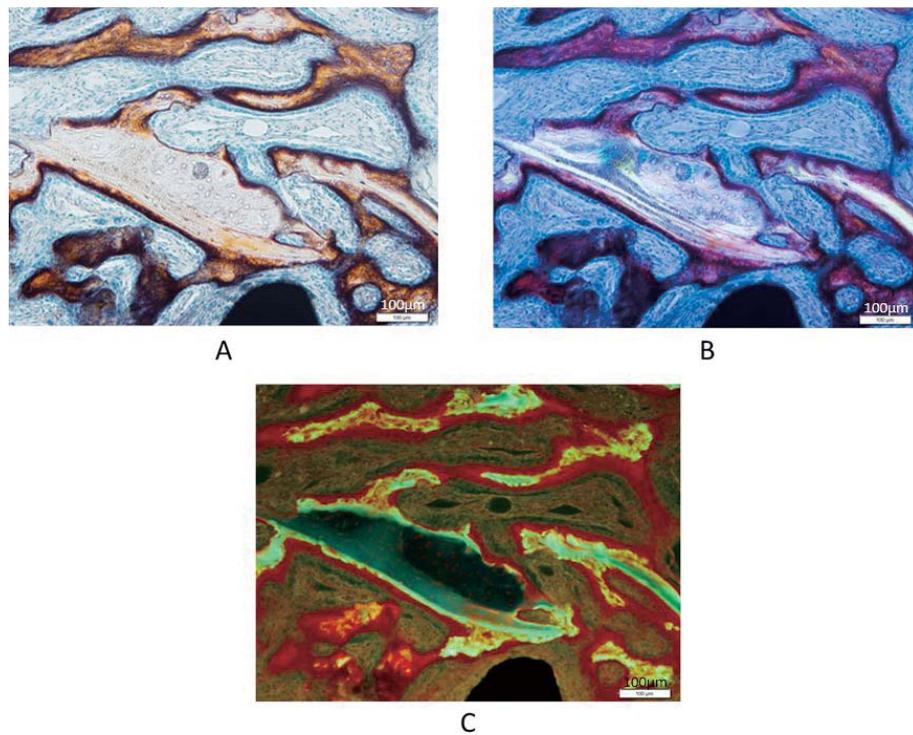
trols, but not from those in the other groups.

#### **Ratios of fibrous and lamellar bone**

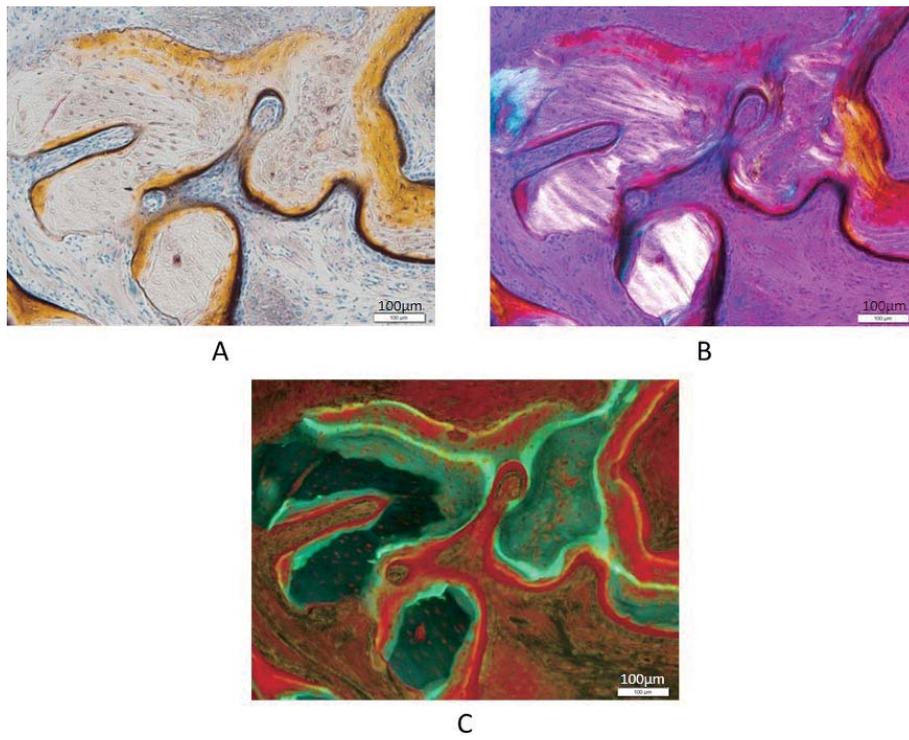
New bone was classified into osteoid, fibrous bone, and lamellar bone, and the ratios of these bone types were determined (Fig. 23). At 2 weeks, osteoid accounted for an average of 70% in the  $\beta$ -TCP and HA groups, and no fibrous or lamellar bone was ob-



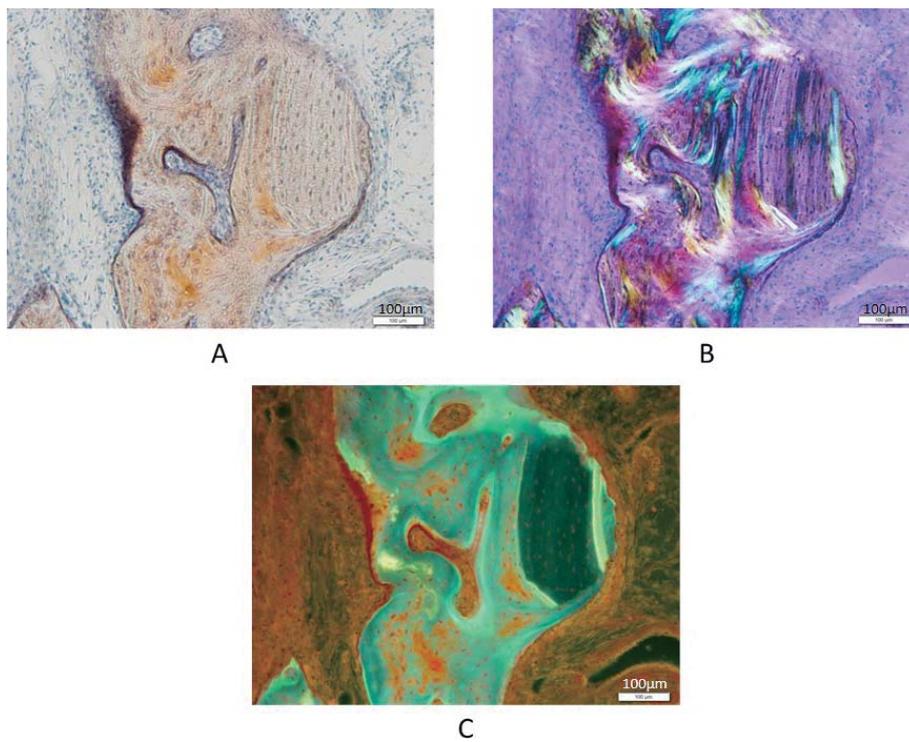
**Fig. 9** Villanueva bone stain with the bone group on the implant side (A), and on the bone side (B).



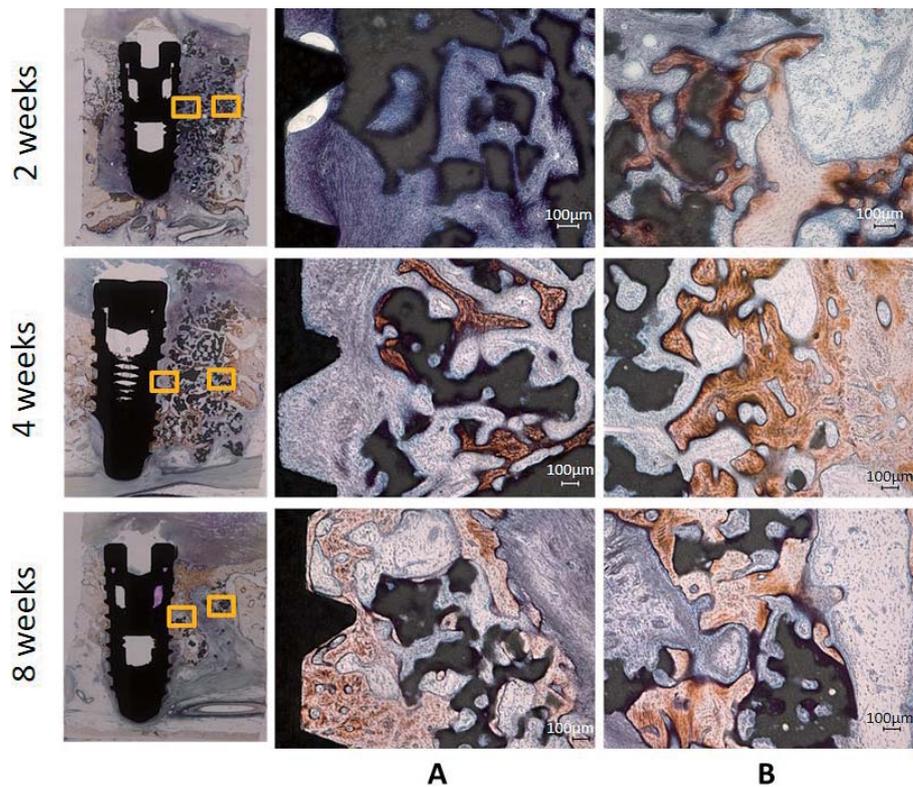
**Fig. 10** Histology of bone defects at 2 weeks in the bone group under natural light (A), polarized light (B), and fluorescent light (C).



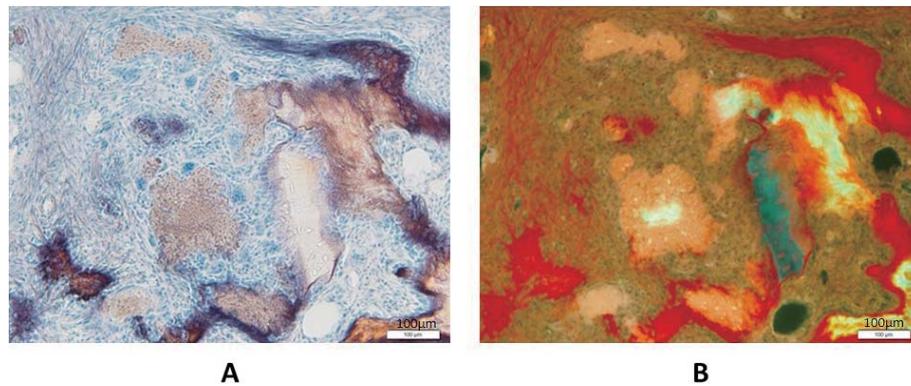
**Fig. 11** Histology of bone defects at 4 weeks in the bone group under natural light (A), polarized light (B), and fluorescent light (C).



**Fig. 12** Histology of bone defects at 2 weeks in the bone group under natural light (A), polarized light (B), and fluorescent light (C).



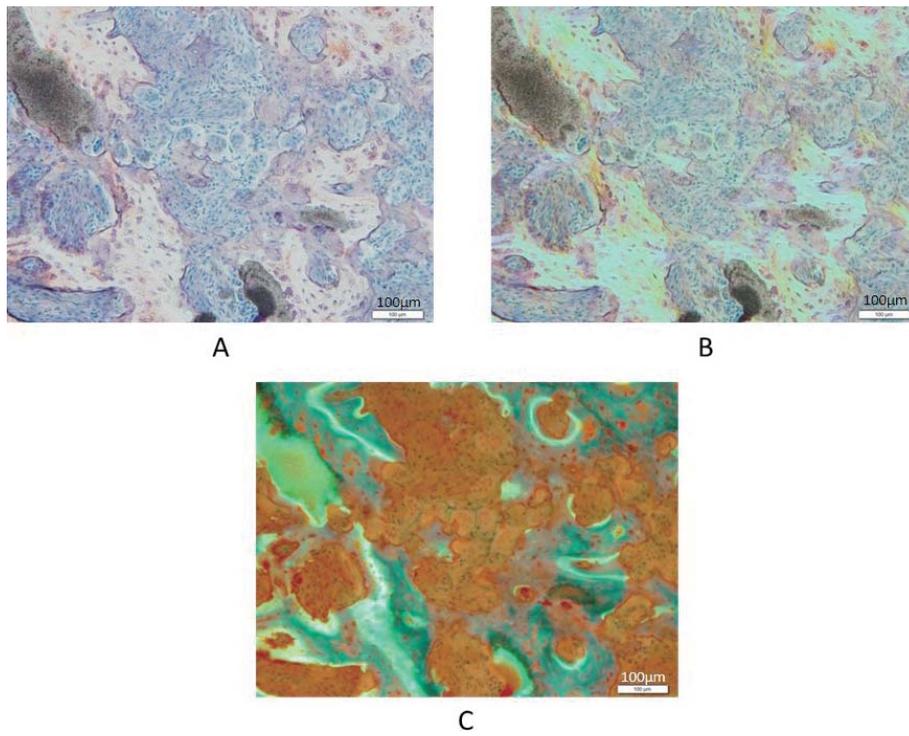
**Fig. 13** Villanueva bone stain with the  $\beta$ -TCP group on the implant side (A), and the bone side (B).



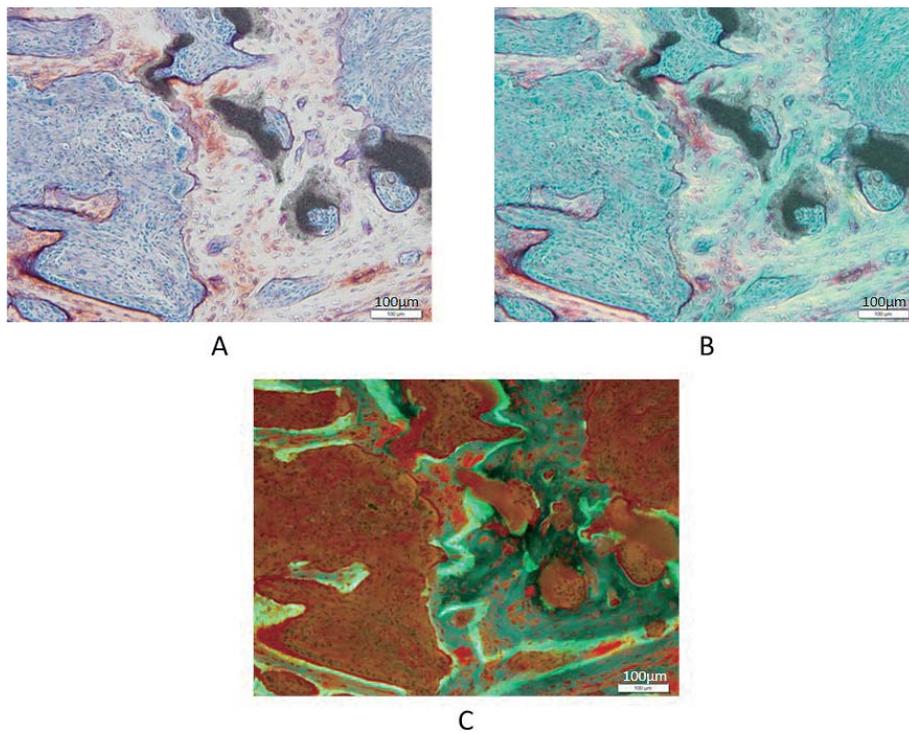
**Fig. 14** Histology of bone defects at 2 weeks in the  $\beta$ -TCP group under natural light (A), and fluorescent light (B).

served. In contrast, although approximately half of new bone was comprised of fibrous and lamellar bone in the control and autologous bone groups, it was mostly fibrous bone in the controls. At 4 weeks, although new bone accounted for 60% or more in all groups and 80–90% in the control and  $\beta$ -TCP gro-

ups, it was mostly fibrous bone. In contrast, approximately half of the new bone was lamellar bone in the autologous bone and HA groups. No osteoid was noted at 8 weeks, and approximately 50% of new bone was lamellar bone in the autologous bone,  $\beta$ -TCP and HA groups, whereas the ratio of osteoid in-



**Fig. 15** Histology of bone defects at 4 weeks in the  $\beta$ -TCP group under natural light (A), polarized light (B), and fluorescent light (C).



**Fig. 16** Histology of bone defects at 8 weeks in the  $\beta$ -TCP group under natural light (A) polarized light (B), and fluorescent light (C).

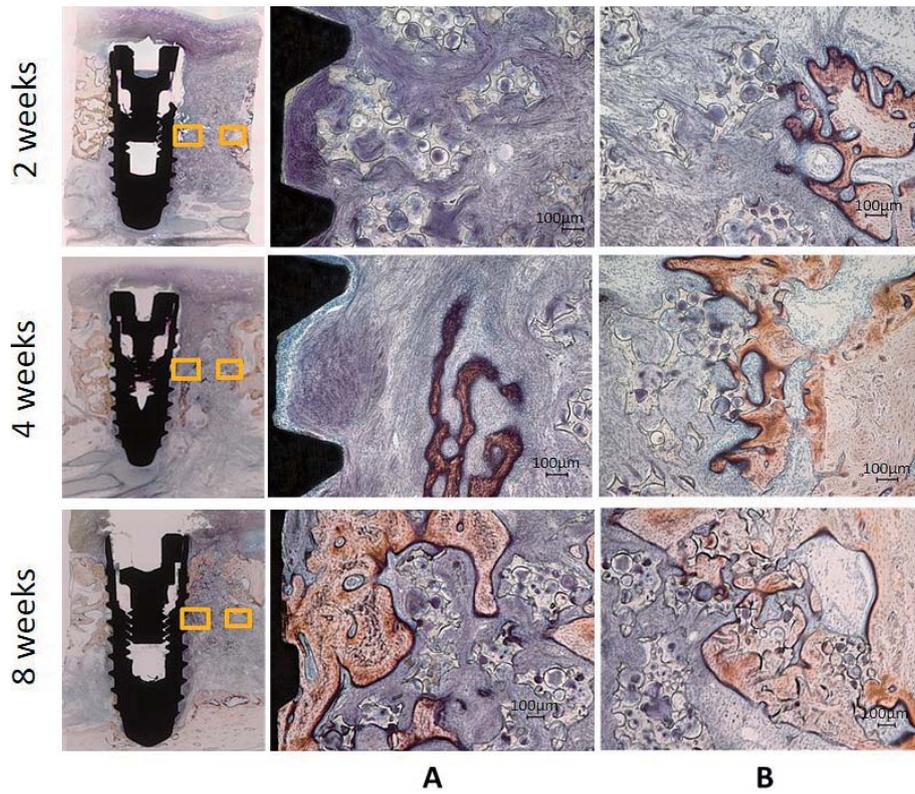


Fig. 17 Villanueva bone stain with the HA group on the implant side (A), and bone side (B).

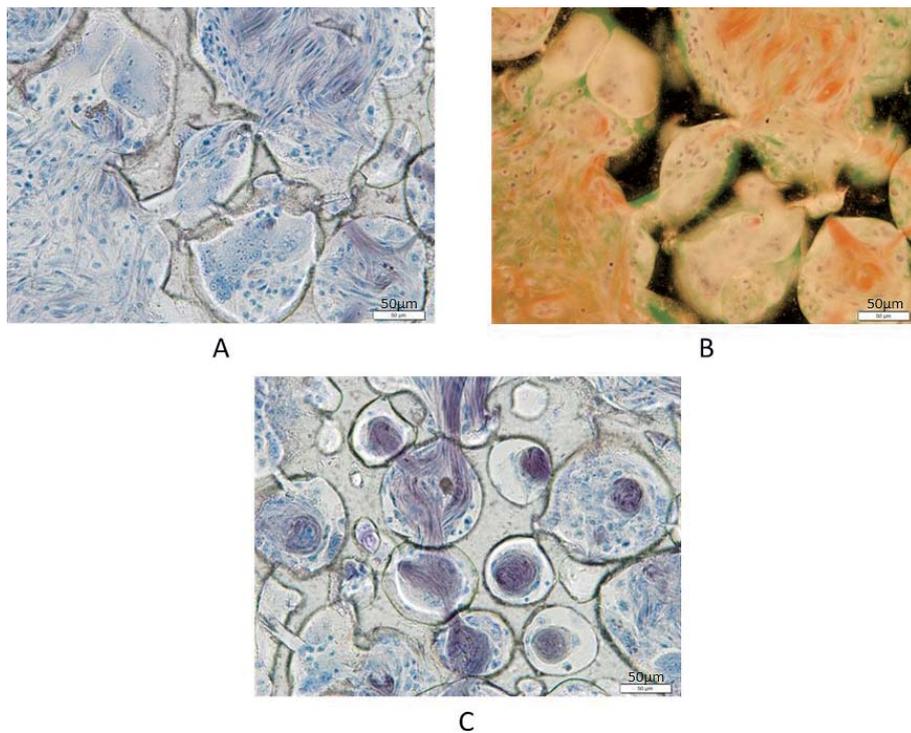
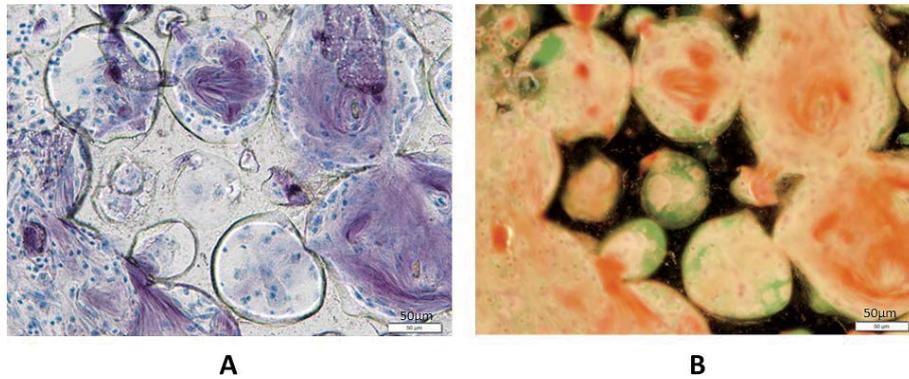
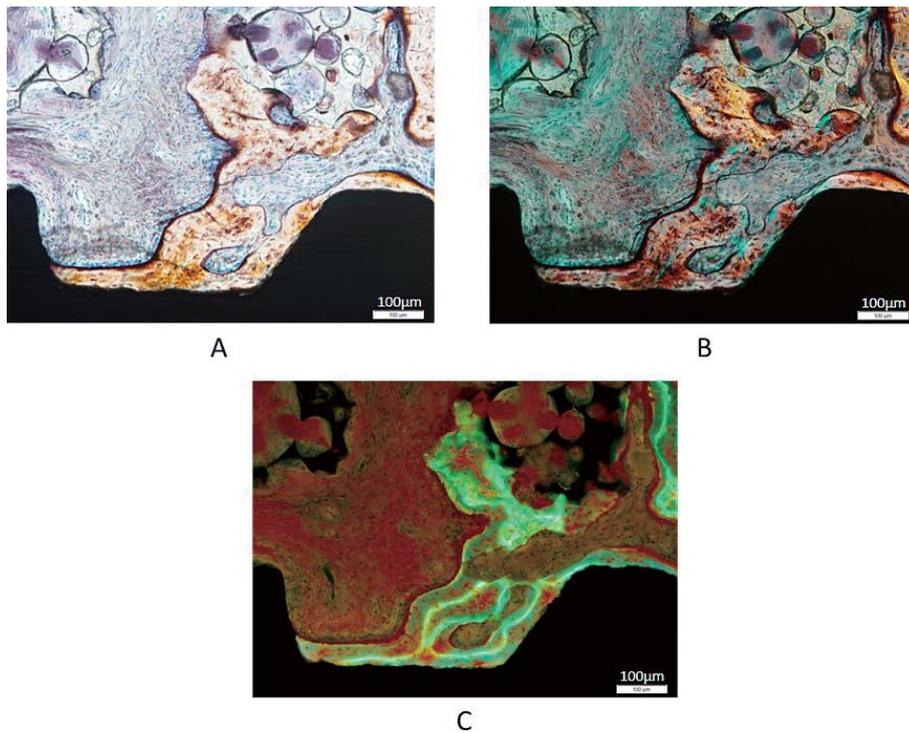


Fig. 18 Histology of bone defects at 8 weeks in the HA group under natural light (A) polarized light (B), and a magnified image of the central region of the bone defect under natural light (C).



**Fig. 19** Histology of bone defects at 2 weeks in the HA group under natural light (A), and fluorescent light (B).



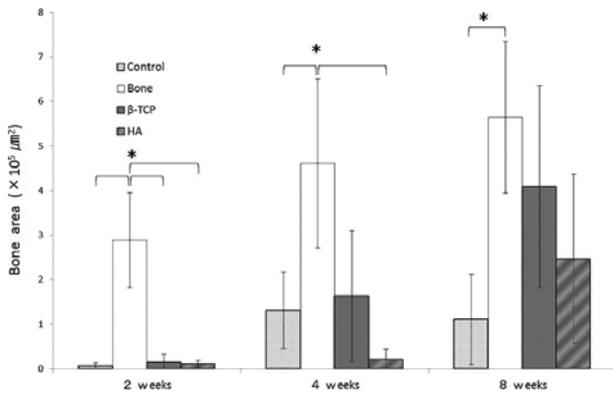
**Fig. 20** Histology of bone defects at 8 weeks in the HA group under natural light (A), polarized light (B), and fluorescent light (C).

creased in the controls.

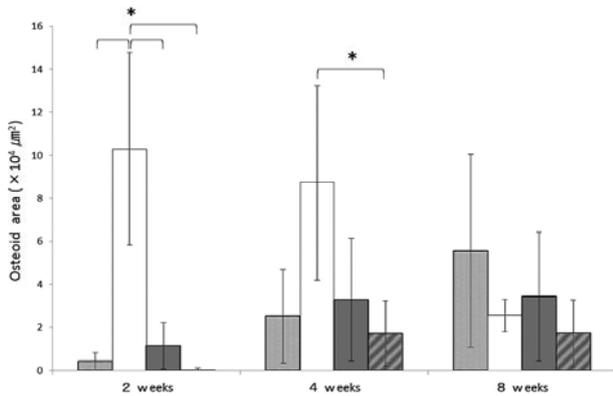
**Number of osteoclasts and osteoblasts (Fig. 24)**

The mean number of osteoclast, at 2 weeks was 28 and 30 in the autologous bone and  $\beta$ -TCP groups, respectively. These numbers were significantly greater in the control and HA groups. No significant difference was noted among the autologous bone,  $\beta$ -TCP and

HA groups at 4 or 8 weeks ; however, the mean number at 2 weeks in the controls was significantly lower than those in the other groups, and almost no osteoclasts were detected at 8 weeks. The number of cells peaked after 2 weeks in the autologous bone and  $\beta$ -TCP groups, and after 4 weeks in the control and HA groups. The mean number of osteoblasts was 88 and 172 at 2 and 4 weeks, respectively, in the autologous

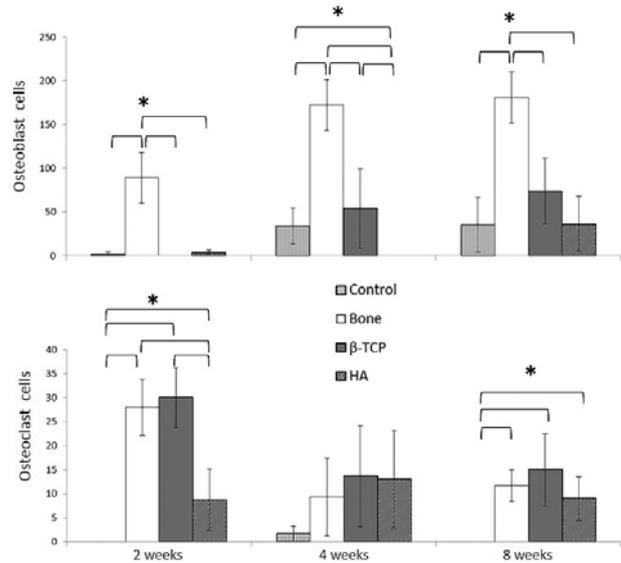


**Fig. 21** Changes in bone formation values (n = 3, \*p < 0.05)

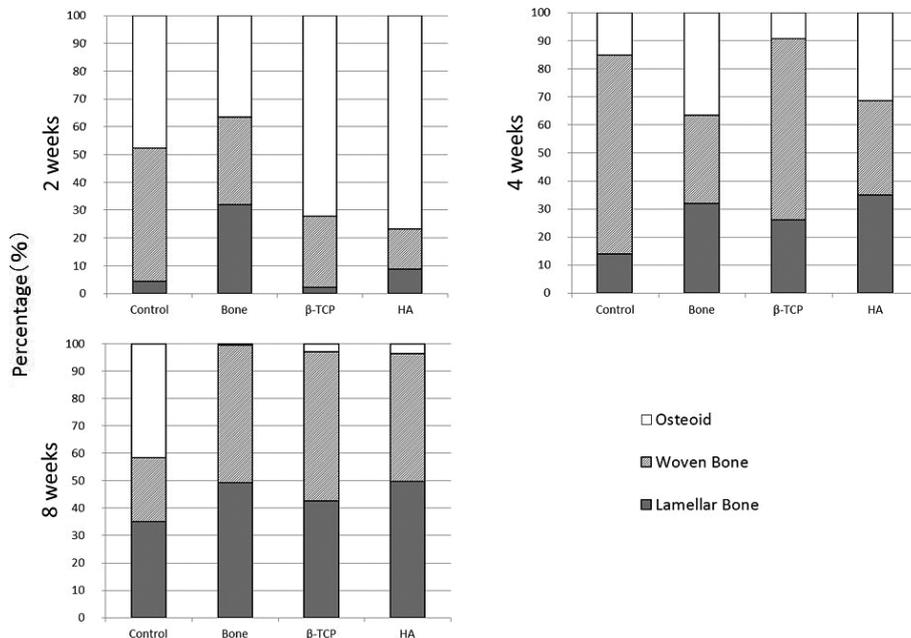


**Fig. 22** Changes in osteoid formation values (n = 3)

bone group. These were significantly higher than in the other groups. The mean number of osteoblasts was significantly lower in the HA group than in the other groups. At 8 weeks, this number was significantly greater in the autologous bone group than in the other groups.



**Fig. 24** Changes in the number of osteoblasts and osteoclasts (n = 3, \*p < 0.005)



**Fig. 23** Ratios of osteoid, fibrous bone, and lamellar bone

## DISCUSSION

### Bone substitutes

Bone defects produced by trauma and bone tumor resection are generally treated with autologous bone grafting, and various bone substitutes are used when the amount of collected autologous bone is insufficient. Calcium phosphate ceramics have an affinity for bone tissue and provide sites that are advantageous for bone formation. HA is commercially available and widely used as a bone substitute. HA has a high affinity for bone tissue and superior osteoconductivity in that it binds to bone.<sup>5</sup> However, it has the disadvantage of remaining in the body for a prolonged period. Previous studies performed in the field have of orthopedics have reported that reconstruction in response to the mechanical demands of bone may not progress in bone defects filled with HA, and the region may become vulnerable and fracture.<sup>6</sup>

In contrast, in the field of dentistry, HA remaining in bone defects, which is not absorbed for a prolonged time, provides sufficient osteoconduction and functions as a scaffold for new bone for a long time. Therefore, it is considered useful for creating space to prevent soft tissue invagination into defective regions during the new bone formation process.<sup>7</sup> Jarcho *et al.*<sup>8-9</sup> also demonstrated that HA particles adhered or chemically bound to new bone.  $\beta$ -TCP has been developed for applications in the dental field. Yokoyama *et al.*<sup>10</sup> performed various biological tests and confirmed its safety in the human body.  $\beta$ -TCP has recently been applied clinically, and the course of its absorption and substitution by new bone in the body has been reported.<sup>11</sup>  $\beta$ -TCP is expected to play the role of a scaffold for bone formation, and the cells involved in bone formation are thought to readily aggregate because new blood vessels extend through the micro-pore structure.

### Implant stability quotient

The ISQ of implants was measured in order to determine the state of healing. The change in ISQ, which is measured as a value between 1 and 100, depends on the height, quality, and force of binding to the implant of bone around the structure. The ISQ of successfully

placed implants was  $65 \pm 5$  in many cases. ISQ either does not change over time or may even decrease slightly when the value at the time of placement is 65 or greater, and increases over time when the value at placement is 50–60. Furthermore, treatment is more likely to fail when the value at the time of placement is 50 or lower, suggesting that loading in the early phase is possible when ISQ is 60 or greater, and immediate loading is possible when ISQ is 60–65 or greater. Based on the ISQ values, the healing process may have been normal in all groups.

### Bone histomorphometric observations

Villanueva bone staining enables microscopic observations of completely different bone properties in the same region of a preparation under natural, polarized and fluorescent lights. Polarized light microscopy is useful for investigating bone quality, such as that of lamellar and fibrous bone, and bone distribution.<sup>12, 13</sup> Under fluorescent light, calcified bone emits a deep green color, poorly calcified bone emits an orange color, and osteoid emits a red color. The bone-labeling materials TC and CL emit yellow and yellow-green colors, respectively. Since these antibiotics are incorporated through chelate bonding into regions in which osteoid is being calcified, these bone-labeling materials were administered before and after implant placement, respectively, to distinguish bone formed before and after placement.  $\beta$ -TCP and HA were absorbed and dispersed, respectively; however, both materials were surrounded by new bone. These bone-labeling materials were deposited along granules, suggesting that both  $\beta$ -TCP and HA offered superior osteoconduction.

### Bone histomorphometric findings

In the healing process of bone defects, such as extraction sockets, blood clots that fill the defects are generally covered by an epithelium of fibrous connective tissue within 1–2 weeks of treatment, thereby inducing the organization of blood clots. Callus formation from the floor and walls of the cavity are also observed from this time, and the cavity is saturated with callus within 2–4 weeks. After 4 weeks, new bone remodeling progresses to a situation close to normal

bone.<sup>14</sup> In our study, the osteoid mass increased with time and bone mass also increased after 4 weeks in the controls. However, the ratio of lamellar bone in new bone was low, and new bone was mostly comprised of osteoid and fibrous bone, suggesting that callus formation was still progressing at 8 weeks.

In the autologous bone group, the osteoid mass decreased with time from 2 weeks, and the ratio of osteoid in new bone was 1% or lower at 8 weeks, suggesting that the callus formation period had been completed and the process had transitioned to bone remodeling at 8 weeks. In contrast, the osteoid and new bone masses in the  $\beta$ -TCP and HA groups were not significantly different from those in the controls. However, while osteoid still accounted for approximately 30% of new bone at 8 weeks in the controls, it only accounted for 5% or less in the  $\beta$ -TCP and HA groups, while lamellar bone accounted for 40% or more. Narrow spaces between the bone substitutes that filled the bone defects may have readily been saturated with callus and rapidly transitioned to new bone remodeling.

While bone substitutes make spaces, residual bone substitutes may interfere with new bone formation.<sup>15</sup> In our study, new bone formation was slower in the  $\beta$ -TCP and HA groups than in the autologous bone group. Although the number of osteoclasts at 2 weeks was significantly greater in the autologous bone and  $\beta$ -TCP groups than in the other groups, no significant difference was noted among the autologous bone,  $\beta$ -TCP and HA groups at 4 or 8 weeks. This suggests that the absorption of grafted bone or bone substitutes by osteoclasts was activated at 2 weeks. In contrast, the number of osteoblasts was significantly greater only in the autologous bone group, and no significant difference was noted among the other groups, suggesting that the cell induction ability was low in the  $\beta$ -TCP and HA groups.

These results demonstrated that although both  $\beta$ -TCP and HA adequately acted as scaffolds for new bone formation, they may have delayed its formation because of inferior cell induction activity, such as osteoblast induction. Non-absorbable HA is thought to remain and persistently interfere with and delay new bone formation.  $\beta$ -TCP is considered superior to HA

for new bone formation because it is absorbable. New bone formation was observed earlier than in the  $\beta$ -TCP group. In addition, the number of osteoclasts was significantly greater after 2 weeks, whereas no significant difference was observed in the number of osteoblasts from the HA group.

These results suggest that the absorption of  $\beta$ -TCP granules reduced the inhibition of new bone formation, making them superior for new bone formation. Nakayama *et al.*<sup>7</sup> reported that  $\beta$ -TCP was superior for new bone formation because granules were absorbed earlier and scaffolds for bone formation and conduction disappeared; however, it was inferior to HA in maintaining residual ridge morphology. Morikawa *et al.*<sup>6</sup> demonstrated that the mechanical strength of new bone was reduced by residual granules, suggesting that absorbable bone substitutes, such as  $\beta$ -TCP, are superior for regions unrelated to residual ridge morphology, such as the area around implants. When HA is used for "space making", a longer time is needed for bone healing, which may increase the risk of infection and impair the clinical outcome.

Kubo *et al.*<sup>16</sup> showed that a mixture of a bone substitute with 75% or more autologous bone induced new bone formation equivalent to that induced by autologous bone grafting alone. Therefore, it is recommended to avoid the use of an artificial bone substitute alone, and use it in combination with autologous bone. Increasing the amount of autologous bone placed in regions around implants and on the mucosal surface far from the existing bone, where the mobilization of cells, such as osteoblasts, takes time, and decreasing the amount in regions near the existing bone, rather than mixing at an equal ratio for the entire bone defect, may reduce the required amount of autologous bone, while at the same time providing a result close to that of autologous bone alone. This may be useful in creating spaces.

## CONCLUSION

We filled experimental alveolar bone defects around implants with two types of artificial bone substitute, porous hydroxyapatite and  $\beta$ -tricalcium phosphate, and histologically investigated their influence on new bone formation. We found that  $\beta$ -TCP induced new

bone formation earlier than HA. These results suggest that a combination with autologous bone is desirable when HA is used as an artificial bone substitute.

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