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Measurement of interspaces after filling bone defects with different sizes of β-tricalcium phosphate granules

– Comparison between experimental animals and defect models –

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We investigated the granule interspaces created for vascular formation when a bone defect is filled with four sizes of β-TCP granules. Bone defects in experimental animals and models were filled with the granules. Images were obtained from the samples using micro-CT imaging, and the average volume of the granule interspaces was measured by 3D image analysis software. When a bone defect was filled with granules, the larger granules created lager interspaces. It is thought that maintenance of the granule interspaces when filling the defect may favor microvascular development and new bone formation. Therefore, we speculated that granule resorption might be faster with a smaller granule size. However, it is also thought that smaller interspaces might lead to less microvascular formation, further delaying new bone formation. (J Osaka Dent Univ 2015 ; 49 : 149–156)

Key words : Granule interspaces ; β-Tricalcium phosphate ; Bone defect

INTRODUCTION

Following tooth loss, alveolar bone is resorbed over time.[1–5] In cases with high levels of absorbed alveolar bone, bone augmentation by use of various artificial materials is often performed in combination with oral implant treatment.[6–9] Beta-tricalcium phosphate (β-TCP) is an artificial bone supply material used for bone augmentation. It has a high level of osteoconductivity, as it is absorbed relatively quickly and replaced with autologous bone,[10] and has recently been applied in the fields of orthopedic medicine[11–15] and dentistry.[16–21] When β-TCP granules are used for filling bone defects, microvascular formation advances in the granule interspaces, which is followed by bone formation to encircle the granules.[22, 23] Accordingly, the granule interspaces are considered important for microvascular formation and subsequent new bone formation.

We used granules of different sizes in animal experiments to measure granule interspaces and investigated whether adequate space required for microvascular formation could be obtained when the granules were added *in vivo.* In addition, we measured the interspaces in a bone defect model and compared the results with those obtained in the animal experiments. We also analyzed the usefulness of a bone defect model as a substitute for animal experimentation from the standpoint of animal welfare.

MATERIALS AND METHODS

We used four types of β-TCP granules with different diameters for measurements ; Type P (50–150 μm) (Kyocera Medical, Kyoto, Japan), Type S (200–500 μm), Type M (500–1000 μm) and Type L (1000–2000 μm) (BIORESORB® Macro Pore, ORALTRONICS, Bremen, Germany) (Fig. 1).

Experiment 1 : Granule interspace measurement in animals

Experimental animals

We used five adult male crab-eating monkeys (*Macaca fascicularis*) with body weights of approximately 5 kg, and healthy permanent dentition. The animals were given a standard primate diet (PS-A, Oriental Yeast, Tokyo, Japan) and water. Granule interspaces

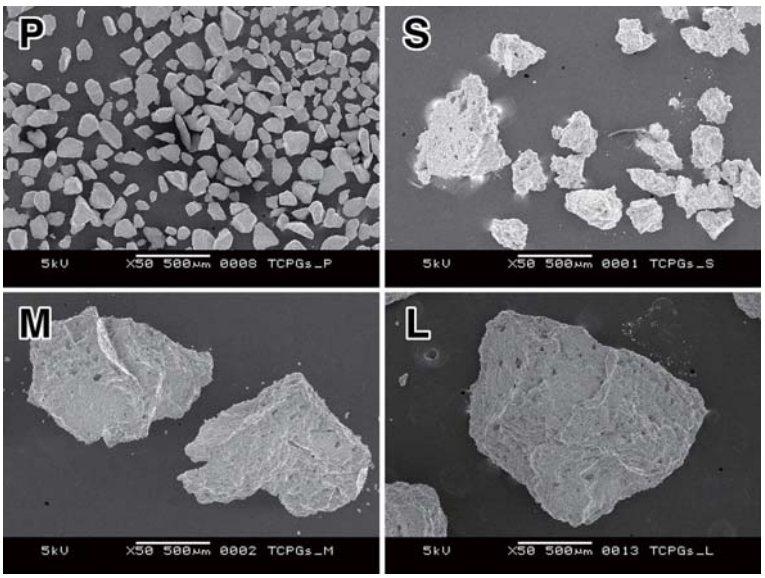

Fig. 1 SEM images of the four β-TCP granule types, P, S, M and L (Bar = 500 μm).

in the bilateral mandibular molar region were measured. The present experiment was approved by the Animal Research Committee of Osaka Dental University (Approval numbers 13-02001, 14-02001) and performed under the provisions of the Regulations for Animal Experiments of Osaka Dental University.

Surgical procedures

The five monkeys were given general anesthesia with 0.1 mL/kg xylazine hydrochloride (Selactal® 2% injection, Bayer Yakuhin, Tokyo, Japan) as a sedative and 0.2 mL/kg Ketamine hydrochloride (Ketalar®, Daiichi Sankyo Propharma, Tokyo, Japan) as an anesthetic. The bilateral mandibular molars (second premolar, first molar, and second molar) were extracted so as to minimize surgical invasion to the surrounding tissue. For one week after the procedure, the mouths were rinsed daily with a 0.01% benzethonium chloride solution (Neostelin Green Gargle 0.2%®, Nippon Shika Yakuhin, Shimonoseki, Japan) and the animals were given a soft diet. As an antimicrobial agent for preventing infection, 15 mg/kg lincomycin hydrochloride hydrate (Lincocin® injection, Pfizer Japan, Tokyo, Japan) was intramuscularly administered.

At 8 weeks after tooth extraction, the animals were again anesthetized using the same method as that for tooth extraction. Gingiva in the experimental region was incised and detached to expose the bone, and a total of four bone defects, two on each side, were formed by drilling 6 mm into the socket margin of the buccal side using a 3.5 mm implant drill (ITI implant system, STRAUMANN, Basel, Switzerland) under irrigation with physiological saline (Otsuka normal saline injection, Otsuka Pharmaceutical, Tokushima, Japan) (Fig. 2). Immediately after drilling, granules in physiological saline were placed in the defects, using Type L and S granules on one side and Type P and M granules on the opposite side. After filling the defects, a gingival flap was sutured over the wound. Similar to that after tooth extraction, intraoral rinsing was performed for 1 week, a soft diet was provided, and an antibacterial agent was administered intramuscularly. Sutures were removed one week after the operation and the soft diet was replaced with a primate diet.

Sample preparation

The monkeys were euthanized by an overdose of 50 mL/kg pentobarbital sodium salt (Somnopentyl® injection, Kyoritsu Seiyaku, Tokyo, Japan) 2 weeks after granule placement and acrylic resin was injected through the bilateral carotid artery.[24, 25] All of the experimental portions were soaked in 10% neutral buffer formalin solution and extracted using a large diamond

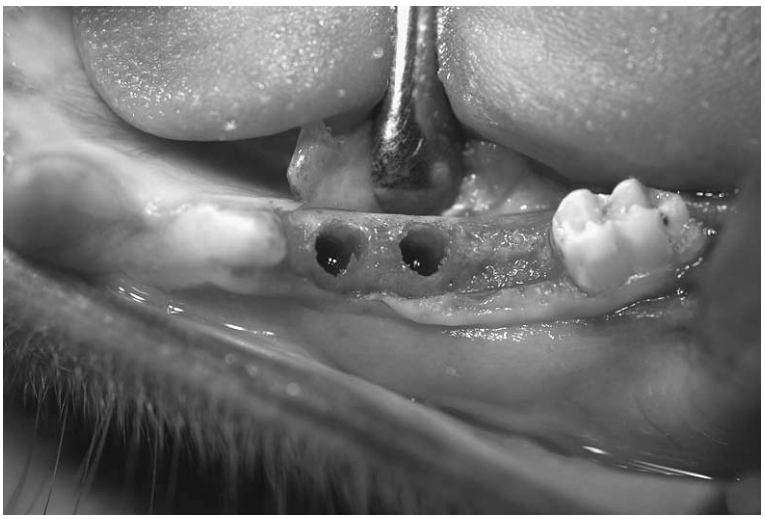

Fig. 2 Bone defects in an experimental animal (left side).

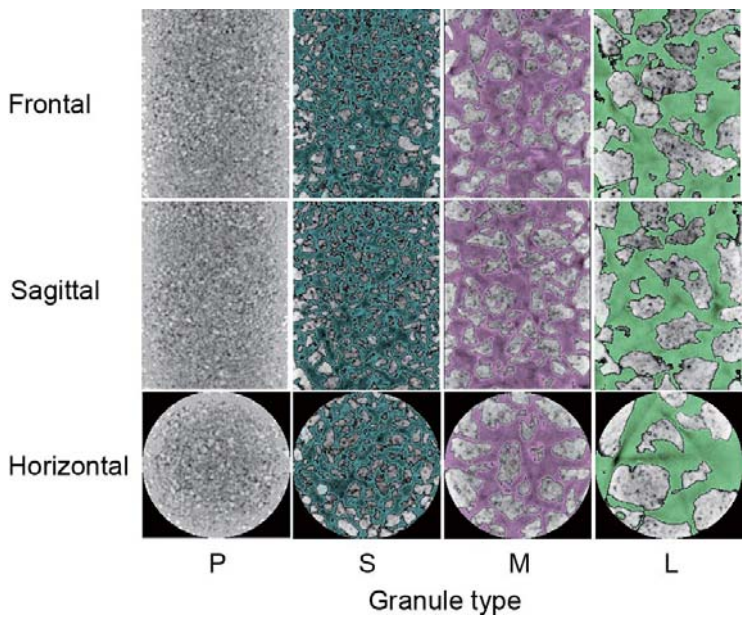

Fig. 3 Two-dimensional micro-CT images of specimens of the P, S, M and L granule types in frontal, sagittal, and horizontal sections. Interspaces in the granule are colored.

band saw (BS-310 CP, EXAKT, Norderstedt, Germany). Each bone defect was then cut into a block using a small diamond band saw (BS-3000, EXAKT).

Granule interspace measurement

The blocks were radiographed using a micro focus X-ray CT system (micro-CT) (SMX-130 CT ; SHIMADZU, Kyoto, Japan) with a tube voltage of 45 kV, a tube current of 120 μA, and a slice thickness of 27.5 μm. Slice images of only the bone defects were then extracted and processed using 3-D image analysis software (VGStudio MAX® Ver. 1.2.1, Volume Graphics, Heidelberg, Germany). For displaying only the granule interspaces in the images, a columnar portion 3 mm in diameter and 5 mm in height was extracted by removing the surrounding area contacting the socket wall with a margin of 0.5 mm. The extracted portion was divided into the granules and inter-

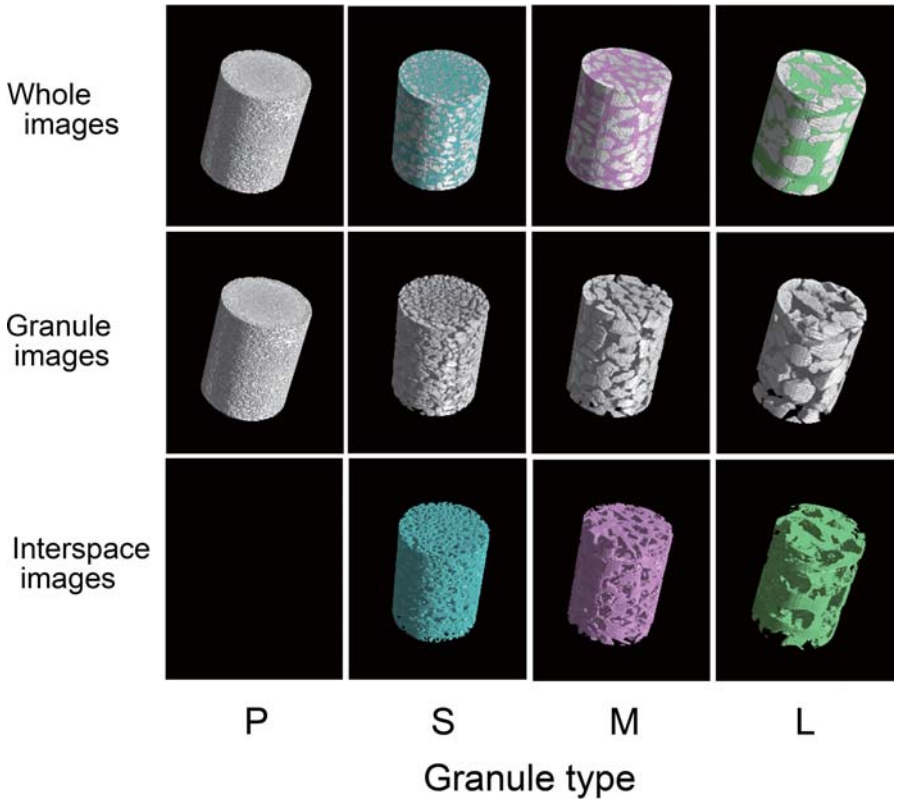

Fig. 4 Three-dimensional micro-CT whole images, granule images and interspace images (colored) of the P, S, M and L types.

spaces. To measure the interspaces, the mean for each of the three axes was calculated (Figs. 3 and 4).

Experiment 2 : Granule interspace measurement in bone defect model

Bone defect model

A polypropylene tube with a 4.5-mm inside diameter was cut into 8-mm lengths. The tubes were attached to an acrylic plate on a cut end to create a bone defect model (Fig. 5). Forty such models were prepared. Two methods of granule filling, wet and dry, were utilized. The same as in the animal experiments, granule (Types P, S, M and L) in physiological saline were used for wet filling, and those without saline were used for dry filling. The four different types of granules using the two filling methods were applied to five models each (Fig. 6).

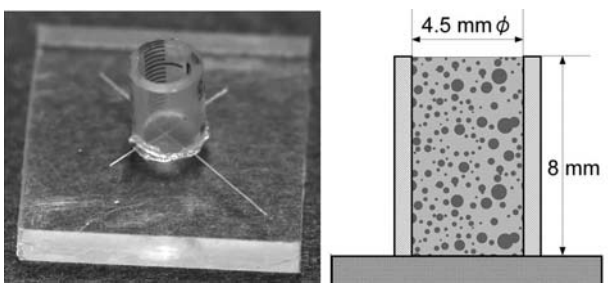

Fig. 5 Photograph and schematic illustration of the bone defect model.

Granule interspace measurements

Micro-CT images of the samples were exposed with a tube voltage of 35 kV, a tube current of 120 μA, and a slice thickness of 21.8 μm. Slice images of the granule filled areas were processed into 3-D images using 3-D image analysis software. For displaying the granule interspaces in the images, a columnar portion 4 mm in diameter and 5 mm in height was highlighted by removing 0.5 mm of the surrounding area contact-

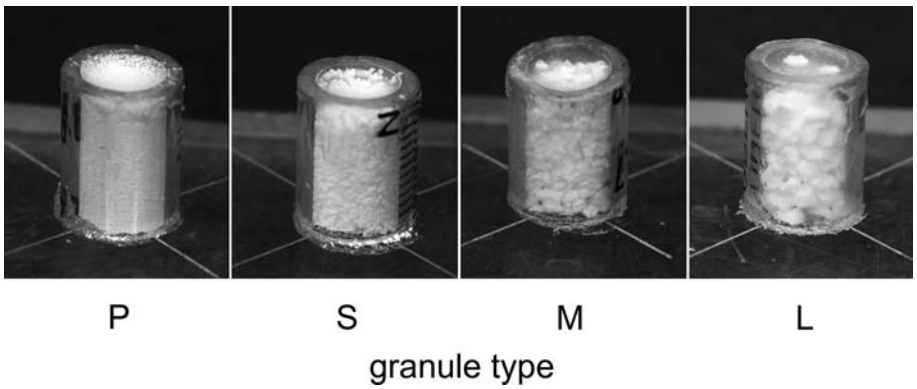

Fig. 6 Photographs of the bone defect model filled with dry granules.

ing the inner wall of the model. The extracted portion was then divided into granules and interspaces. The mean values of the interspaces were calculated for the X, Y and Z directions. Moreover, the mean of each granule interspace measurement was compared with those obtained in the animal experiments and tested by one-way ANOVA.

RESULTS

Experiment 1

The mean size of the interspaces with granules placed into the bone defects of the experimental animals was 110.8 ± 22.7, 173.2 ± 20.9, and 247.8 ± 20.8 μm for Types S, M and L, respectively. We were unable to measure the interspaces of defects filled with Type P granules with the image analysis software used in this study (Table 1, Fig. 7).

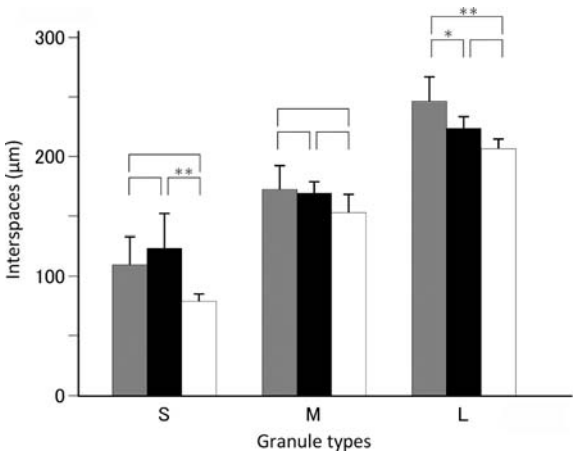

Fig. 7 Average interspace between granules for the animal experiment (■), wet filling model (■) and dry filling model (□) (*$p<0.05$, **$p<0.01$)

Table 1 Measurements of granule interspaces.

Experiment	Type	Average	X direction	Y direction	Z direction
Animal	S	110.8 ± 22.7	111.2 ± 22.3	114.0 ± 23.4	107.6 ± 23.5
	M	173.2 ± 20.9	174.2 ± 25.5	172.2 ± 16.1	174.0 ± 25.1
	L	247.8 ± 20.8	240.8 ± 19.2	242.4 ± 21.2	261.6 ± 26.6
Wet filling	S	125.0 ± 28.2	126.2 ± 28.1	125.8 ± 28.2	122.6 ± 27.8
	M	170.0 ± 9.5	172.6 ± 9.9	171.8 ± 9.4	165.2 ± 9.6
	L	225.0 ± 9.9	224.2 ± 12.5	227.2 ± 9.2	223.4 ± 9.6
Dry filling	S	80.0 ± 5.3	81.4 ± 6.1	81.2 ± 6.1	77.2 ± 4.3
	M	153.2 ± 15.2	155.6 ± 14.6	155.6 ± 16.8	149.4 ± 14.1
	L	208.4 ± 7.8	207.6 ± 8.5	208.4 ± 10.1	209.4 ± 5.9

(μm, Mean ± SD, n = 5)

Experiment 2

Wet filling

The mean granule interspace size with wet filling was 125.0 ± 28.2, 170.0 ± 9.5, and 225.0 ± 9.9 μm for Types S, M and L, respectively, while, Type P could not be measured (Table 1, Fig. 7).

Dry filling

The mean granule interspace size with dry filling was 80.0 ± 5.3, 153.2 ± 15.2, and 208.4 ± 7.8 μm for Types S, M and L, while that of Type P could not be measured (Table 1, Fig. 7).

DISCUSSION

Animal experiment

It is thought that the early healing stage for bone repair around an extraction socket and implant (postoperative weeks 1–2) is when microvascular formation occurs, followed by initiation of new bone formation.[26, 27] We measured the granule interspaces in the animal experiments at postoperative week 2 (the microvascular formation stage) using a micro-CT device.

Bone defect model

In order to model bone defects in humans, radiolucent samples are needed to obtain a CT radiograph image of only the granules. Furthermore, it is necessary to know the dimensions of the samples during formation in the bone defects. Therefore, in the present study, we used radiolucent polyethylene tubes cut to a fixed length and with a thickness size similar to the diameter of an implant drill. Because we assumed that the bone defects in humans would be filled with blood, we fashioned the bone defect model with one end sealed to avoid leakage of physiological saline.

Granule filling

When using granular artificial materials to fill bone defects in a clinical setting, the granules are frequently mixed with blood in the bone defect area. Moreover, as dry granules are difficult to use, physiological saline is routinely added. In order to standardize the method of filling for both the animal experiment and the defect model, we first filled the bone defect model with physiological saline and then added the granules.

Measurement of granule interspaces

In the bone defect model, the interspaces were smaller with dry filling than with wet filling for all granule sizes. We believe that the physiological saline, used in place of blood, might have better retained the interspaces in the wet filling method. On the other hand, with dry filling, granular compaction might have resulted from movement during transportation to the micro-CT device. Moreover, the granule interspaces in the defect model with wet filling were similar in size to the defects in the animal experiment at 2 weeks after filling. These findings indicate that the method of filling was adequate to simulate clinical conditions.

In both the animal experiments and the bone defect model, larger granules tended to result in larger interspaces. A previous study noted that interspaces 50 μm or larger were required for microvascular formation,[28] while others have reported that microvascular formation developed between the granules, resulting in new bone formation, when granular materials were used in clinical situations.[22, 23] Accordingly, it is thought that maintenance of the granule interspaces when filling the defect may favor microvascular development and new bone formation. In view of these findings, it is thought that new bone formation advances from microvascular formation, as the interspaces of the Type S, M and L granules used in the present study were of adequate size. Moreover, the granule interspaces could be secured reliably when the granules were wet with blood or physiological saline when filling.

On the other hand, we found it impossible to measure interspaces when using Type P, the smallest granules. Based on the micro-CT images, the granule interspaces appeared to be about 20 μm. It was previously reported that the surface area might be larger for smaller granules, even when the total volume of granules filled was the same regardless of granular size.[29] It was also noted that granule resorption was faster with a larger surface area of β-TCP.[30] Therefore, we speculated that granule resorption might be faster with a smaller granule size. However, it is also thought that smaller interspaces might lead to less microvascular formation, further delaying new bone for-

mation. Accordingly, when very small granules are used for filling in hope of early resorption, it is necessary to maintain the granule interspaces prior to filling to ensure adequate space for microvascular formation by promoting granule resorption. In addition, combining the granules with a scaffold such as collagen may be effective.

Alternative to animal experiments

In the present study, the sizes of interspaces created with the Type S, M and L granules in the bone defect model compacted by wet filling after 2 weeks were nearly the same as those in the animal experiment. Therefore, we think that wet granules used for filling in a bone defect model provide the same interspace environment as those applied to an experimental animal. Accordingly, we propose that a bone defect model is useful as a substitute for conventional animal experiments in regard to new bone formation in the granule interspaces. In a future study, we intend to further examine the usefulness of such a model for investigating microvascular formation and new bone formation in relation to granule interspaces.

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