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Three-dimensional evaluation for augmented bone using guided bone regeneration

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Objective: This study evaluated new bone regeneration beyond the skeletal envelope within an occlusive titanium cap on rabbit calvaria using microfocus computed tomography images.

Methods: In 10 rabbits, the calvaria was exposed and a circular groove was prepared. After penetrating the marrow, a standard hemispherical titanium cap was placed in the groove and covered with a cutaneous flap. After 1 or 3 months, the animals were killed and the calvariae and titanium caps were dissected. After taking microfocus computed tomography images of the specimens, histological sections were made. The specimens were observed using three-dimensional images constructed from the microfocus computed tomography images, and the histological sections were examined to compute bone parameters.

Results: The three-dimensional images and histological specimens showed that new bone formed in flat, cup-like, and dome shapes. The bone parameters trabecular thickness and the proportion of marrow space to the capacity of the titanium cap increased, whereas bone density decreased, and there were significant differences between the 1- and 3-month groups.

Discussion: First, a cylinder of new bone formed from the existing bone. Gradually, bone formed along the cap wall and the new tissue formed in a crater indented centrally. Finally, the new tissue formed in the shape of a dome.

Conclusion: Trabecular bone formed along the wall of the titanium cap, and bone filled the inside of the cap within 3 months.

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Guided bone regeneration has been proposed as an effective therapy to facilitate alveolar bone healing (1). Its usefulness has been proven experimentally (2–8) and clinically in dental implants (9–11). The possibility of placing dental implants is limited by the presence of an adequate bone volume for anchorage. A number of studies have examined the application of guided bone regeneration around an implant, and these studies have proved that it is a useful technique (12–17). Numerous articles have clearly demonstrated that the growth of bone above the external borders of the skeleton is possible using guided bone regeneration (18–22). In these articles, histological specimens evaluated guided bone regeneration in two dimensions. Previously, we evaluated the effects of an occlusive titanium cap on bone generation beyond the skeletal envelope (23, 24). However, two-dimensional histological specimens are unable to capture a precise representation of the three-dimensional structure of bone.

This study performed a three-dimensional examination of guided bone regeneration using three-dimensional microfocus computed tomography. Three-dimensional microfocus

computed tomography has two important features. First, the spatial resolution is 4 μm, allowing detailed structure evaluation. Second, non-destructive evaluation is possible (25–27). We used this process to examine newly formed immature bone and tissue beyond the skeletal envelope in rabbit calvaria in an occlusive titanium cap. We investigated the detailed structure of the new bone and analyzed three-dimensional construction images, without damaging the sample. Bone parameters were also analyzed.

Materials and methods

Animals

Ten adult male Japanese white rabbits weighing 2.5–2.8 kg were used. Before the experiment, the health of the rabbits was monitored for 2 weeks. The rabbits were kept in a standard cage in an experimental animal room (24°C, 55% humidity, 1 atmosphere, 12 h light/dark cycle.) and were fed a standard laboratory diet and water. This study was approved by the Animal Experimentation Committee of Nihon University School of Dentistry.

Titanium cap

The experimental device was a custom-made standardized stiff hemispherical titanium cap with a smooth surface (Ti > 99.5%, JIS H6400, Sankin, Tokyo, Japan). The cap was 4 mm high, 8 mm in diameter, and 0.2 mm thick. The cap was cleaned in 0.1 w/v% germitol water (Maruishi Pharmaceutical Co. Ltd, Osaka, Japan) in an ultrasonic bath to remove contaminants and was sterilized using ethylene oxide gas.

Anesthesia and surgery

All operations were conducted using sterile techniques. General anesthesia was induced by injecting pentobarbital sodium (Nembutal®, 0.4 ml/kg: Abbot Laboratories, North Chicago, IL, USA) via an ear vein and maintained with gas inhalation with halothane (Fluothane®, 1.5–2.0 % vol %: Takeda Chemical Industries Ltd, Osaka, Japan). The forehead of the rabbit was shaved. Approximately 1.8 ml of lidocaine HCl containing 1 : 80,000 epinephrine (2% Xylocaine®, Astra Japan Ltd, Fujisawa Pharmaceutical Co. Ltd, Osaka, Japan) was used as local anesthetic to reduce hemorrhaging under the skin over the calvaria.

A flap was cut using a midsagittal incision (#15 surgical blade, Feather, Osaka, Japan), and exfoliated from the forehead. The periosteum was incised and lifted to expose the calvaria on both sides of the midline. The skull was prepared on each side of the midline using a trephine drill (Bone trephine 131001, Technica, Tokyo, Japan) with an inner diameter of 8 mm under profuse irrigation with sterile saline. The circular groove did not penetrate the calvaria. This groove ensured a tight seal between the edge of the titanium cap and the surface of the skull. The depth of the groove was standardized by drilling into the bone until the cutting edge of the trephine was just below the bone surface to a depth not exceeding 0.5 mm. Nine small holes were drilled with a no. 2 round burr to induce bleeding from the marrow space within the circle. A standardized titanium cap was pressed into the circular grooves on each side of the midline. The periosteum was replaced, covering as much of the titanium cap as possible, and sutured with absorbent suture (5–0 Opepolix® II, Azwell, Osaka, Japan). Each reflected flap was repositioned to cover the titanium cap and sutured with interrupted sutures (4–0 silk Mani® suture, Mani Co. Ltd, Tochigi, Japan). As the periosteum is not elastic, it was impossible to cover the top of the cap (approximately 4 mm above the bone surface). Postoperatively, the rabbits received 25,000,000 U Penicillin G (Sigma-Aldrich, St. Louis, MO, USA), in a volume of 0.1 ml/kg, given as a single intramuscular injection.

The 10 rabbits were divided into two equal groups, and killed after 1 or 3 months. The rabbits were killed with an overdose of pentobarbital, and were fixed by perfusion fixation with 10% neutral buffered formalin. The skin, muscular layer, and periosteum were cut open and exfoliated, and the soft tissues removed. The calvarial bone with the titanium caps was dissected, and specimens were made. The specimens were fixed in 10% neutral buffered formalin for 1 month. The titanium caps were removed from the specimens from the left parietal bone. After three-dimensional microfocus computed tomography images had been taken, histological specimens were produced to identify new bone. Specimens from the right side were used for other experiments.

Three-dimensional microfocus computed tomography images

Three-dimensional microfocus computed tomography (micro-CT; SMX-130CT, Shimadzu, Japan) was used to observe the new bone in the titanium cap, at a voltage of 60 kV, an electric current of 55 μA, in 23.4-μm-thick layers, with a field of view (XY) of 11.9 mm. The resolution of one computed tomography tomogram slice was 512×512 pixels. Care was taken to keep the samples wet at the time of imaging.

After photography, histological specimens were prepared.

Producing histological specimens

After obtaining microfocus computed tomography images of the specimens, they were immersed in an ethanol series, and decalcified for 1 month before being harmonized in 5% aqueous sodium sulfate solution for 24 h, and embedded in paraffin. Then, they were cut carefully along the sagittal plane, which passed through the center of the original circle, using a trephine drill with a thickness of 6 μm. The sections were stained with hematoxylin and eosin. New bone was identified using optical microscopy (VANOX-S AHBS-514, Olympus, Tokyo, Japan).

Making and evaluating three-dimensional images

To obtain a three-dimensional image of the new bone (i.e. the calcified area of new bone is new bone in the narrow sense) and calvarial bone (existing bone) from the histological specimens, three-dimensional image construction

software (TRI/3D bon, Ratoc System Engineering, Tokyo, Japan) was used. While performing the image analysis, we calculated the trabecular thickness (TT), tissue volume (TV, volume of new bone and marrow bone cavity), bone volume (BV, volume of new bone), bone density (BV/TV), proportion of new bone to the capacity of the titanium cap (BV/CV), and the proportion of marrow space to the capacity of the titanium cap (TV/CV).

For these parameters, the average and standard deviation were computed at 1 and 3 months. The Mann–Whitney *U*-test was used for comparisons, and a probability of less than 5% was defined as significant.

Results

The three-dimensional construction images

Three basic shapes were defined as in the histological specimens: flat, cup-like, and domed (Figs 1–3).

One-month group

In the histological specimen of the 1-month group, the blood clots were considered to be bleeding that originated from the perforated marrow in the titanium cap. The ostoblasts were found around the trabecular bone (Fig. 4).

The three-dimensional images were constructed based on the histological specimens. In the 1-month group, there were marked differences in new bone formation between individuals. In three images, new bone was seen along the inside of the titanium cap to about half of the cap height. The new bone was cup-like shaped, with an indentation at the apex. In the other two images, flat new bone formed from the existing bone. Trabecular bone formed in the space surrounding the new and existing bone. The amount of trabecular bone decreased gradually in the upper part of the titanium cap. Continuity was observed between the wall-like new bone formed along the wall of the titanium cap and the trabecular bone formed in the central part of the cap. The trabecular bone was thick on the side with existing bone, and became thinner near the apex of the cap. In the upper part of the titanium cap, the new bone formed a network of crossing beam-like structures.

Three-month group

In the histological specimens of the 3-month group, the new bone in the titanium cap consisted of wall-like new bone, trabecular bone, and

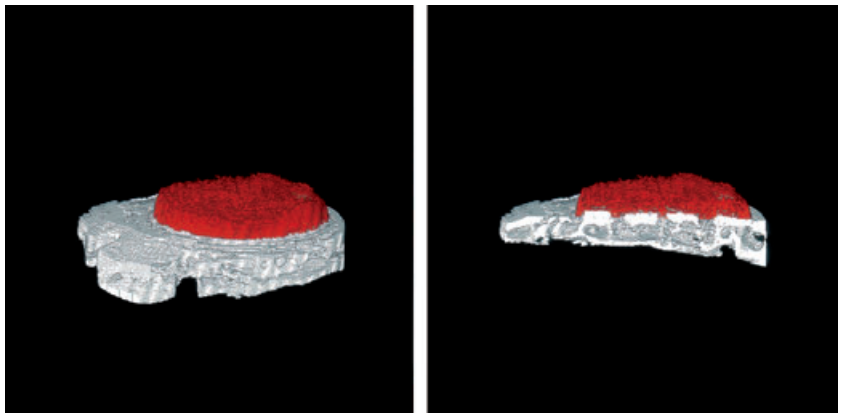

Fig. 1. Micro-computed tomography image showing a small flat area of new bone formed from existing bone (× 2.5) (red, new bone; white, existing bone).

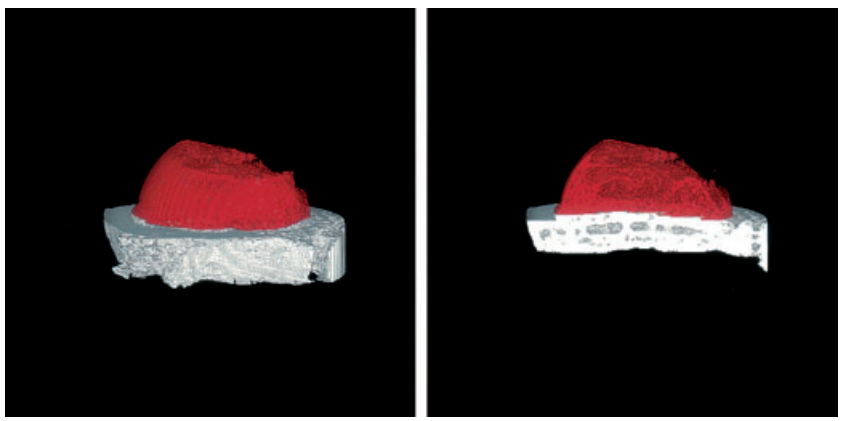

Fig. 2. Micro-computed tomography image showing newly formed bone with a cup-like shape (red, new bone; white, existing bone).

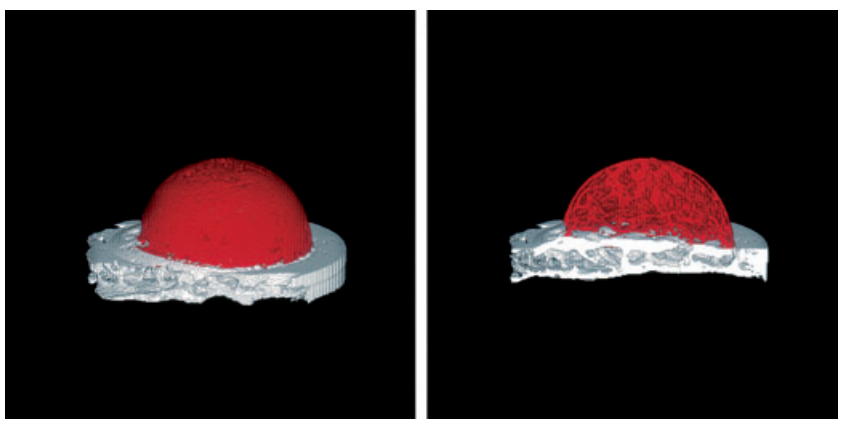

Fig. 3. Micro-computed tomography image of newly formed bone with a dome-like shape (red, new bone; white, existing bone).

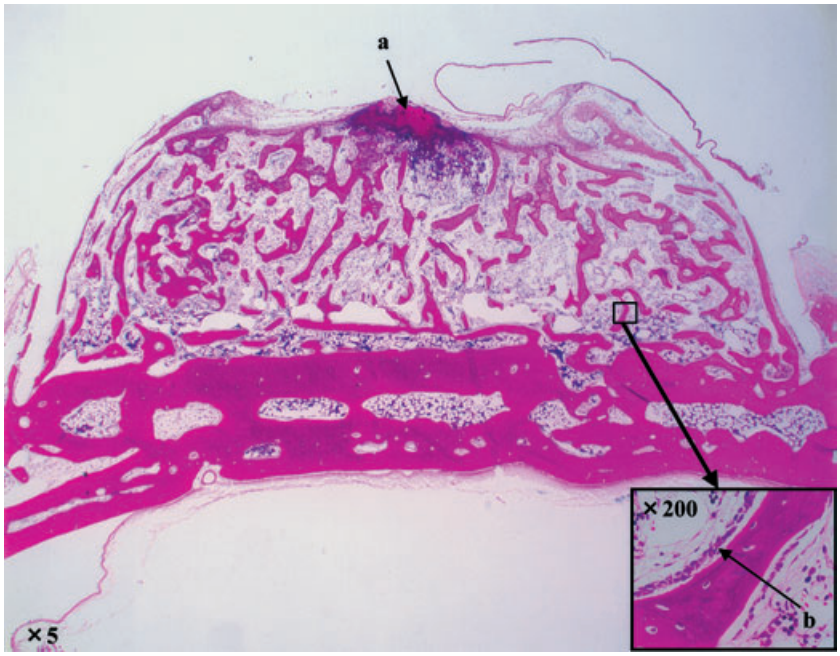

Fig. 4. Histological image of the 1-month group. a, blood clot; b, many osteoblasts around the trabecular bone.

lamellar-like new bone. The trabecular bone was thicker in the 3-month group than in the 1-month group. The amount of the trabecular bone in the upper part of a cap increases as compared with the side of the existing bone. Around the trabecular bone, a small number of osteoclasts was scattered (Fig. 5).

The three-dimensional images were constructed based on the histological specimens. In the 3-month group, the new bone formed a dome along the wall of the titanium cap to near the summit of the cap in four of five images. In the remaining image, new bone had formed to about two-thirds of the cap height. Although continuity between the wall-like new bone and trabecular bone was not apparent in one specimen from the 3-month group, continuity was confirmed in all of the specimens from the three-dimensional reconstructions. The trabecular bone was thick, forming beam-like branches. The bone marrow cavity was larger, and the ratio of bone marrow to newly formed tissue was higher in the 3-month group.

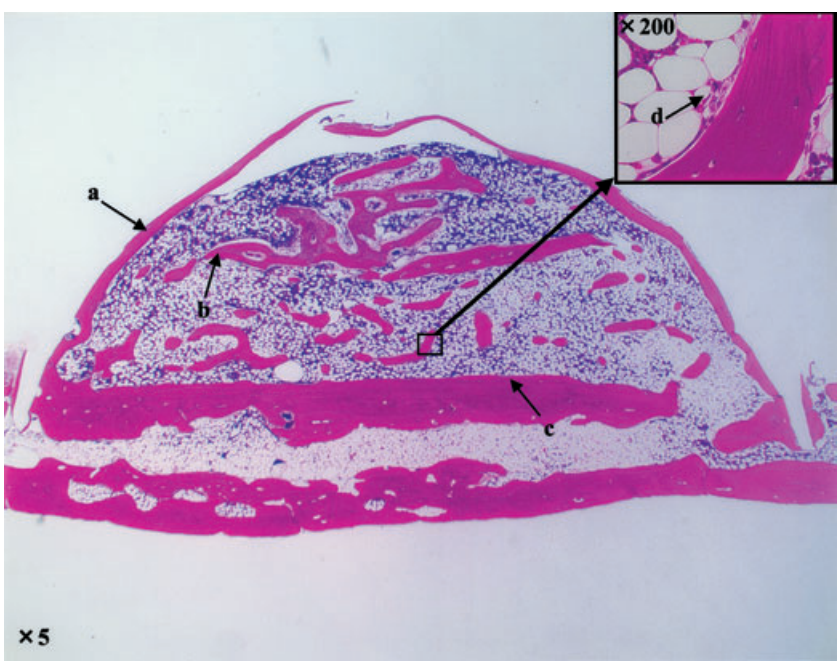

Fig. 5. Histological image of the 3-month group. a, wall-like new bone; b, trabecular bone; c, lamellar-like new bone; d, small number of osteoblasts around the trabecular bone.

Bone parameters

The Mann–Whitney *U*-test showed no significant difference ($p > 0.05$) in bone volume and BV/CV between the 1- and 3-month groups. By contrast, TT, TV and TV/CV were significantly higher at 3 months (TT: 77.0 ± 4.1% vs. 87.9 ± 6.5%; TV: 40.6 ± 19.8% vs. 75.5 ± 17.4%; TV/CV: 38.0 ± 19.3% vs. 65.5 ± 13.3%; all $p < 0.05$), whereas BV/TV decreased significantly ($p < 0.05$) at 3 months (29.8 ± 6.8% vs. 43.1 ± 7.3%) (Figs 6–8, Table 1).

Discussion

We used microfocus computed tomography to examine new bone that formed beyond the skeletal envelope in rabbit calvaria, and evaluated the bone using a bone parameter analysis. The results showed that the amount of marrow was greater in the 3-month group than in the 1-month group.

Three-dimensional construction images

Numerous studies have documented new bone formation beyond the skeletal envelope (18–24, 28–34). The following four characteristics of guided bone regeneration seem to influence the predictability of the results: barrier material of sufficient stiffness, healthy vascularized bone bed, immobilization of the membrane in a submerged position, and an appropriate healing time (29). Various materials have been used to form the enclosed space,

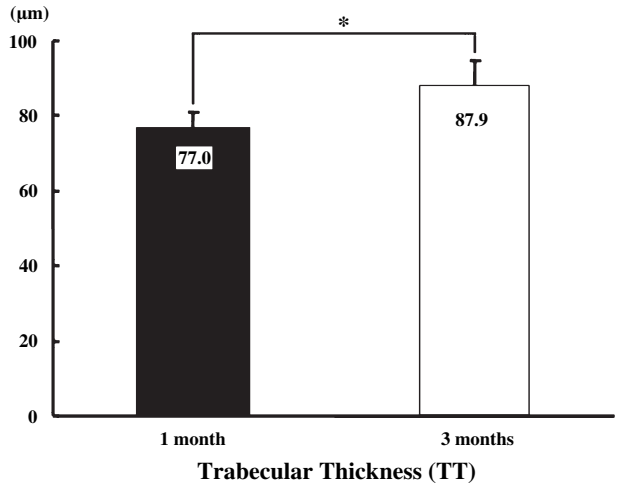

Fig. 6. Trabecular thickness (TT). *Mann–Whitney *U*-test, $p < 0.05$.

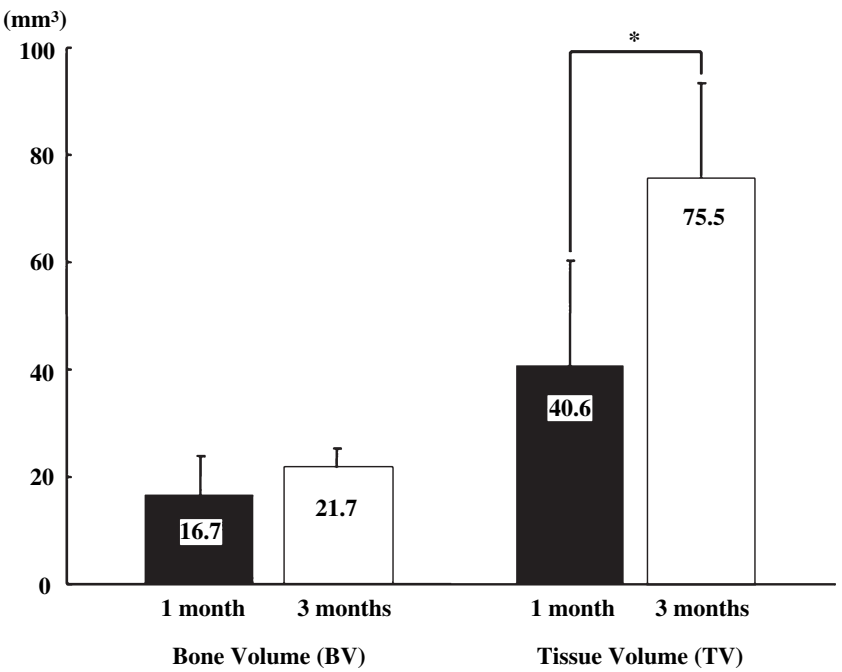

Fig. 7. Bone volume (volume of new bone) (BV) and tissue volume (volume of new bone and bone marrow space) (TV). *Mann–Whitney *U*-test, $p < 0.05$.

including hemispherical domes made of polyactic acid (18, 22, 33), expanded polytetrafluoroethylene membrane (29–31), and hemispherical titanium caps (20, 23, 24, 28, 32, 34). It is essential that the membranes have biocompatibility, sufficient stiffness and stability, space, an adequate blood supply, and total occlusion (20, 35). In this study, the skull bone was prepared using a trephine drill and nine small holes were drilled within this circle. A standardized titanium cap was placed snugly in circular grooves on each side of the midline. The titanium cap was biocompatible, and had sufficient stiffness and stability. The occlusiveness was considered equal to that reported by Minegishi *et al.* (23) and Yamada *et al.* (24). Furthermore, no connective tissue derived from epithelium formed in the titanium cap, and blood was supplied via the nine holes drilled into the marrow space. Schmid *et al.* (31) investigated the relationship between bone formation and membrane permeability and found that membrane permeability was not necessary in the guided generation of new bone. Therefore, the new bone in our experiment was not of epithelial origin, but originated from the perforate marrow. Schmid *et al.* (22) also reported that an environment suitable for bone formation is very important for rebirth in the initial stage, and if such an environment is not established by the time of early healing, little regeneration will occur, even after a prolonged period. Majzoub *et al.* (32) placed a titanium dome in rabbit calvaria and perforated the external cortical surface of the skull mechanically. They noticed that perforating the bone appeared to accelerate bone regeneration initially. Rompen *et al.* (34) placed a titanium dome in rabbit parietal bone after drilling nine 0.8-mm-diameter holes and reported that stimulation of the blood supply and providing access for bone-forming cells via the cortical perforations or blood clots enhances *de novo* bone formation in this experimental model. Furthermore, in histological studies, van Steenberghe *et al.* (28) and Schmid *et al.* (31) reported marked individual differences in bony growth.

For these reasons, it is thought that an environment suitable for bone formation in the initial recovery period is important for the growth of new bone, and that a blood supply (Fig. 4) to the occluded space from the marrow is also important. Moreover, environmental differences were thought to cause the individual differences in new bone formation.

The new bone that formed within the titanium cap was distinct from the wall-like new bone that formed along the titanium cap: trabecular bone formed in the central part of the titanium cap, and new lamellar-like bone followed the existing bone in the 1-month group, but seldom did by 3 months. In this study, the wall-like new bone that formed along the titanium cap was thin. As it is difficult for blood vessels to penetrate this wall-like new bone (33), this is thought to explain why this wall-like new bone

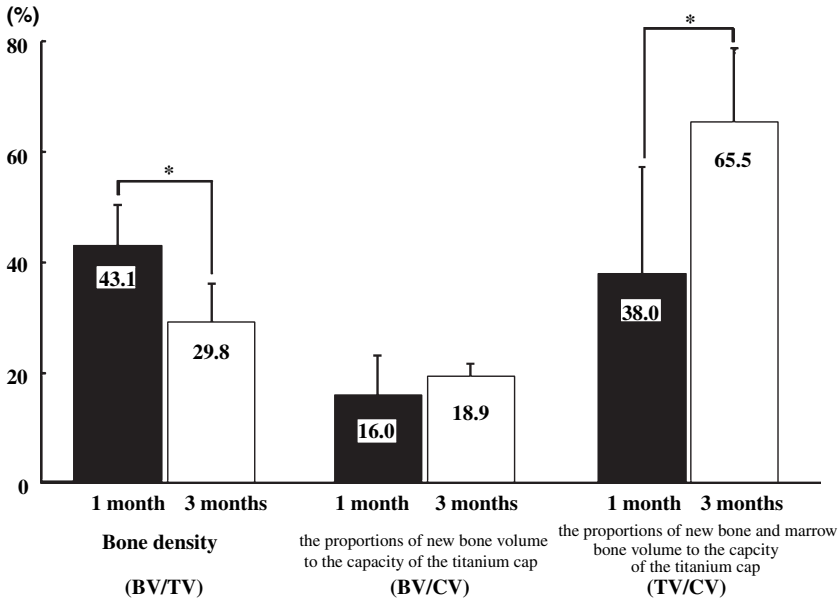

Fig. 8. Bone density (BV/TV), the proportions of new bone volume to the capacity of the titanium cap (BV/CV), and the proportions of new bone and marrow bone volume to the capacity of the titanium cap (TV/CV). *Mann–Whitney *U*-test, $p < 0.05$.

Table 1. The results of bone parameters

	1 month	3 months	Mann–Whitney *U*-test
TT (μm)	77.0 ± 4.1	87.9 ± 6.5	*
BV (mm^3)	16.7 ± 7.2	21.7 ± 3.4	
TV (mm^3)	40.6 ± 19.8	75.5 ± 17.4	*
BV/TV (%)	43.1 ± 7.3	29.8 ± 6.8	*
BV/CV (%)	16.0 ± 7.2	18.9 ± 2.4	
TV/CV (%)	38.0 ± 19.3	65.5 ± 13.3	*

TT, trabecular thickness; BV, bone volume; TV, tissue volume; BV/TV, bone density; BV/CV, proportion of new bone to the capacity of the titanium cap; TV/CV, proportion of marrow space to the capacity of the titanium cap.
There were significant differences between 1- and 3-month group in TT, TV, BV/TV and TV/CV.
*Mann–Whitney *U*-test, $p < 0.05$.

did not become much thicker in the 3-month group. Moreover, only small amounts of trabecular bone formed in the central part of the titanium cap, although this area had a rich blood supply, originating from the marrow, which permitted free traffic of blood cells and osteoblasts. The trabecular bone became thicker by 3 months, although the volume of the marrow cavity also increased in the newly formed tissue. Lundgren *et al.* (20) reported that for new marrow, about twice as much trabecular bone was formed. In this study, it is thought that since the wall-like new bone was connected with the trabecular bone, which formed the central part of the titanium cap, the latter became thicker, and its connection was lost with growth of the marrow bone, and the number of connections decreased. In the specimen in the 3-month group in which a connection formed between the wall-like new bone and the trabecular bone, no bone was found in the central part of the titanium cap (Fig. 5). The three-dimensional image based on microfocus computed tomography demonstrated connections between the trabecular bone within the titanium cap, the existing bone, and the new wall-like bone that formed along the wall of the titanium cap. It is thought that the trabecular bone resulted from perforating the existing bone and the ensuing bleeding into the cap (Fig. 4). Furthermore, the formation of lamellar-like new bone, which was observed at 1 month, was decreased at 3 months. This occurred because osteoblasts (Fig. 4) were stimulated by the supply of blood, and bone formation from cortical bone was promoted by the existence of a blood clot (Fig. 4) (34). Consequently, the lamellar-like new bone that followed the existing bone was superfluous in the 1-month group. We postulated that since new bone was converted into general bone structures in the 3-month group, the quantity of stratified new bone decreased. A bone structure rich in marrow bone containing trabecular bone developed between two sheets of cortical bone. As a result, although growth of each of the three types of new bone was promoted, overall marrow bone predominated in the titanium cap.

Bone parameters

For an objective comparison of the new bone, morphometric bone parameters were calculated, including trabecular thickness (TT), bone volume (i.e. new bone) (BV), tissue volume (i.e. new bone plus the marrow cavity) (TV), bone density (BV/TV), the ratio of new bone to the capacity of the titanium cap (BV/CV), and the ratio of new tissue volume to the capacity of the titanium cap (TV/CV). Overall, the trabecular bone was thicker in the 3-month group than in the 1-month group, both histologically and in the three-dimensional images. TT increased significantly at 3 months. Although osteoblasts were seen on the trabecular bone at 1 month (Fig. 4), they were not seen at 3 months. Therefore, it is thought that osteoblasts contributed to the thickness of the trabecular bone at 1 month, but the number of osteoblasts decreased (Fig. 5), except near the apex of the titanium cap, so the trabecular bone adjacent to the existing bone did not increase in thickness.

Between 1 and 3 months, BV and BV/CV were similar, whereas TV significantly increased. As TV significantly increased, while BV remained

relatively constant, BV/TV decreased significantly in the 3-month group, indicating that there was more low-density bone at 3 months. Moreover, as the marrow cavity became wider as TV increased significantly, while BV remained constant, TT increased significantly at 3 months. Therefore, there was a large amount of thin trabecular bone in the 1-month group that united to form thick trabecular bone by 3 months. Therefore, the cavity of the marrow bone, which became larger, occupied the area around the wall-like new bone, and the number of connections between the wall-like new bone and the trabecular bone decreased. As a result, connections between the wall-like new bone and trabecular bone were difficult to detect at 3 months.

BV/CV differed little between 1 (16.0 ± 7.2%) and 3 (18.9 ± 2.4%) months. In addition, our unpublished data indicate that the ratio of trabecular bone in the titanium cap remained similar at 1, 3, and 6 months. Therefore, the proportion of trabecular bone within the titanium cap does not depend on the length of the postoperative period. Three-dimensional images constructed from microfocus computed tomography images are excellent in that the evaluation is non-destructive, and the form of a sample can be comprehended in three dimensions.

Conclusion

Using microfocus computed tomography to examine bone regeneration beyond the skeletal envelope in rabbit calvaria, at least within the limitations of this 10 rabbit experimental study, we made the following conclusions.

1 The specimens could be examined in three dimensions using three-dimensional images constructed from microfocus computed tomography images.

2 Within the titanium cap, lamellar-like new bone and new wall-like bone formed, followed by trabecular bone. This bone was rich in marrow bone.

3 With time, the new bone that formed along the wall of the titanium cap changed shape from flat, to cup-like, and to dome shaped.

4 A quantitative comparison of the groups at 1 and 3 months was possible by calculating bone parameters such as trabecular thickness, new bone volume, the proportions of new bone and marrow cavity, and the bone pattern.

We hope to achieve precise bone formation using graft materials. Therefore, microfocus computed tomography is useful for evaluating bone formation and bony tissue in three dimensions.

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