Coating Dental Implants with Synthetic Bone Mineral for Early New Bone Formation \emph{in Vivo}

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Abstract: This study focused on the post-implantation formation of new bone in the peri-implant region of synthetic bone mineral (SBM)-coated implants in rat femur models and aimed to elucidate the effects of SBM surface treatment on early bone formation and bone tissue quality. Twenty-four 8-week-old Wistar rats were randomly assigned to four experimental groups: two-week implantation with blasting-treated implant (6 rats; control group) or SBM-treated implant (6 rats; experimental group); and four-week implantation with blasting-treated implant (6 rats; control group) or SBM-treated implant (6 rats; experimental group). After implantation, the following data were collected and compared: pull-out strength, bone mineral density (BMD), BMD color images, and histological characteristics. Comparisons of the two- and four-week data indicated that the pull-out strengths and BMD of the SBM group were significantly higher than those of the control group. BMD color imaging and histological observations indicated that, in comparison with the control group, the new bone formed in the peri-implant region of the SBM group had greater width and higher BMD. Our results will aid the development of implant surface treatments that facilitate early bone formation. Further, the investigated SBM could be applied as a bone prosthetic filling material in regions with bone defects.

Key words: Dental implants, Surface-coated materials, Apatites, Bone density, Rats

Introduction

When performing implant surgery on elderly or osteoporosis patients, it is impossible to ensure sufficient cancellous bone support because the bone mineral density (BMD) and bone tissue may have deteriorated significantly. Thus, it is necessary to carefully examine whether dental implant surgery is indicated in such cases. Autologous bone transplantation is clinically effective in cases in which the width, height, or amount of maxillary and mandibular bone at the site of implantation is insufficient due to bone resorption. In fact, it is considered the gold standard for bone augmentation\textsuperscript{1,2}. However, there are several problems with autologous bone transplantation, such as the high degree of invasiveness at the harvest site and limitations related to the amount of bone that can be obtained. Alternatively, demineralized and non-demineralized freeze-dried bone allografts (DFDBA and FDBA, respectively) are commercially available for allogenic bone transplantation, which is thought to have an extremely low risk of infection. Meanwhile, hydroxyapatite and β-tricalcium phosphate (β-TCP) of various morphologies are used as bone substitutes. However, despite their superior bone conductivity, these calcium phosphate-based materials alone cannot be easily used as bone filler because they lack bone-inducing capacity. Therefore, they are often used in combination with autologous bone transplantation or other bone filling methods (such as guided bone regeneration and platelet-rich plasma therapy). As a result, significant efforts are being devoted to the development of improved methods and materials, including new biomaterials as well as regenerative methods that use biomaterials as a scaffold.

The synthetic biomaterials used for bone reconstruction include tricalcium phosphate and hydroxyapatite. These materials are already in clinical use\textsuperscript{3,4}. Tricalcium phosphate is a widely used biocompatible ceramic in orthopedic and dental applications. On the other hand, hydroxyapatite has been shown to exhibit excellent osteoconductivity. Given these factors, LeGeros developed a synthetic bone mineral (SBM) that is identical to actual bone by combining the biological trace metal elements Mg\textsuperscript{2+}, Zn\textsuperscript{2+}, Mn\textsuperscript{2+}, and F\textsuperscript{−} with a bioresorbable carbonate apatite\textsuperscript{5-9}. SBM, which contains various minerals necessary for organisms, has the advantage of containing useful elements for bone remodeling. Before determining the effects of preimplant surgical administration of this SBM, it was first experimentally tested as an oral supplement in patients with osteopenia/osteoporosis\textsuperscript{5,9}. The SBM supplement promoted bone formation and metabolism and improved bone tissue quality throughout the body. This suggests that ingesting SBM may help to shorten the healing period for implant surgery. However, while SBM has been studied as a supplement, no one has investigated the response when SBM is implanted during peri-implantation. Thus, in the present study, we evaluated the usefulness of surface treating an implant with SBM with the aim of accelerating bone formation and improving bone tissue quality by focusing on the newly generated bone in the peri-im-
implants. A rat femur model was used for this purpose.

This study was conducted with the aim of improving the healing of dental implants, but the place of implantation is the femur. Since there is no cancellous bone in the diaphysis of the femur, the femur was selected to observe neoplastic bone formation.

Materials and Methods

Animal specimens

The study protocol was approved by the Ethics Committee of the Nihon University School of Dentistry at Matsudo Animal Care and Use Committee (AP19MAS002-2). The care and use of rats were in compliance with the guidelines of the National Institutes of Health regarding the care and use of animals for experimental procedures (NIH Publications, 2011 revision) and the investigator’s Institutional Animal Care and Use Committee. Twenty-four 8-week-old Wistar rats (Sankyo Labo Service Corp., Tokyo, Japan) were used in the study. The relevant institutional and national guidelines were followed for the care and use of the animals. The twenty-four rats were equally divided into two-week and four-week implantation groups, each of which was randomly divided into blasting-treatment (control) and SBM-treatment (experimental) groups. Each group comprised six rats.

Body weight of rat specimens

The body weights of all the rats were measured at the following times: one week before surgery (age: 8 weeks), at the time of surgery (9 weeks), one week after implantation (10 weeks), two weeks after implantation (11 weeks), three weeks after implantation (12 weeks), and four weeks after implantation (13 weeks). Rats in each group (n=6) were weighed during each period.

SBM preparation and characterization

The SBM (SBM #126) used for the implant coatings was prepared using a modified hydrolysis method reported by LeGeros et al. Briefly, the method consisted of hydrolyzing a mixture of dicalcium phosphate dihydrate (CaHPO4·2H2O), MgCl2, and ZnCl2 in a solution containing NaHCO3 and NaF. The reaction temperature was 95°C. The pH was not adjusted, and the reaction time was 16 h. The obtained product was rinsed with double-deionized water and dried at 100°C in a low-temperature oven. Mg, Zn, and F were added in higher concentrations to the SBM than those corresponding to normal rat bone to take advantage of their positive effects on bone remodeling. The SBM was characterized using the following techniques: X-ray diffraction to determine the phase composition and crystal lattice size; Fourier-transform infrared spectroscopy to determine the presence of functional groups; thermogravimetry and differential scanning calorimetry to determine the carbonate content; and inductively coupled plasma atomic emission spectroscopy to determine the Ca, P, Mg, Zn, Na, and K concentrations. Srinivasan et al. reported that normal rat bone mineral and SBM have similar apatite structures in the above analysis methods.

Surface treatment of implants

A total of twenty-four Ti-6Al-4V (wt%) cylindrical implants measuring 1.2 mm in diameter and 4.0 mm in height, with a hole located 0.5 mm from the upper end for pull-out strength tests, were used. All twenty-four implants were subjected to blasting with 110-μm abrasive particles, cleaned with an ultrasonic device, and autoclaved, as per the method described by Sato et al. and Watanabe et al. They were then subjected to ultrasonic cleaning in double-distilled water. Twelve of these implants were assigned to the control group. The remaining twelve implants were coated with the SBM after the blasting treatment and assigned to the experimental group. The method used to form the SBM coating on the implants was that described by Rohanizadeh et al. In brief, 0.4 g of the SBM powder was mixed in 100 ml of 0.3% H3PO4, after which the deposited supernatant was collected. The implants were made to stand vertically in a petri dish containing the supernatant and left in that position for 24 h in a thermostatic chamber heated to 75°C.

Implant placement protocols

All rats underwent implant surgery on their femurs at 9 weeks under general anesthesia, which was administered via an intraperitoneal injection of metedomidine, midazolam, and butorphanol. Implantation was performed at a position of 15 mm from the epiphysyal on the mesial side of the femur. One operator prepared a 2.5-mm deep hole in the femur using a 1.2-mm diameter drill. The implant was then inserted into the hole to a depth of 2.5 mm using a drill at a speed of 500 rpm under saline irrigation to avoid heating the bone. The remaining 1.5 mm of the implant was covered by muscle rather than bone and used for connecting the load cell to measure the pull-out strength. The incision wound was sutured after the surgery. Disinfection was performed after the surgery, and 4.4 mg/kg of carprofen was subcutaneously administered for three days as postoperative management. Two and four weeks after implantation, the animals from the respective groups were sacrificed using carbon dioxide gas. One animal from each group was randomly selected for histological observation with Villanueva–Goldner stain. The pull-out strength and BMD were analyzed for the remaining five animals in each group. In consideration of the 3R principle (Replacement, Reduction, and Refinement) of animal experiments under the Act on Welfare and Management of Animals, we used a single rat from each group as a representative for that group for the histological observations.

Pull-out strength test

Pull-out strength tests were performed to evaluate the adhesive strength between the implant body and the surrounding bone. The extracted samples were mechanically anchored to a baseplate using a self-curing resin. The setup was adjusted using a level to align the test area with the load cell such that the exerted force was perpendicular to the test area as possible, thereby minimizing shear forces. A 110-mm-long stainless-steel wire was threaded through the hole at the top of the implant and connected to the load cell, with the distance between the top of the implant and the load cell being 50 mm. A universal testing machine (TG-5k; Minebea Mitsumi Inc., Kanagawa, Japan) was used for the pull-out tests, which were performed at a cross-head speed of 1.0 mm/min. The pull-out strength was determined as the peak force required to detach the implant from the bone.

BMD measurements and BMD color imaging

BMD measurements and BMD color imaging were conducted to quantify and visualize the bone density around the implant body. After the pull-out test, the femur was subjected to microcomputed tomography (μCT) scanning using an R-mCT2 system (Rigaku Corp., Tokyo, Japan) with a 90-kV anode electrical current and 30-μm resolution. The isotropic voxel resolution was 30 × 30 × 30 μm. To verify new bone formation around the implant, an area of 1.5 mm2 around the bone where the 1.2-mm implant had been placed was scanned at a depth of 0.5–1.0 mm from the inner cortical bone. Thus, a cuboid of the peri-implant bone with base dimensions of 1.5 × 1.5 mm and a height of 0.5 mm was used to analyze the BMD. The R-mCT Image Analysis (Rigaku Corp., Tokyo, Japan) software was used to generate three-dimensional models.
from the scanned data. The TRI/3D-BON image analysis software (Ratoc System Engineering Co., Ltd., Tokyo, Japan) was used to calculate the BMD of the peri-implant bone cuboid and generate color images depicting the BMD intensity, with the colors blue and light blue; green and yellow; and orange and red representing low, medium, and high BMD values, respectively.

**Histological microscopy observations**

Histological microscopy observations were performed to visually evaluate bone formation over time with respect to the surface treatment. The femur was cut at the midpoint of its long axis using a diamond disk (Isomet; Buehler Ltd., Lake Bluff, IL, USA). The bone tissue was then dehydrated in a series of 70–80% ethanol solutions and then in 100% acetone. Next, it was embedded in methyl methacrylate acrylic resin (Osteoresin Embedding Kit; Fujifilm Wako Pure Chemical Corp., Osaka, Japan) and cured. The embedded specimens were cut into 30–40-μm-long sections perpendicular to the long axis of the implant using a diamond disk, polished to a final thickness of 30 μm, and subjected to Villanueva–Goldner staining. Further, non-demineralized specimens from the diaphyseal region were obtained. Finally, new bone formation around the implant was observed with a microscope by an observer blinded to the sample and sample group.

**Statistical analyses**

All the values shown in the figures represent the mean ± standard deviation. Student’s t-test was used for the statistical analyses of the body weight, pull-out strength, and BMD. The null hypothesis was that there is no difference between the control and SBM groups. The analyses were performed using SPSS (Version 22.0, Chicago, IL, USA). Differences with p < 0.05 were considered statistically significant.

**Body weight**

Fig. 1 shows the results of the average body weight measurements for the two groups (n=6) at each observation time. The SBM and control groups showed no marked difference in body weight during each observation period. Despite slight weight loss during the week of implant surgery, weight gain was observed with the recovery of the sutured region in all experimental groups. There was no significant difference in the fluctuation of body weight between the groups.

**Results**

**Body weight**

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**Pull-out strength**

The results of the average pull-out strength tests (n=5) are shown in Fig. 2. The femurs of euthanized rats showed no particular tissue overgrowth, fractures, or cracks. Two weeks after implantation, the pull-out force of the control group was 1.8 ± 0.4 N, while that of the SBM group was 18.6 ± 2.7 N, indicating that the SBM group had significantly higher strength (p < 0.01). Four weeks after implantation, the pull-out force of the control group was 4.4 ± 1.3 N while that of the SBM group was 24.4 ± 5.1 N. Thus, even after four weeks, the SBM group had significantly higher strength (p < 0.01). Intragroup comparisons indicated that the values for the control group were significantly higher four weeks after implantation than two weeks after implantation (p < 0.05). Similarly, the values for the SBM group four weeks after implantation were significantly higher than those two weeks after implantation (p < 0.05).

**BMD measurements**

The results of the average BMD measurements (n=6) are shown in Fig. 3. The BMD value for the SBM group two weeks after implantation was 770.0 ± 49.7 mg/cm\(^3\), which is approximately 1.8 times greater than that in the control group (437.8 ± 71.0 mg/cm\(^3\); between-group comparison, p < 0.01). Moreover, the BMD value of the SBM group four weeks after implantation was 957.9 ± 40.0 mg/cm\(^3\), which is approximately twice that for the control group (489.1 ± 37.9 mg/cm\(^3\); between-group comparison, p < 0.01).
Thus, the SBM group showed a statistically significant increase in BMD at 2–4 weeks after implantation (within-group comparisons, \( p < 0.05 \)).

**BMD color imaging**

The results of dividing the BMD for the new bone that formed in the peri-implant regions into six levels are shown in Fig. 4. BMD color imaging shows the results of one representative case in each group (\( n=6 \)). The femurs of euthanized rats showed no particular tissue overgrowth, fractures, or cracks. In the case of the control group (Fig. 4(a)), the implant was covered with new bone with a low BMD two weeks after implantation, as indicated by the predominance of blue. Although the colors corresponding to the BMD for the new bone in some locations near the cortical bone (Fig. 4(a), left side) ranged from red to green, the width of this region was small. Four weeks after implantation (Fig. 4(b)), the predominant colors were red, yellow, green, and blue, indicating that the BMD was slightly higher than that after two weeks. However, the width of the new bone remained nearly unchanged. In the case of the SBM group, the peri-implant region was green to yellow in color after two weeks (Fig. 4(c)), indicating that the implant was surrounded by new bone with a moderately high BMD, while for some locations, the color ranged from orange to red. We observed new-growth bone with a slightly higher BMD in the region between the peri-implant region and the bone marrow. Four weeks after implantation (Fig. 4(d)), the color of the area around the implant ranged from yellow to orange, indicating new bone growth with a slightly higher BMD. Some parts were red, suggesting a higher BMD than that observed in the case of the SBM group two weeks post implantation (Fig. 4(c)). Thus, it can be concluded that new-growth bone with a high BMD was present in the area extending from the peri-implant region to the bone marrow.

**Histological microscopic observations**

Histological microscopic observations shows the results of one representative case in each group. Moderate- and high-magnification images of the control group two weeks after implantation are shown in Fig. 5(a) and (b), respectively. The black circle visible in the images is the implant. The femurs of euthanized rats showed no particular tissue overgrowth, fractures, or cracks. In Fig. 5(a), parts of the peri-implant region (upper portion and lower-right portion) are stained green, indicating calcified new-growth bone. In Fig. 5(b), the peri-implant region is lined with bone marrow, which seems to be forming osteoid. This is stained red (arrows). Moderate- and high-magnification images of the control group four weeks after implantation are shown in Fig. 5(c) and (d), respectively. Fig. 5(c) shows the formation of calcified new-growth bone in the peri-implant region. The amount of new-growth bone, which is stained green and leads to the formation of the cortical bone on the upper side of the implant, is greater in Fig. 5(c) than in Fig. 5(a). Further, the amount of osteoid formed within the marrow in the peri-implant region (arrows) in Fig. 5(d) is higher than that seen in Fig. 5(b), which corresponds to the control group two weeks post implantation.

Moderate- and high-magnification images of the SBM group two weeks post implantation are shown in Fig. 6(a) and (b), respectively. In Fig. 6(a), the amount of calcified bone formed in the peri-implant region and the amount of bone marrow within the cortical bone adjacent to the upper side of the implant is higher than those in the case of the control group (Fig. 6(a)). Further, nearly the entire region around the implant is...
Figure 5. Villanueva–Goldner staining of femur cross-section of specimen in control group after (a, b) two and (c, d) four weeks; (b) and (d) are magnified images of the area around the implant in (a) and (c), respectively. The arrows indicate osteoid formation within the bone marrow. The black circular area in the images is the implant.

Figure 6. Villanueva–Goldner staining of femur cross-section of specimen in synthetic bone mineral (SBM) group after (a, b) two and (c, d) four weeks; (b) and (d) are magnified images of the area around the implant in (a) and (c), respectively. The arrows indicate osteoid formation within the bone marrow. The black circular area in the images is the implant.
surrounded by osteoid and new bone growth. In addition, the amount of osteoid (arrows) formed within the bone marrow in the peri-implant region in Fig. 6(b) is higher than that for the control group (Fig. 5(b)). Moderate- and high-magnification images of the SBM group at two- and four weeks post implantation are shown in Fig. 6(c) and (d), respectively. Newly formed calcified bone (stained green) running along the wall for three-fifths of the implant from the cortical bone side of the peri-implant region can be observed in Fig. 6(d). Finally, the formation of osteoid (arrows; stained red) within the calcified bone (stained green) can be observed in Fig. 6(d).

Discussion

It has been reported that the factors that have a determining effect on the outcome of implantation surgery are the time required for new-growth bone to form in the peri-implant region and the time at which implant fixation occurs\(^{15}\). It is also known that, instead of smoothly worked implant surfaces, roughly processed implant surfaces are more likely to ensure osteoblast adhesion and differentiation, which, in turn, ensures early implant-to-bone fusion. Currently, implants subjected to a range of surface treatments are available commercially. The objective of this study was to evaluate the effects of SBM surface treatment on early bone formation as well as improvements in bone tissue quality.

The body weights of all rats were measured starting at the age of 8 weeks, when they were in preliminary care. The increases in body weights of all animals were low from the time of surgery until one week post operation; however, they subsequently gained weight, and their body weights were normal, indicative of normal growth, suggesting successful implantation.

Owing to its exceptional corrosion resistance, Ti does not normally corrode when used in oral cavity environments\(^{16}\). Nevertheless, in acidic environments in which fluorine is present, hydrogen fluoride, which corrodes Ti, is produced in minute amounts\(^{16}\). For example, when fluorine was included in the surface coatings for teeth with a concentration of approximately 9000 ppm, the pH of the environment was approximately 2.5. It has been reported that Ti corrodes at this pH\(^{16}\). Therefore, in the present study, we paid particular attention to the pH of the SBM used in the coating. Nakagawa et al. reported that immersion of Ti in a 0.1% aqueous sodium fluoride solution (fluoride concentration of 453 ppm) resulted in an increase in potential without causing corrosion when the pH was higher than 4.4 owing to a change in the corrosion potential, while corrosion occurred when the pH was lower than 4.3\(^{16}\). Thus, referring to the corrosion potential, we performed preliminary experiments to investigate the pH of a mixture of SBM in a 0.4% H₃PO₄ solution. With 0.4 g of SBM, the pH was 4.51. Based on Nakagawa’s results\(^{16}\), this should not cause corrosion of Ti. Indeed, no corrosion was observed by electron microscopy several days after coating the mixture on Ti alloy. However, longer term corrosion experiments should be conducted in the future to confirm the long-term corrosion behavior.

The pull-out strengths of the samples in the SBM group were 10.3 and 5.5 times higher than those in the control group two and four weeks after implantation, respectively. In addition, the pull-out strength of the samples in the SBM group increased by 1.3 times between two and four weeks after implantation. The results of the BMD analysis indicated that the new-growth bone around the SBM-coated implants was 1.8 and 2 times denser than that of the control group two and four weeks after implantation, respectively. In addition, the BMD of the SBM group increased 1.2-fold between weeks two and four. Similar results were obtained in the pull-out strength tests. Thus, it was revealed that early-stage bone formation occurs after implantation. This conclusion is in line with the results of a study by Watanabe et al.\(^9\), who observed accelerated bone formation after the ingestion of the SBM as a supplement.

BMD color imaging and histological observations of the control group showed a slight increase in BMD and the area of new-growth bone surrounding the implant from two weeks to four weeks post-implantation. Compared to the control group, the SBM group had an increased area of new-growth bone at two weeks post-implantation, and the BMD of the new-growth bone was markedly higher. In addition, at four weeks post-implantation, new-growth bone with high BMD was observed in the SBM group over a wide area, ranging from the peri-implant region to the entire bone marrow. Thus, BMD color imaging and histological observations revealed that bone formation was faster in the SBM group than in the control group, and that the SBM group had significantly higher absolute values of BMD and a larger area of bone formation than the control group.

The elements and minerals present in the SBM, such as Mg, Zn, Mn, and various fluorides, contribute to the synthesis of collagen and other proteins, which regulate calcitonin secretion and alkaline phosphatase activity and decrease osteoclast activity. This helps in the maintenance of bone tissue\(^{18,19}\). Mg helps to limit cytokine formation as well as the number of osteoclasts produced\(^20\). It has been reported that the administration of elements such as Ca, Zn, Mn, and Cu to patients with osteoporosis can help prevent decreases in their bone density\(^20\). Ito et al. performed in vivo experiments and found that the release of Zn activates osteoblasts and suppresses osteoclasts\(^20\). Fluorine increases the amount of collagen generated and aids alkaline phosphatase activity, which in turn promotes the differentiation of osteoblasts\(^20\). Thus, the formation and spread of new-growth bone within the bone marrow in the specimens of the SBM group can be attributed to the release of the Zn contained in the SBM. Watanabe et al.\(^9\) administered SBM to rats by including it in their food and found that the oral ingestion of the SBM was effective in the promotion of bone formation. Sato et al. reported that, when they embedded implants that caused mechanical friction in the femurs of rats that had ingested SBM as a supplement, new bone formation was promoted in the peri-implant region\(^9\). Srinivasan et al. reported that SBM was effective as a supplement in maintaining bone health in subjects experiencing adverse effects on their bone microstructure and bone density, such as estrogen and mineral deficiencies\(^9\). Therefore, when considered together, the results of the above-described studies suggest that SBM can not only replenish minerals but also promote early bone metabolism. Therefore, it is suitable for facilitating bone formation.

The surface characteristics of implants have a determining effect on osteoblasts and bone formation on their surface. The organic responses that occur when an implant is embedded include the adhesion of proteins onto the implant surface. These become mature bones as they differentiate into stem cells and proliferate. Therefore, several research groups are attempting to develop implant surface processing methods that can aid early bone fusion.

The findings of this study indicate the potential benefits of surface-treating implants with SBM prior to peri-implantation. We hope that this study will aid the development of surface processing methods that facilitate early bone formation. Further, the investigated SBM should find application as a bone prosthetic filling material for use in regions with bone defects. We believe that surface treating implants with
SBM can shorten the healing period after implant surgery, increase patient expectations regarding the treatment outcome, allow implant surgery for elderly patients and those with osteoporosis (i.e., for whom it is not usually indicated), and improve the diet, esthetics, and quality of life of patients. Further research should be conducted to explore the benefits of implanted SBM in more detail.

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Conflicts of Interest

The authors have declared that no conflict of interests exists.

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