Effect of a Synthetic Bone Mineral Supplement on Bone Growth in the Distal End of the Rat Femur

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Abstract
Aims: Rough-surfaced dental implants are considered optimum surfaces for osseointegration, but have a high incidence of peri-implantitis. In contrast, machined surface dental implants do not cause peri-implantitis, but enable less osteoblast proliferation and differentiation, which is important in bone forming around the implant. We previously found that synthetic bone mineral (SBM) developed for osteoporosis, accelerates bone forming around rough-surfaced implants in rats. However, the effect of SBM has not been investigated in normal rats without implants. Thus, this study investigated whether SBM is influence in eliciting bone forming in normal rats without implants.

Methods: twenty-four female Wistar normal rats (7 weeks of age) were randomly assigned to receive a control group fed a diet without SBM (n = 12, control) or an experimental group fed a diet with SBM (n=12, experimental). The rats were sacrificed at 11 and 13 weeks. Bone mineral density (BMD) and fluorescent staining were assessed at the distal end of the femur. Between-group differences in BMD at 11 and 13 weeks of age were analyzed.

Results: BMD in cortical bone and cancellous bone was significantly higher in rats who received SBM than those who did not at both 11 and 13 weeks of age. Fluorescence microscopy image of rats who received SBM demonstrated more green fluorescence, compared with rats who did not receive SBM, at both 11 and 13 weeks of age.

Conclusions: Rats who received SBM exhibited increased BMD relative to rats who did not receive SBM, which indicated that the intake of SBM was effective in bone forming.

Keywords:
Animal study, Dietary supplement, Machine surface implant, Synthetic bone mineral, Bone formation

Background
Dental implant is an effective modality to restore esthetic appearance and masticatory function because of tooth loss. Nevertheless, osseointegration between implant and bone requires 3–6 months (1). Therefore, there is an ongoing effort to improve the interface between bone and implant, in order to accelerate the process of osseointegration and improve its quality. These efforts have been primarily focused on chemically improving the interface by incorporating inorganic phases onto or into the titanium oxide layer, or physically improving the interface by increasing the level of roughness (2, 3). It is known from previous studies that some types of rough-surfaced implants, such as those coated with hydroxyapatite, reportedly have a higher incidence of peri-implantitis (4). When peri-implantitis occurs, the beneficial aspects of rough-surfaced implants become factors that exacerbate inflammation around the implant fixture. The risk of peri-implantitis is lower with machined implants which plaque is difficult to adhere (5, 6). Machine surfaces enable less osteoblast proliferation and differentiation, which are important factors for the long-term success of implants (7). Thus, interventions are needed to accelerate bone forming around machined surface dental implants. In the

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past, Ogawa (8) reported that titanium surfaces treated with ultraviolet light develop a unique electrostatic status and act as direct cell attractants to effectively reduce the osseointegration period without the aid of ionic or organic bridges; this imparts a novel physicochemical functionality to titanium (9). Vibratory stimulation can also improve bone-to-implant contact ratio (10).

In general medicine, systematic approaches to improve bone forming have been investigated; these include supplemental therapy for osteoporosis (11, 12). LeGeros developed a calcium phosphate-based supplement that incorporates fluoride (F), carbonate, magnesium (Mg) and zinc (Zn); known as synthetic bone mineral (SBM), this supplement promotes bone forming and inhibits bone resorption in osteoporosis patients (13, 14). In this context, bone forming has been reported around rough-surfaced implants when using SBM (11). However, the effect of SBM has not been investigated in normal rats without implants. In this study, we assessed the effect of SBM on bone forming in normal rats, focusing on the growth plate at the distal end of the femur, where bone forming is most active.

Table 1. Mineral compositions (Wt %) of diets without and with synthetic bone mineral (SBM)

|                  | AIN-93M (Diet without SBM) | AIN-93M + SBM (Diet with SBM) |
|------------------|----------------------------|--------------------------------|
| Calcium (Ca)     | 0.51                       | 0.74                           |
| Phosphate (P)    | 0.3                        | 0.48                           |
| Magnesium (Mg)   | 0.05                       | 0.35                           |
| Zinc (Zn)        | 0.003                      | 0.036                          |
| Fluorine (F)     | 0                          | 0.005                          |
| Carbonate (CO\textsuperscript{3}) | 0          | 0.12                           |
| Natrimum (Na)    | 0.1                        | 0.13                           |
| Kalium (K)       | 0.35                       | 0.75                           |
| Chlorine (Cl)    | 0.16                       | 0.17                           |

SBM: synthetic bone mineral

AIN-93M, developed by the American Institute of Nutrition and prepared by the Oriental Yeast Co., Ltd. (Tokyo, Japan), was used as the control diet. The experimental diet consisted of AIN-93M and SBM. The SBM was prepared in accordance with the protocol published by LeGeros (13). SBM was then added to AIN-93M; the final mineral composition was prepared according to Mijares protocol (14). The compositions of the diets with and without SBM are shown in Table 1.

Animal experiment

The study protocol was approved by the Ethical Committee of Nihon University School of Dentistry at Matsudo (AP16MD004–2). Twenty-four 6-week-old female Wistar rats (Sankyo Labo Service, Tokyo, Japan) were included in the study. After consuming a diet without SBM for 1 week during environmental acclimation, 7-week-old rats were randomly allocated into one of two groups: a control group fed a diet without SBM (n=12, control group) or an experimental group fed a diet with SBM (n=12, experimental group). Rats were housed individually; food and water were given ad libitum, and temperature and relative humidity were maintained at 20°C ± 1°C and 50% ± 1%, respectively.

Six rats in each group were randomly selected for euthanasia at 11 and 13 weeks of age. Seven days before they were euthanized, four of these eight rats in each group were randomly selected for intraperitoneal injection of 20
mg/kg of calcein, in order to visualize new bone formation with fluorescent labeling. Four of six rats were allocated for bone mineral density (BMD) analysis. The remaining two rats were allocated for fluorescence microscopy observations.

**BMD and BMD color imaging**

Imaging conditions for micro computed tomography (micro-CT, R_mCT2; Rigaku, Tokyo, Japan) were as follows: tube current: 160 μA; tube voltage: 90 kV; field of view: 10 mm; shooting time: 26 s; and voxel size: 30 μm × 30 μm × 30 μm. Micro-CT images of the femur and phantoms for CT value proofreading were taken at 11 and 13 weeks of age. The measurement region in the distal femur (6.0 mm × 5.0 mm × 3.0 mm) was 0.5 mm from the datum line connecting the two ends of the epiphyseal growth plate on the femur (Fig. 1); this was referred to as the epiphyseal region. Additionally, measurements in the epiphyseal region were divided into cortical bone and cancellous bone. R_mCT Image Analysis software (Rigaku, Tokyo, Japan) was used to generate three-dimensional models with the scanned data. A TRI/3D-BON image analyzer (Ratoc System Engineering, Tokyo, Japan) was used to calculate the BMD of the distal femur metaphysis cuboid; it was also used to generate color images depicting BMD intensity, with blue/light blue, green/yellow, and orange/red representing low, medium, and high BMD values, respectively.

**Fluorescence microscopy observation**

The femur was cut in the epiphyseal region using a diamond disk (Isomet; Buehler Ltd., Lake Bluff, IL, USA). The bone tissue was dehydrated in a 70%-80% ethanol series followed by 100% acetone; it was then embedded in methyl methacrylate acrylic resin (Osteoresin Embedding Kit, Wako Pure Chemical Industries, Tokyo, Japan) and cured. Embedded specimens were cut into 30 μm sections perpendicular to the long axis of the implant using a diamond disk; they were then polished to a final thickness of 20 to 30 μm, and unstained, non-demineralized specimens from the diaphyseal region were obtained. New bone formation around the implant was observed with a fluorescence microscope (BZ-9000, KEYENCE Co., Osaka, Japan).

**Statistical analysis**

Between-group differences in BMD at 11 and 13 weeks of age were analyzed by the Mann-Whitney U test. All statistical analyses were performed using the statistical package PASW Statistics (Version 18.0, SPSS, Chicago, IL, USA). *P* values <0.05 were considered statistically significant.

**Results**

**Bone mineral density**

Results of BMD analyses in the cortical bone are shown in (Fig. 2A). The experimental group at 11 weeks of age was 1477.5 ± 24.03 mg/cm³, nearly 1.8-fold greater than that of the control group (841.22 ± 34.60 mg/cm³, *P* < 0.05). The experimental group at 13 weeks of age was 1552.3 ± 39.73 mg/cm³, nearly 1.8-fold greater than that of the control group (870.55 ± 24.06 mg/cm³, *p* < 0.05).

Results of BMD analyses in the cancellous bone are shown in (Fig. 3A). The experimental group at 11 weeks of age was
Fig. 2  Bone mineral density (BMD) and BMD color imaging in cortical bone. (A): BMD was significantly greater in the experimental group than in the control group at 11 and 13 weeks of age (P < 0.05). Both groups showed statistically significant increases in BMD from 11 to 13 weeks of age. The asterisks represent significant differences (P < 0.05). (B): BMD color imaging of the control group at 11 and 13 weeks of age showed mainly blue and yellow regions (a and d). However, BMD color imaging of the experimental group at 11 and 13 weeks of age showed mainly orange and red regions (b and c).

Fig. 3. Bone mineral density (BMD) and BMD color imaging in cancellous bone. (A): BMD was significantly greater in the experimental group than in the control group at 11 and 13 weeks of age (P < 0.05). Both groups showed statistically significant increases in BMD from 11 to 13 weeks of age. The asterisks represent significant differences (P < 0.05). (B): BMD color imaging of the control group at 11 and 13 weeks of age showed mainly blue and yellow regions (a and d). However, BMD color imaging of the experimental group at 11 and 13 weeks of age showed mainly orange and red regions (b and c).
1191.3 ± 8.41 mg/cm³, nearly 1.8-fold greater than that of the control group (664.3 ± 25.50 mg/cm³; p < 0.05). The experimental group at 13 weeks of age was 1245.8 ± 16.70 mg/cm³, nearly 1.8-fold greater than that of the control group (683.6 ± 27.88 mg/cm³; p < 0.05).

Bone Mineral Density color imaging

Color images of BMD in the cortical bone of the control group were primarily blue and green at 11 and 13 weeks of age (Fig. 2B-a and c); corresponding images were primarily yellow, with some red and blue, in the experimental group at the same time points (Fig. 3B-b and d). Color images of BMD in the cancellous bone of the control group were primarily blue and green at 11 and 13 weeks of age (Fig. 3B-a and c); corresponding images were primarily yellow, with some green, in the experimental group at the same time points (Fig. 3B-b and d). Given that blue/light blue, green/yellow, and orange/red represent low, medium, and high BMD, respectively, these results show that the BMD of both cortical and cancellous bone was higher in the experimental group than in the control group at both time points.

Fluorescence microscopy

Fluorescence microscopy image demonstrated more green fluorescence (showing bone forming) in the experimental group than in the control group at both 11 and 13 weeks of age (Figure. 4).
Discussion

This animal study revealed that, at 11 and 13 weeks of age, rats that were fed SBM showed increased quantitative BMD of the distal femur in both cortical and cancellous bone, compared with control rats that were not fed SBM: this showed that SBM intake was effective in causing bone forming. Additionally, increased BMD was observed qualitatively in BMD color images of rats in the experimental group at 11 and 13 weeks of age, where the majority of areas showed increased bone forming. This finding could be partially explained by the results of fluorescence microscopy image, which revealed the dynamics of bone remodeling through incorporation of calcine fluorescent dye. Green fluorescence strongly appeared in the experimental group at 11 weeks and 13 weeks; this suggested that rats that were fed SBM showed a greater extent of bone forming.

The sole difference between the two groups was whether they had been fed SBM. Thus, the difference of BMD mineral composition, 7-fold greater Mg content and 12-fold greater Zn and F content in the experimental group, may primarily affect bone forming. F, Zn, and Mg play important roles in bone forming and resorption. Mijares (14) stated that the effect of SBM may be explained in terms of individual and combined effects of F, Zn, and Mg on bone cell activities, such as bone forming and bone resorption when released from the SBM, as well as their effects when incorporated in newly formed bone. This might explain why rats in the experimental group had greater BMD than those in the control group.

Machined surfaces are beneficial to reduce the risk of peri-implantitis. Because of the reduced rates of osteoblast proliferation and differentiation associated with machined implants, approaches for accelerating bone forming are needed. SBM supplementation was originally developed for the prevention and treatment of osteoporosis in aged individuals. The ability of SBM supplementation to promote bone forming, as shown in this study, supports further research regarding its therapeutic abilities in patients with implants, as well as those with osteoporosis.

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