Original

Effect of a Dietary Supplement on Peri-Implant Bone Strength in a Rat Model of Machined Surface Implants

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(Accepted for publication, December 21, 2018)

Abstract: Efforts to improve the bone-implant interface to accelerate and improve the quality of osseointegration have generally focused on chemically improving the interface by incorporating inorganic phases on or into the titanium oxide layer or physically improving it by increasing the level of roughness. However, some types of rough-surfaced implants, such as those coated with hydroxyapatite (HA), may result in a higher incidence of complications. Once peri-implantitis occurs, the factors promoting bone integration can turn into risk factors, exacerbating inflammation around the implant fixture. Thus, it is extremely important to study approaches for accelerating bone formation around the machined surface dental implants. Peri-implant bone formation may be enhanced by systemic approaches, such as the use of osteoporosis supplements, to promote bone metabolism. The present study aimed to investigate if peri-implant bone mineral density (BMD) was improved after oral synthetic bone mineral (SBM) intake, which facilitates improved secondary stability of the machined surface dental implants and shortens the healing period. Twenty-four 7-week-old female Wistar rats were randomly assigned to receive a standardized diet with or without SBM (diet with SBM group and diet without SBM group, respectively; n = 12 for both). The rats underwent implant surgery at 9 weeks of age under general anesthesia. The main outcome measures BMD, pull-out strength, real-time PCR and Fluorescence microscopy observations of the implant from the femur were compared at 2 and 4 weeks after implantation using the Mann-Whitney U test. At 2 and 4 weeks after implantation, BMD, pull-out strength, real-time PCR and fluorescence microscopy observations were both significantly greater in the diet with SBM group than in the diet without SBM group. This study demonstrated that SBM could be effective in accelerating peri-implant bone formation for machined surface implants during the healing period after implantation.

Key words: Animal study, Dietary supplement, Implant, Machined surface implant

Introduction

Dental implant treatment is an effective modality to restore esthetic and masticatory functions lost due to tooth loss. However, osseointegration between the implant and bone requires 3-6 months1. Therefore, efforts are being made to improve the bone-implant interface to accelerate and improve the quality of osseointegration. These efforts have focused on improving the interface chemically by incorporating inorganic phases on or into the titanium oxide layer, or physically by increasing the level of surface roughness2,3. In a consensus report published in 2009, it was concluded that “moderately rough and rough surfaces provided enhanced bone integration compared with smooth and minimally rough surfaces”4-6. However, some types of rough-surfaced implants, e.g., those coated with hydroxyapatite (HA), were reported to show a higher incidence of complications, i.e., peri-implantitis7.

Simion et al. reported in their 12-year retrospective follow-up study concluded that when implants with machined surfaces are used, the risk of peri-implantitis in the posterior maxilla was less than with rough surface implants. However, machined surfaces show less osteoblast proliferation and differentiation, than the rough surface implants, which are important factors for the prognosis of implant treatments8,9. These disadvantages could be addressed by using approaches aimed at accelerating bone formation around machined surface dental implants. In the past, Ogawa10 reported that machine surface implants treated with ultraviolet light developed a unique electrostatic characteristic, acting as direct cell attractants to effectively reduce the osseointegration period without the aid of ionic or organic bridges, which is a novel physico-chemical characteristic of titanium11. Ultraviolet photofunctionalization has also been reported to improve the bone-to-implant contact ratio12,13.

In addition to these local approaches, systemic approaches such as the use of osteoporosis medication to promote bone metabolism may improve peri-implant bone formation after the surgery14-16. LeGeros developed synthetic bone mineral (SBM), a calcium-phosphate-based supplement incorporating magnesium (Mg), zinc (Zn), fluoride (F), and carbonate, to promote bone formation and inhibit bone resorption in osteoporosis17,18. On the basis of this development, one study revealed that SBM accelerated bone formation in normal rats both...
the compositions of the diets with and without SBM are shown in Table 1. The experimental diet consisted of AIN-93M, developed by the American Institute of Nutrition Committee and prepared by the Oriental Yeast Co, Ltd. (Tokyo, Japan), was used as the control diet. The experimental diet consisted of AIN-93M + SBM. The SBM was prepared according to LeGeros’ protocol [13]. Briefly, a mixture of dicalcium phosphate dihydrate (CaHPO$_4$ • 2H$_2$O) and SBM was used as the control diet. The experimental diet consisted of AIN-93M and SBM. The null hypothesis was that bone formation, evaluated by pull-out strength, bone mineral density (BMD), relative gene expression, and fluorescence microscopy findings, around the machined surface dental implants would not differ between rats fed diets with and without an SBM supplement.

### Materials and methods

#### Animal diet

AIN-93M, developed by the American Institute of Nutrition Committee 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 according to LeGeros’ protocol [13]. Briefly, a mixture of dicalcium phosphate dihydrate (CaHPO$_4$ • 2H$_2$O) and magnesium and zinc chlorides (MgCl$_2$ and ZnCl$_2$, respectively) was hydrolyzed in double-distilled water containing dissolved potassium carbonate and sodium fluoride. SBM was then added to AIN-93M, the mineral composition of which was adjusted using Mijares’ method [15]. The compositions of the diets with and without SBM are shown in Table 1.

#### Animal experiment

The study protocol was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo (AP15MD004-1). Twenty-four 6-week-old Wistar rats (Sankyo Labo Service Corp., Tokyo, Japan) were included in the study. After consuming a diet without SBM for 1 week to acclimate to the changes in their environment, the 7-week-old rats were randomly allocated into one of two groups: a control group fed a diet without SBM (n = 12) or an experimental group fed a diet with SBM (n = 12). The 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.

All rats underwent implant surgery on their femurs at 9 weeks of age while under general anesthesia administered via an intraperitoneal injection of medetomidine, midazolam, and butorphanol. One operator prepared a hole in the femur 1.2 mm in diameter and 2.5 mm deep using a 1.2-mm-diameter drill. The implants were then inserted into the hole to a depth of 2.5 mm using a drill at a speed of 500 rpm, with 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 the connection to the load cell to measure the pull-out strength. The incision wound was sutured after completion of the surgery.

Seven days before euthanasia at 2 and 4 weeks, one randomly selected rat received a 20 mg/kg injection of calcein intraperitoneally for visualization of new bone formation using fluorescent labeling. Six rats in each group were randomly selected for euthanasia at 2 weeks and 4 weeks after implantation. Four of these six rats were allocated for pull-out testing, BMD analysis, and quantitative real-time PCR. The remaining two rats were allocated for fluorescence and histological microscopic observations.

#### Pull-out strength

The pull-out test was conducted to evaluate the adhesive strength between the implant body and the bone. The titanium specimens were mechanically anchored to a baseplate with self-curing resin. The setup was adjusted using a level to align the test area with the load cell such that the direction of the exerted force was as perpendicular 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 50 mm separating the top of the implant and load cell. An Instron universal testing machine (TG-5k; MinebeaMitsumi Inc., Kanagawa, Japan) was used for the pull-out test with a 1.0-mm/min cross-head speed. Pull-out strength was determined as the peak force applied to detach the implant from the bone.

#### BMD and BMD color imaging

BMD and BMD color imaging were performed to quantify and visualize the bone density around the implant body. After the pull-out test, the femur was subjected to microcomputed tomography (mCT) scanning performed with an R_mCT2 device (Rigaku Corp., Tokyo, Japan) using a 90-kV anode electrical current at a 30-μm resolution. The isotropic voxel resolution was 30 μm × 30 μm × 30 μm. To verify new bone formation around the implant, a 1.5-mm$^2$ area surrounding the bone where the 1.2-mm implant was placed was scanned at a depth of 0.5-1.0 mm from the inner cortical bone. Thus, a cuboid of peri-implant bone with a 1.5 × 1.5-mm base and a 0.5-mm height was used to analyze BMD. R_mCT Image Analysis software (Rigaku Corp., Tokyo, Japan) was used to generate three-dimensional models using the scanned data. A TRU/3D-BON image analyzer (Ratoc System Engineering Co., Ltd., Tokyo, Japan) was used to calculate the BMD of the peri-implant bone cuboid and generate color images depicting BMD intensity, with blue and light blue, green and yellow, and orange and red representing low, medium, and high intensity, respectively.

### 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                       | 0.1                            |
| Zinc (Zn)        | 0.003                      | 0.036                          |
| Fluorine (F)     | 0                          | 0.005                          |
| Carbonate (CO3)  | 0                          | 0.12                           |
| Natrium (Na)     | 0.1                        | 0.1                            |
| Kalium (K)       | 0.35                       | 0.35                           |
| Chlorine (Cl)    | 0.16                       | 0.16                           |

SBM: synthetic bone mineral
and high BMD values, respectively.

**Quantitative real-time PCR assay**

Quantitative real-time PCR assay was performed to evaluate the metabolic activity of osteoblasts by the effect of SBM. After mCT scanning, osseous tissues surrounding the implant were used for quantitative real-time PCR (qRT-PCR) testing to explore the potential molecular mechanisms at 2 and 4 weeks after implantation of the fixture. Total RNA was isolated using TRIzol® Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer’s instructions. At an absorbance of 260 nm, RNA concentration was detected using a Nano Drop spectrophotometer (ND-1000; Thermo Fisher Scientific Inc., Waltham, MA, USA). Reverse transcription was performed, and complementary DNA (cDNA) was synthesized using up to 1 μg of isolated RNA, by a Prime Script RT reagent Kit (Takara Bio Inc., Tokyo, Japan) according to the manufacturer’s protocol. The qRT-PCR assay was performed using an SYBR Premix Ex Taq (Takara Bio Inc., Tokyo, Japan).

GAPDH was employed as a housekeeping gene, and the primer sequences used in this study are listed in Table 2. The relative gene expression of an alkaline phosphatase, collagen type 1, and osteocalcin were used as outcomes.

**Fluorescence microscopy observations**

Fluorescence microscopic observation was carried out in order to visually evaluate the bone formation over time due to the effect of SBM. The femur was cut at the midpoint of the long axis 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, embedded in methyl methacrylate acrylic resin (Osteoresin Embedding Kit; Fujifilm Wako Pure Chemical Corp., Osaka, Japan), and cured. Embedded specimens were cut into 30-μm sections perpendicular to the long axis of the implant using a diamond disk, polished to a final thickness of 20 to 30 μm, and unstained, nondemineralized specimens from the diaphyseal region were obtained. New bone formation around the implant was observed with a fluorescence microscope (BZ-9000; Keyence Corp., Osaka, Japan).

**Body weight**

Weight measurements were carried out to evaluate the health of the rats. All 24 rats were weighed at 7, 9, and 11 weeks; the 12 surviving rats were also weighed at 13 weeks to confirm growth and good physical condition. Rats were also weighed before euthanasia.

**Statistical analyses**

Between- and within-group differences in BMD, pull-out strength, and relative gene expression at 2 and 4 weeks after implantation were analyzed by using the Mann-Whitney U test. Changes in body weight were separately analyzed by using the Friedman test to verify whether weight significantly changed over time in each group. 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**

**Pull-out strength**

Results of the pull-out strength test are given in Fig. 1. The pull-out
strength in the diet with SBM group at 2 weeks after implantation was 0.55 ± 0.14 N, approximately 9.9 times greater than that in the diet without SBM group (0.06 ± 0.14 N; between-group comparison, P < 0.05). The pull-out strength in the diet with SBM group at 4 weeks after implantation was 2.14 ± 0.38 N, also approximately 3.4 times greater than that in the diet without SBM group (0.61 ± 0.25 N; between-group comparison, P < 0.05). Both diet groups showed statistically significant increases in pull-out strength 2 to 4 weeks after implantation (within-group comparisons, P < 0.05).

**Bone mineral density**

BMD results are shown in Fig. 2. The BMD in the diet with SBM group at 2 weeks after implantation was 320.62 ± 25.35 mg/cm³, approximately 1.2 times greater than that in the diet without SBM group (248.43 ± 19.07 mg/cm³; between-group comparison, P < 0.05). The BMD in the diet with SBM group at 4 weeks after implantation was 454.77 ± 25.07 mg/cm³, approximately 1.1 times greater than that in the diet without SBM group (396.85 ± 17.70 mg/cm³; between-group comparison, P < 0.05). Both diet groups also showed statistically significant increases in BMD from 2 to 4 weeks after implantation (within-group comparisons, P < 0.05).

**BMD color imaging**

The BMD color image in the diet without SBM group was primarily...
Figure 4. The qRT-PCR findings. The Alp-expression level in the diet with SBM group at 2 weeks after implantation was approximately 2.2 times greater than that in the diet without SBM group (P < 0.05). The Alp-expression level in the diet with SBM group at 4 weeks after implantation was approximately 1.6 times greater than that in the diet without SBM group (P < 0.05). Both diet groups showed no statistically significant increases from 2 to 4 weeks after implantation (P < 0.05).

The col 1-expression level in the diet with SBM group at 2 weeks after implantation was approximately 1.4 times greater than that in the diet without SBM group (P < 0.05). The col 1-expression level in the diet with SBM group at 4 weeks after implantation was approximately 1.6 times greater than that in the diet without SBM group (P < 0.05). The diet with SBM group showed statistically significant increases in col 1-expression level from 4 weeks after implantation (P < 0.05).

The ocn-expression level shows no significant difference in the diet with SBM group and the diet without SBM group at 2 and 4 weeks after implantation (P < 0.05). Both diet groups show no significant increase in ocn-expression level from 2 to 4 weeks after implantation (P < 0.05). * p < 0.05
blue and green at 2 and 4 weeks after implantation (Figs. 3-A and C), but the image in the diet with SBM group was primarily yellow, with some red and blue, at the same time points (Figs. 3-B and D). Given that blue/light blue, green/yellow, and orange/red represent low, medium, and high BMD, respectively, these results indicate that the BMD of peri-implant bone was higher in the diet with SBM group than in the diet without SBM group.

Quantitative real-time PCR assay

The qRT-PCR data are presented in Fig. 4. The Alp-expression level in the diet with SBM group at 2 weeks after implantation was 1.09 ± 0.23, approximately 2.2 times greater than that in the diet without SBM group (0.48 ± 0.23; between-group comparison, P < 0.05). The Alp-expression level in the diet with SBM group at 4 weeks after implantation was 1.69 ± 0.26, approximately 1.6 times greater than that in the diet with SBM group (0.64 ± 0.17; between-group comparison, P < 0.05). Both diet groups showed no statistically significant increases from 2 to 4 weeks after implantation (within-group comparisons, P < 0.05).

The col 1-expression level in the diet with SBM group at 2 weeks after implantation was 0.66 ± 0.25, approximately 1.4 times greater than that in the diet without SBM group (0.44 ± 0.12; between-group comparison, P < 0.05). The col 1-expression level in the diet with SBM group at 4 weeks after implantation was 1.32 ± 0.25, approximately 1.6 times greater than that in the diet with SBM group (0.59 ± 0.34; between-group comparison, P < 0.05). The diet with SBM group showed a statistically significant increase in col 1-expression level from 4 weeks after implantation (within-group comparisons, P < 0.05).

The ocn-expression level was not significantly higher in the diet with SBM group than in the diet without SBM group at 2 and 4 weeks after implantation (P < 0.05). Both diet groups showed no statistically significant increase in ocn-expression level from 2 to 4 weeks after implantation (within-group comparison, P < 0.05).

Fluorescence microscopy observation

Fluorescence microscopy imaging in the diet with SBM group demonstrated more green fluorescence (indicating bone formation) than that in the diet without SBM group at both 2 and 4 weeks after implantation (Fig. 5). As the rats aged from 2 to 4 weeks (Figs. 5 a and c, and 5 b and d, respectively), the green fluorescence became more pronounced in both diet groups.

Body weight

Body weight comparisons are given in Fig. 6. The body weights in both diet groups increased significantly over the feeding period (Fig. 6; Friedman test, P < 0.0001). The Mann-Whitney U test indicated no significant body weight difference between the groups at 2 (10 weeks of age) and 4 weeks (12 weeks of age) after implantation. * p < 0.05

Discussion

This animal study revealed that in a rat model of machined surface implants, dietary supplementation with SBM resulted in greater peri-implant BMD and pull-out strength in comparison with the results associated with a normal diet, suggesting that SBM could contribute to bone formation around implants. These findings highlight the potential of oral supplements to accelerate bone formation after machined surface dental implant surgery in patients. The rats that received SBM supplementation showed 9.9 and 3.4 times greater pull-out strength at 2 and 4 weeks after implantation, respectively, than those without SBM supplementation. Because the presence of SBM in the feed was the only difference in the intervention between the groups, the observed difference in pull-out strength might be attributable to the promotion of bone formation by SBM.

Given that the body weights of both groups significantly and steadily
increased during the feeding period, the rats seemed to grow normally. Some increased bone formation may be explained by the growth of the rats; however, similar growth curves were observed in both groups, suggesting that SBM worked mainly by reinforcing the bone, not by increasing body weight. To investigate the influences on pull-out strength in greater depth, BMD measurements, BMD color imaging data, and fluorescence microscopy observations were analyzed. BMD and pull-out strength in the diet with SBM group were about 1.2–1.1 times those in the diet without SBM group at both 2 and 4 weeks. As the degree of increase in pull-out strength and BMD were similar, the increase in BMD may have resulted in the increased pull-out strength.

The SBM-containing diet had seven times more Mg and 12 times more Zn than the diet without SBM, and also included F, which was absent from the control diet. Mg, Zn, and F play important roles in bone formation and resorption. Mg deficiency results in bone loss; Zn has been shown to reduce cathepsin and carbonic anhydrase mRNA expression, inhibiting osteoclast development; and F has been shown to promote osteoblast differentiation by increasing total collagen content and alkaline phosphatase activity. Mijares et al. stated that the mechanism underlying the effects of SBM may be attributable to the individual and combined effects of Mg, Zn, and F on bone cell activities such as bone formation and resorption when released from SBM or when incorporated into newly formed bone. These reports might explain why SBM-fed rats demonstrated greater BMD than those fed a normal diet.

BMD color images also indicated that new bone growth around the implants was greater in the diet with SBM group than in the diet without SBM group. Color imaging (Fig. 3B and D) visually suggested that the implants were surrounded and fixed more quickly in the diet with SBM group, likely resulting in the observed rapid increase in pull-out strength. This finding is consistent with the results of the fluorescence microscopy imaging (Fig. 5), which showed the dynamics of bone remodeling via the administration and subsequent incorporation of calcine fluorescent dye. This might also be one reason why osteoblast relative gene expression growth around the implants was greater in the diet with SBM group than in the diet without SBM group.

There is an ongoing effort to improve the interface between bone and implant in order to accelerate and improve the quality of osseointegration. However, once peri-implantitis occurs, the factors benefitting bone integration can turn into risk factors exacerbating inflammation around implant fixtures. SBM was originally developed for the prevention and treatment of osteoporosis. Therefore, from the perspective of implant treatment, the SBM supplement appears to be a promising subject for further study. Within its limitations, this study concludes that the dietary supplement SBM developed for osteoporosis was effective in accelerating peri-implant bone formation of machined surface implants during the healing period after implantation.

Acknowledgments
This work was supported by JSPS KAKENHI Grant Number 17K17220 and NIH NIAMS research grant no. R01 AR056208 (Y. Zhang). I would like to thank Professor Zhang of the Department of Biomaterials and Biomimetics, New York University College of Dentistry, New York, USA who provided the synthetic bone mineral.

Conflict of Interest
The authors declare no conflict of interest.

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