

## Bone Reactions around Pure Titanium Implants in Rats Fed Low-calcium Diet

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<Original Article>

## Bone Reactions around Pure Titanium Implants in Rats Fed Low-calcium Diet

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Key words: dental implant, rat, low-calcium diet, bone morphometric analysis

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### ABSTRACT

We investigated the bone response after placement of implants in the tibiae of Rats Fed Low-calcium Diet. Female Jcl-Wister rats at 14 weeks old were fed a low-calcium diet or a normal diet for 8 weeks, after which pure titanium implants were placed in the proximal metaphysis of the tibia. Animals were euthanized on days 7, 14, 28, 56, and 84 after implantation. Polished specimens without decalcification were prepared for histological observation. The same specimens were used to examine unit bone mass around the implant and the bone-implant contact ratio with a bone morphometric measuring system. In cortical bone, there was no significant difference in the ratio of bone-implant contact. However, in cancellous bone, the ratio in the experimental group tended to be lower than that in the control after day 56. In the experimental group, cancellous trabecula became porous, while the newly formed bone in contact with the implant was thin and the unit bone mass tended to be lower as compared to the control. Based on these findings, we concluded that attention should be given to porosity of the cancellous trabecula and associated reduction of the implant bearing capacity in patients with osteoporosis who undergo implant therapy.

### INTRODUCTION

Prosthetic treatment using dental implants is often performed in daily dental practice and the clinical performance has been reported to be favorable [1]. Such prosthetic treatment with an implant is often given when teeth are lost in middle-aged and older

patients, who are frequently complicated with various diseases. Among those, it is considered that osteoporosis, a bone metabolic disease, might have effects on the outcome of implant therapy.

Dao et al. [2] and Fujimoto et al. [3] studied the relationship between clinical implant performance and osteoporosis, and suggested that implant use is

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not always a risk factor for osseointegration. In a histological study of experimental animals, Motohashi et al. [4] and Yamazaki et al. [5] studied reactions in bone adjacent to implants placed in the tibiae of an osteopenic model of ovariectomized rats. According to their findings, there was no difference in bone contact ratio in cortical bone, whereas it was significantly decreased in cancellous bone. Meanwhile, Yamazaki et al. [5] found that the bone contact ratio in an experimental group was relatively lower than that in the controls 56 days after implant placement in the cortical bone. As for unit bone mass, both studies reported that the experimental groups showed significantly lower levels. In view of those findings, we prepared osteopenic rat models by feeding them with a low-calcium diet. An implant body was placed in the proximal metaphysis of tibia for more closely investigating the effects of osteoporosis on bone tissue around implants. Histological and morphometric analyses were performed.

## MATERIALS and METHODS

### *Experimental animals and implant body*

Hashiguchi et al. [6] measured bone mineral density in the lumbar spines of rats to study the effects of a low-calcium diet on bone metabolism. They reported that bone mineral density rapidly decreased for 4 weeks from immediately after the start of low-calcium feeding, whereas it scarcely changed after 4 weeks. In the present study, we considered that production of an osteopenic model with fully-advanced density decrease was necessary, thus implant placement was performed at 8 weeks after the start of low-calcium feeding.

For experimental animals, 50 female 10-week-old Jcl-Wistar rats with a body weight of approximately 200 g were acquired (CLEA, Japan Inc.). After rearing them with a normal diet until 14 weeks of age, they were divided into 2 groups of 25 rats each; the low-calcium diet group (experimental group), provided with a low-calcium diet (Ca: 0.02 g/100g, P: 0.55 g/100g, low-Ca MM-3, Oriental Yeast Co., Ltd.), and the normal diet group (control group) (Ca: 1.66 g/100g, P: 1.24 g/100g, MM-3, Oriental Yeast Co., Ltd). The implant body was placed in the proximal metaphysis of the tibia after the groups had been given their respective diets for 8 weeks (22 weeks of age). The diets were not changed until the end of the experiment. For the experimental implant, a pure titanium implant body with a cylindrical shape 1.0 mm in diameter and 2.0 mm in length (material: JIS grade II titanium wire, Platon Japan) was used (Fig. 1).

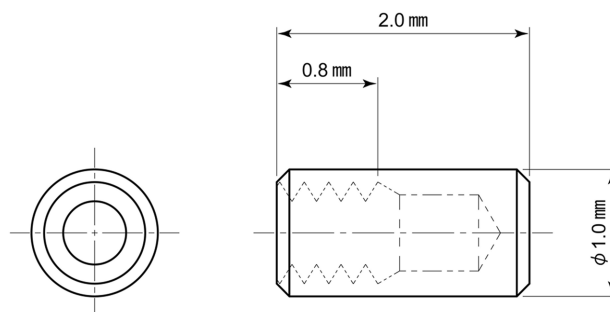


Fig. 1 Design of implant body.

### *Implant placement procedure*

A sterile implant body was placed in the proximal metaphysis of the tibia. For the surgery, the rats were put under general anesthesia by an intraperitoneal injection of 40 mg/kg of sodium pentobarbitone (Nembutal, Dai Nippon Sumitomo Pharma) after inhalation anesthesia with diethylether. After shaving hair off of the right lower leg, an incision was made to expose the proximal metaphysis of the tibia. While irrigating the inner bone surface about 10 mm distal from the knee joint with sterile physiological saline, a perforation was made using a #1/2 round bar. A socket was formed vertical to the cortical bone surface using #90 and #100 hand reamers, then the hole was rinsed with sterile physiological saline to remove bone fragments produced during drilling. The implant body was placed to make its upper edge aligned with the height of the superior edge of the cortical bone (Fig. 2, 3). After placement, the muscle and skin layers were sutured using resorbable thread (Vicryl 5-0, Johnson & Johnson, KK). After the surgery, 20 mg/kg of the antibiotic Flumarin (Shionogi & CO., Ltd., Japan) was administered by intramuscular injection for 3 days for infection control.

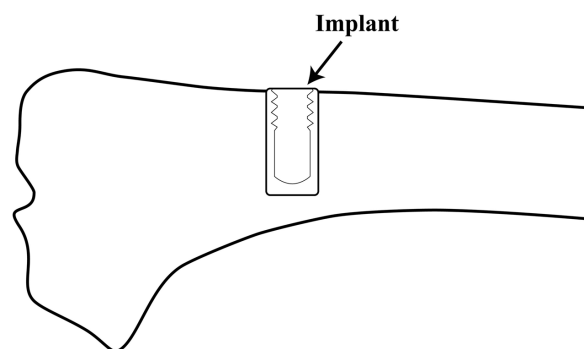


Fig. 2 Schematic diagram of implant surgery.

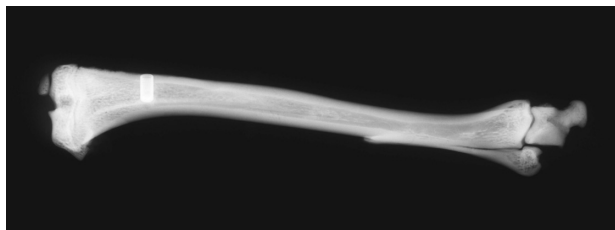


Fig. 3 Representative rat tibia from control group on day 28 after implant placement. Soft X-ray image taken in direction vertical to the long axis of the implant.

#### Preparation of specimens

Five rats in each group were euthanized and their tibias extracted on days 7, 14, 28, 56, and 85 after implant placement. The extracted bones were fixed, dehydrated, and embedded in MMA resin using an ordinary method. The specimens were ground to a thickness of  $50\ \mu\text{m}$  in the direction parallel to the long axis of the implant body using a grinder (EXAKT, Germany). After the removal of resin, each specimen was stained with toluidine blue for light microscopy and morphometry.

#### Bone morphometry

Specimens stained with toluidine blue were examined using a morphometric system (Histometry RT CAMERA, System Supply Co., Ltd., Japan), with the measuring area shown in Figure 4. The measuring area was set based on the method of Motohashi et

al. [4]. The cross-section was considered to be the plane parallel to the direction of the long axis of the implant body. The measuring points are shown below.

Points a, f: The points on the measured section where the vertical line drops from the uppermost margin of the cortical bone on both sides of the implant body and crosses the implant body.

Points b, e: The points where the vertical line drops from the uppermost margin of cancellous bone and crosses the implant body.

Points c, d: The lowermost edges of the implant body on the side of the medullary cavity.

Cortical bone area: The area corresponding to the implant body that reaches from point a to b, and from point e to f.

Cancellous bone area: The area corresponding to the implant body that reaches from point b to point e, via points c and d.

Morphometric determinations were made using the histological images for the following items.

Unit bone mass: The ratio (%) of calcified bone mass area ( $\mu\text{m}^2$ ) as compared to the medullary cavity tissue area ( $\mu\text{m}^2$ ) after subtracting the area of the implant.

Bone contact ratio: The ratio (%) occupied by the total length ( $\mu\text{m}$ ) of the implant surface in contact with the bone as compared to the circumferential length of the implant body ( $\mu\text{m}$ ) in the cortical and cancellous bone areas noted above.

Significant differences for the examined items between the groups were analyzed using Mann-Whitney's U test.

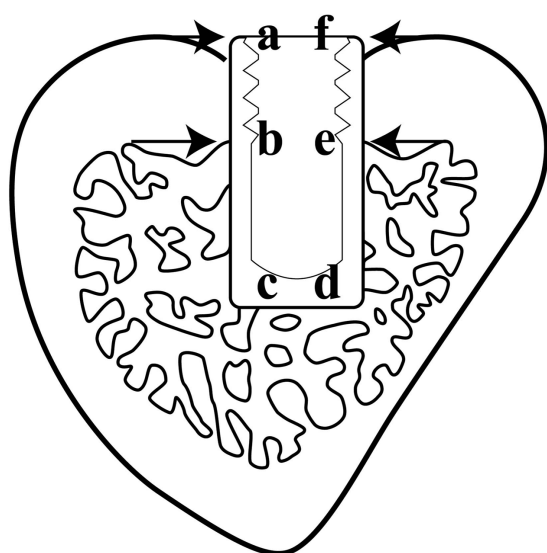


Fig. 4 Measuring area

Points a, f: Points where the vertical line drops from the uppermost margin of the cortical bone on both sides of the implant body and then crosses the implant body.

Points b, e: Points where the vertical line drops from the uppermost margin of cancellous bone to the implant body and then crosses the implant body.

Points c, d: The lowermost edges of the implant body on the side of the medullary cavity.

Cortical bone area: Area corresponding to the implant body ranging from point a to b, and from point e to f.

Cancellous bone area: Area corresponding to the implant body ranging from point b to e via points c and d.

## RESULTS

Postoperative infection was not observed in any of the rats, and there were no significant differences in regard to body weights between the experimental and control groups.

## PATHOHISTOLOGICAL FINDINGS

### *Control group (normal diet)*

#### (1)Day 7

A large number of fragments possibly produced at the time of implant placement were observed in the bone marrow. Thin and immature newly-formed bone was in contact with some parts of the implant surface. Abundant cancellous trabeculae were observed in the medullary cavity in comparison to the experimental group (Fig. 5-a).

#### (2)Day 14

Immature newly-formed bone without a lamellar structure was observed along the implant surface. The

thickness and quantity of the bone was increased more than on day 7. The quantity of cancellous bone was also increased (Fig. 5-b).

#### (3)Day 28

The findings were similar to those on day 14 (Fig. 5-c).

#### (4)Day 56

Newly-formed bone was in contact with most parts of the implant circumference. The bone was increased in thickness and a lamellar structure was observed in all of the specimens. The thickness of the newly-formed bone around the implant was increased and calcification had advanced more than that in the experimental group (Fig. 5-d).

#### (5)Day 84

The findings were similar to those on day 56, with lamellar bone observed in most parts around the implant. In the medullary cavity, the trabeculae were indistinct. The medullary cavity was replaced partly by proliferating newly-formed bone (Fig. 5-e).

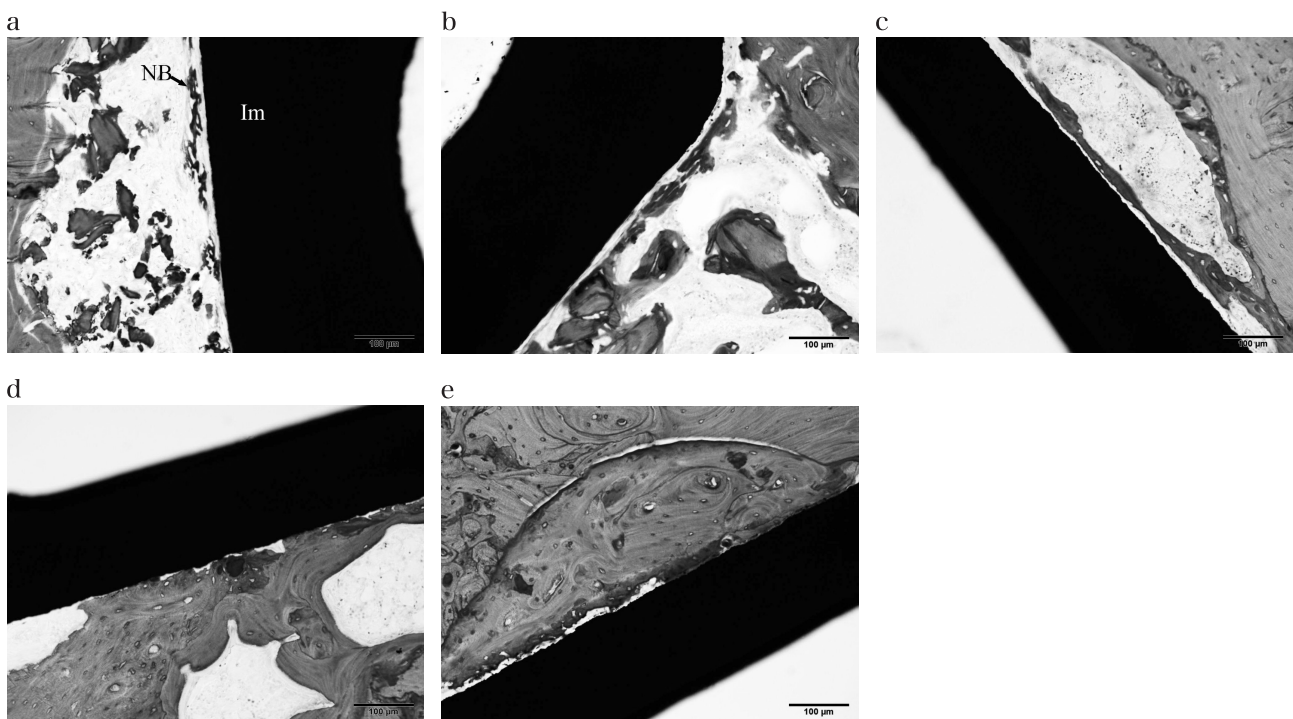


Fig. 5 Control group (Toluidine blue stain, x200.)

a: 7 days. Thin and immature newly formed bone was partly in contact with the implant surface. (NB: New Born, Im: Implant)

b: 14 days. Immature new bone without a lamellar structure was formed along the implant surface.

c: 28 days. The findings were similar to those on day 14.

d: 56 days. Newly formed bone was in contact nearly all around the implant. The newly formed bone had increased thickness and showed a lamellar structure.

e: 84 days. Newly formed bone with a lamellar structure nearly surrounded the implant. The medullary cavity was partially replaced by proliferating newly formed bone.



**Experimental group (low-Ca diet)****(1)Day 7**

Bone fragments possibly produced at the time of implant placement were observed in the bone marrow. Thin and immature newly-formed bone was in contact with some parts of the implant surface. The cancellous bone in the medullary cavity was thinner and more porous than that of the control group (Fig. 6-a).

**(2)Day 14**

Newly-formed trabecular bone was produced from the inner surface of cortical bone and trabecula of cancellous bone. This newly-formed bone was immature without a lamellar structure and had formed along the implant surface. The thickness and quantity of the newly-formed bone was increased in comparison to day 7 (Fig. 6-b).

**(3)Day 28**

The findings were similar to those on day 14. However, the ratio of newly-formed bone contacting the implant surface was increased (Fig. 6-c).

**(4)Day 56**

Newly-formed bone was in contact with most parts of the implant circumference. The bone was increased in thickness. In some specimens, the bone had a lamellar structure, while in others the bone remained relatively immature without a lamellar structure around the implant (Fig. 6-d). The existing trabecula of the cancellous bone was decreased and becoming porous.

**(5)Day 84**

The findings were similar to those on day 56. In all specimens, newly-formed lamellar bone was observed in most parts around the implant (Fig. 6-e). The existing trabecula of cancellous bone was further decreased and porosity was also increased.

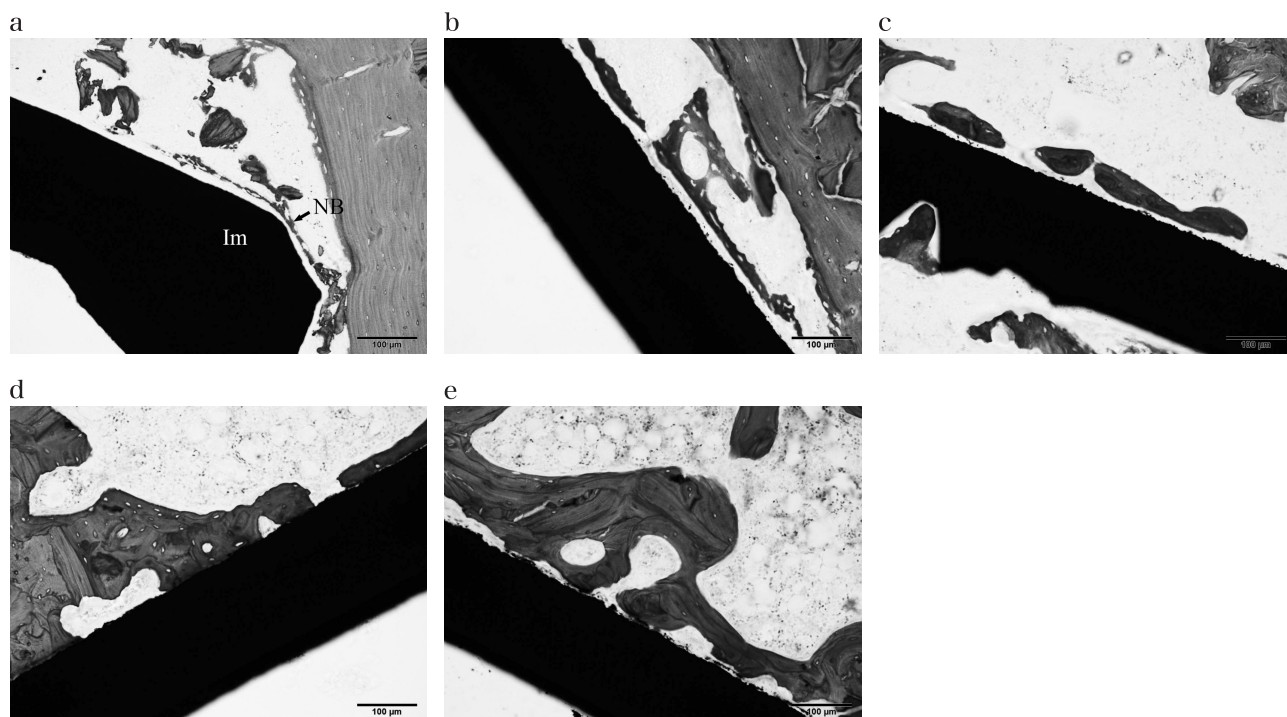


Fig. 6 Experimental group (Toluidine blue stain, x200.)

a: 7 days. Thin and immature newly formed bone are shown in partial contact with the implant surface. Cancellous bone in the medullary cavity was thinner and more porous than that of the control. (NB: New Born, Im: Implant)

b: 14 days. New trabecula was formed from the inner surface of the cortical bone and cancellous trabecula. New bone was formed along the implant surface.

c: 28 days. The ratio of newly formed bone contacting the implant surface was increased.

d: 56 days. In some specimens, a lamellar structure was observed in the newly formed bone around the implant. However, newly formed bone was relatively immature and lacking a lamellar structure in others.

e: 84 days. Newly formed bone with a lamellar structure was observed nearly surrounding the implant.

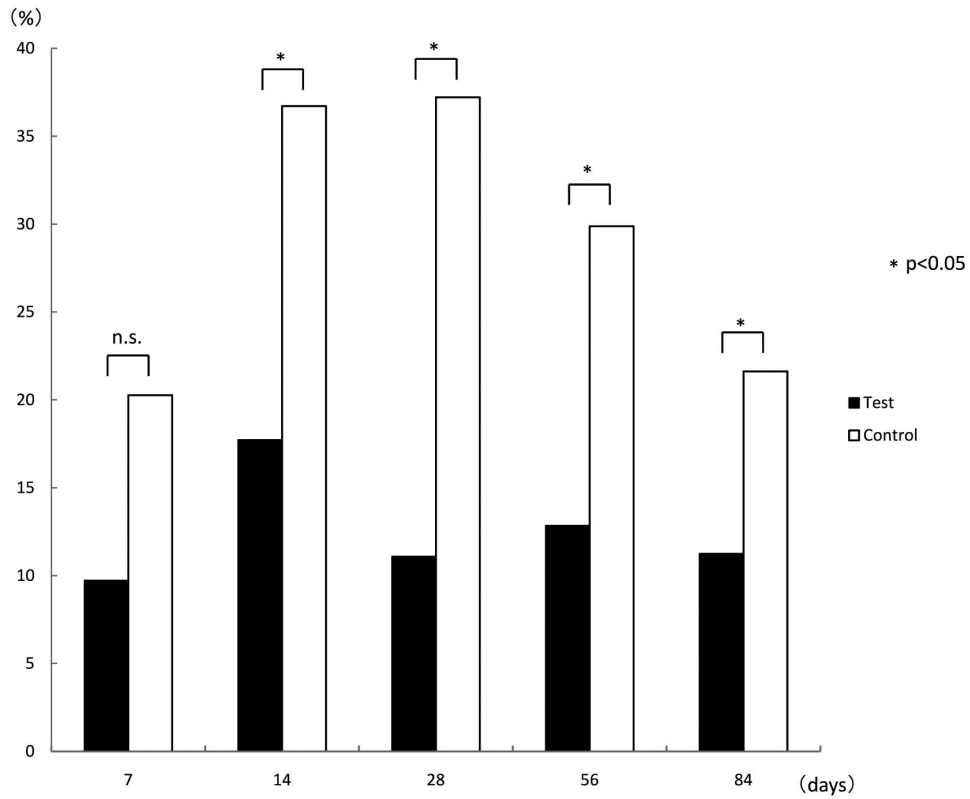


Fig. 7 Unit bone mass (%).

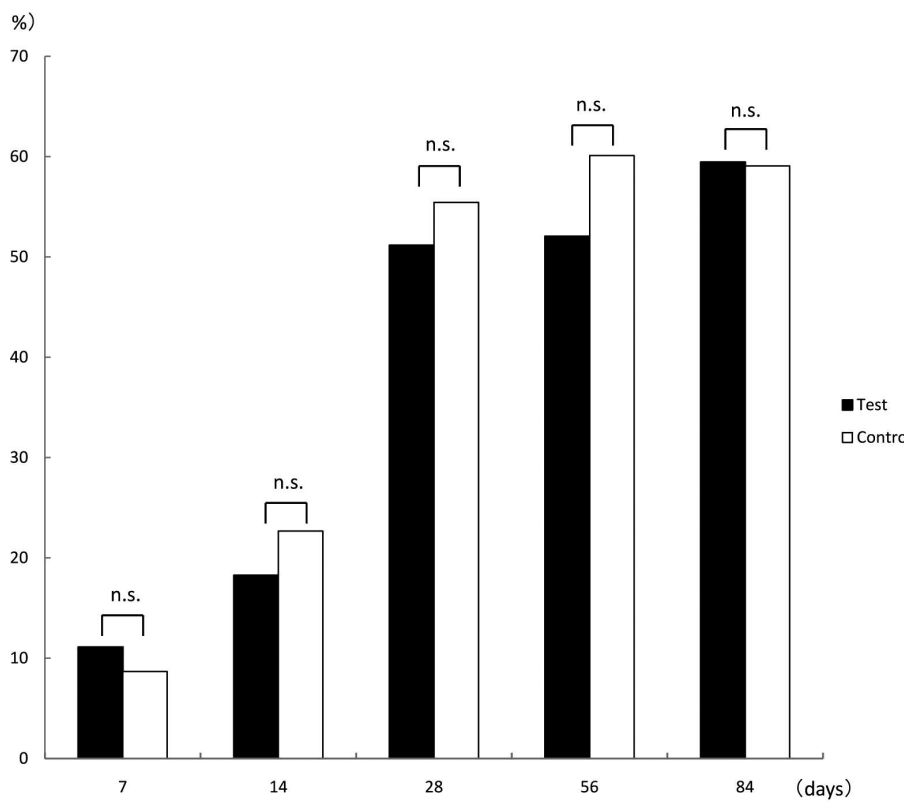


Fig. 8 Bone contact ratio (%) for cortical bone.

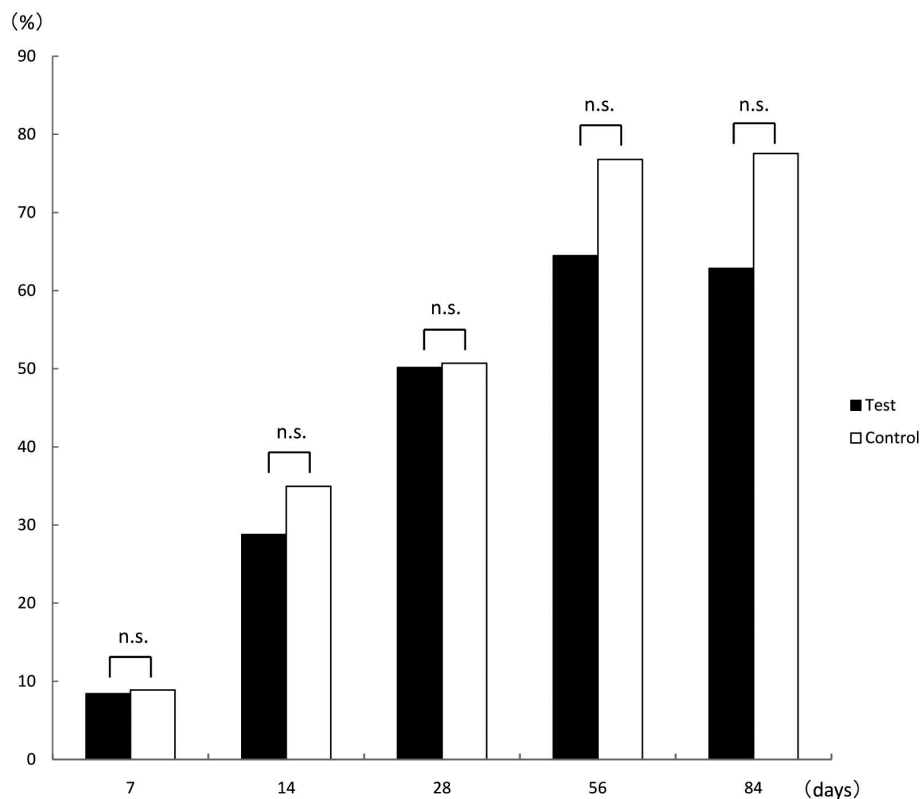


Fig. 9 Bone contact ratio (%) for cancellous bone.

## BONE MORPHOMETRY

### *Unit bone mass*

In the control group, the mean unit bone mass on day 7 was 20.3%, after which it increased to 36.7% on day 14 and continued to increase slightly until a maximum of 37.2% on day 28. Thereafter, it declined to 21.6% by day 84. In the experimental group, the mean unit bone mass on day 7 was 9.7%, after which it reached a maximum of 17.7% on day 14 and decreased to 11.2% by day 84. In both groups, bone mass was sharply increased on day 14. In the control group, bone mass reached the maximum on day 28, while that was reached on day 14 in the experimental group. Thereafter, the levels became lower in both groups. Unit bone mass on postoperative day 7 tended to be lower in the experimental group than the control, though no significant difference was observed. Unit bone mass after day 14 was significantly lower in the experimental group ( $p < 0.05$ ). (Fig. 7).

### *Bone contact ratio*

The bone contact ratio for cortical bone increased over time until day 28 in both groups and then did not fluctuate much thereafter. Moreover, no signifi-

cant difference was observed in the ratio between the groups (Fig. 8). That ratio for cancellous bone also increased over time until day 56 in both groups and did not fluctuate much thereafter. No significant difference was observed in the ratio between the groups. However, the ratio in the experimental group tended to be lower than that in the control group after day 56 (Fig. 9).

## DISCUSSION

Tooth loss in adults can be attributed to caries, periodontal disease, and trauma. When tooth loss occurs, prosthetic treatments with a bridge, artificial denture, or implant are generally performed to restore eating function. Long-term stable prognoses have been reported following prosthetic treatments with dental implants [7,8]. Furthermore, most patients who undergo such treatments are highly satisfied with the results, thus the demand for implant therapy is increasing. A greater number of elderly as compared to younger patients experience tooth loss and are frequently afflicted with other various diseases, among which osteoporosis is one of the principal complications. In Japan, about 10% of the total population or 10 million



patients are estimated to be affected by osteoporosis [9]. With the advent of an aging society, it is anticipated that implant therapy for restoring mastication function in osteoporosis patients will show an increasing trend. Accordingly, in the present study, we investigated the reaction of bone tissue around pure titanium implants to elucidate the conditions that occur in patients with osteoporosis who undergo implant therapy.

For preparing osteopenic rat models, low-Ca feeding [10], ovariectomy [11] and immobilization by neurectomy [12] procedures have been reported. In the present rat experiments, we prepared an osteopenic model by low-Ca feeding for the purpose of alleviating stress on the animals, as they also underwent surgical implant placement. Hashiguchi et al. [6] determined bone mineral density of the lumbar spine in their study of the effects of a low-Ca diet on bone metabolism. They reported that bone density sharply decreased during the 4 weeks after the start of low-Ca feeding and then scarcely changed after week 4. Accordingly, in the present study we set the feeding period as 8 weeks, as our aim was to test rats with fully advanced bone density reduction as an osteopenic model.

In a study of bone reactions around implant bodies in an ovariectomized osteopenic rat model, Motohashi et al. [4] reported that the bone contact ratio for cortical bone was not different as compared to the control, whereas it was significantly decreased in cancellous bone. Meanwhile, Yamazaki et al. [5] reported that the bone contact ratio in cortical bone was lower at 56 days after implant placement in the experimental group as compared to the control group. Moreover, for unit bone mass, both studies found that it was significantly lower in the experimental group. Amano et al. [10] also observed bone reactions around implant bodies in their experimental rats fed with a low-Ca diet for a short period of 7 days before implant placement in the mandible, with a low-Ca diet given continuously after surgery. They reported that the density in the trabecula of the existing cancellous bone declined drastically and that the degree of calcification was also reduced in the experimental group. Furthermore, while the density in the trabecula around the implant body was reduced to about the half in the experimental group as compared to the standard diet group, a layer of newly-formed thin bone was also present and in contact with the implant body. Watanabe [13] used a pathohistological method to observe alveolar bone after 3 weeks of a calcium deficient diet in rats and reported that bone resorption was observed throughout the alveolar bone, bone marrow had expanded, and the reduction in amount of trabecula was

marked. However, there is no known study that observed bone reactions in rats with advanced bone mass reduction induced by long-term feeding of a low-Ca diet before implant placement.

In the present study, low-calcium feeding in the experimental group resulted in a reduction of bone mass, while unit bone mass tended to be lower than that in the control throughout the observation period. In contrast, the bone contact ratio around the implant body by cortical bone was not significantly different between the experimental and control groups. However, with cancellous bone, while no significant difference was observed until day 28, the contact ratio in the experimental group tended to be lower than that in the control on day 56 and 84.

In other studies of the reactions of cancellous bone around an implant body in the tibias of rats, it was reported that immature newly-formed trabecula observed at the early stage after implant placement changed to mature trabecula with a lamellar structure, while overall porosity increased [14,15,16]. It was also noted that these changes might be completed around postoperative day 56. In the present experiment, the bone contact ratio in the cancellous bone reached nearly maximum on day 56 in both the experimental and control groups.

Our histological findings for cortical bone in both groups showed that the implant body surface was in close contact with newly-formed lamellar bone that developed from the existing cortical bone. As for cancellous bone, active osteogenesis was observed over all of the trabecular surface around the implant body in both groups. However, the existing cancellous trabecula and newly-formed trabecula were resorbed over time to become porous. As a result, the thickness of the newly-formed bone around the implant body was less in the experimental group than in the control group.

Liu et al. [17] studied resorption of alveolar bone in rats fed a Ca-deficient diet by determining changes in number of osteoclasts and reported that osteoclasts emerged markedly on the medullary side of the alveolar bone. Otani et al. [18] also reported regarding the progress of alveolar bone resorption in rats fed a Ca-deficient diet. They found that changes in osseous tissues due to a Ca-deficient diet were not developed by inhibition of osteogenesis, but rather because of an increase in bone resorption. Concerning the bone formation and resorption biomarkers, Hashiguchi et al. [6] observed the blood bone metabolism biomarkers and the urinary bone metabolism biomarkers in rats fed low-calcium diet. According to their findings, they reported that bone formation was not decreased and bone resorption was significantly increased in the low-

calcium diet group compared to the control group.

In the present experiment, our histological findings showed that osteogenesis around the implant occurred in a similar manner in the experimental and control groups. However, the cancellous trabecula around the implant became porous and the thickness of the newly formed bone was thinner in the experimental group. Moreover, bone morphometric analysis revealed that unit bone mass in the experimental group tended to decline throughout the experimental period. In contrast, there was no significant difference in bone contact ratio for cortical bone between the groups. However, with cancellous bone, while no significant difference was observed until day 28, the contact ratio in the experimental group tended to be lower than that in the control after day 56. Also, osteogenesis toward the implant body progressed in a similar manner in both groups, which led to the finding of no difference in bone contact ratio for cortical bone. In the experimental group, osteogenesis did not decrease due to the low-calcium diet feeding, while porosity of the existing cancellous bone and bone resorption of the newly formed trabecula progressed further as bone resorption increased. Therefore, the trabecula was thinner as compared to that of the control, which resulted in decreased unit bone mass and reduced newly-formed bone in cancellous bone area contacting the implant body in the experimental group.

For a favorable outcome after implant treatment, it is necessary for the implant body to directly contact the bone without involvement of soft tissue, so as to become firmly fixed in the bone and for maintaining the condition [1,19]. Accordingly, the outcome seems to be greatly affected by the condition of osseous tissue around the implant. As for the reactions of bone tissue between maxillomandibular bone and the implant body in patients with osteoporosis, the pathology of osteoporosis in human maxillomandibular bones must be further studied. Nevertheless, based on the present findings, we concluded that implant bearing capacity is likely to decline in patients with osteoporosis, as the bone contact ratio of cancellous bone tended to be lower than that in the control group, and the unit bone mass decreased and the newly formed cancellous bone was thin in the present osteoporosis model.

## CONCLUSION

We prepared an osteopenic rat model by feeding with a low-Ca diet. In those and control rats, an implant body was placed into the proximal metaphysis of tibia and we investigated the reaction of the bone around the implant body. Our results included

the following.

(1) In rats fed the low-Ca diet, cancellous trabecula around the implant body became porous and the contacting newly formed bone was thin. Also, the unit bone mass of the experimental group tended to be lower than that of the control group throughout the experimental period.

(2) There was no evident effect of the low-Ca diet on the bone-implant contact ratios of the cortical bone.

(3) As for the ratio of contact between cancellous bone and the implant body, a reduction in bone contact caused by the low-Ca diet tended to be observed.

Based on these findings, we concluded that attention should be given to porosity of the cancellous trabecula and associated reduction of the implant bearing capacity in patients with osteoporosis who undergo implant therapy.

The present study was performed in accordance with the Guidelines for Animal Experimentation of Osaka Medical College under the approval of the Committee of Animal Experimentation.

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