**In vitro** investigation of the cell compatibility and antibacterial properties of titanium treated with calcium and ozone

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The purpose of this study was to evaluate the surface modification of calcium ions on roughened titanium as a surface treatment of dental implants for cell attachment, growth, and initial bacterial adhesion. When a surface-roughened, pure titanium disk was immersed in a calcium chloride solution (100 mM) containing 20 ppm ozone for 24 h at 25°C, calcium was detected on the surface by X-ray photoelectron spectroscopy. The calcium-modified, roughened titanium disk had a significantly greater concentration of the initially adhered cells as well as cells cultured over 7 days compared with titanium disks without surface modification. Furthermore, the initial bacterial adhesion on the calcium-ozone treated titanium disk was statistically less than on a pure titanium disk or titanium disk treated without ozone. Dissolved ozone was useful for modifying the surface of roughened titanium with calcium ions and the surface modification may be applicable for dental implants.

**Keywords:** Titanium implant, Surface modification, Cell compatibility, Antibacterial property

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**INTRODUCTION**

Titanium is widely used clinically for dental implants as it features corrosion resistance, high strength, biocompatibility, and osseointegration. In 1952, Brånemark first suggested osseointegration as an important concept for the bonding of titanium with surrounding osseous tissue. Titanium was initially considered to bond directly with surrounding bone tissue. However, electron microscopic studies revealed that fibrous connective tissue partially intermediated the interface between titanium and bone. As a result, several studies have attempted to enhance the binding between metal titanium implants and surrounding bone tissue, and the approaches can be roughly divided into physical and chemical.

In a physical approach, binding is enhanced by increasing the contact area of the implant surface with surrounding bone by surface roughening of the implant for mechanical engagement. Surface roughening is commonly performed by acid or blast treatments and the rate of osseointegration acquisition is increased by these treatments. For a chemical approach, osteoconductive materials, such as hydroxyapatite, are used. Transmission electron microscopy has been used to show that hydroxyapatite has osteoconductivity by directly binding with bone without mediation of fibrous connective tissue. As a result, dental implants made of titanium coated with hydroxyapatite by plasma spraying are available, though problems have been identified such as exfoliation and breaking of the coating layer. Apatite can be a location for bacterial growth. As a result, it is necessary to explore other methods to enhance the binding between titanium and bone.

As apatite spontaneously precipitates on the surface following implant placement, recent studies have been conducted to attain direct binding with bone early by surface treatment of titanium for osteoconductivity. Kim et al. reported an alkali heat treatment method and Ohtsuki et al. reported a hydrogen peroxide treatment. However, the surface structure of titanium is markedly changed by these osteoconductivity treatments as each produces a thermochemical reaction on the surface. As previously noted, commercially available dental implants have a roughened surface. Accordingly, it is advantageous to attain osteoconductivity without changing the roughened structure and we have focused on a calcium surface modification method reported by Ishikawa et al. However, it has not been reported whether osteoconductivity treatment by calcium surface modification is applicable to roughened-surface titanium, a mainstream clinical material. In addition, dental implants of calcium-modified titanium have not been investigated despite being a property required for application to dental implants.

The present study was performed to determine if a
calcium–ozone treatment can be adopted for surface-roughened titanium. Additionally, the cell compatibility and antibacterial property of the dental implant were examined.

MATERIALS AND METHODS
Surface modification of roughened titanium with calcium
Disk-shaped, commercially pure titanium (Grade 4, 99.5% purity, 14 mm in diameter, 1 mm in thickness) was prepared. The surface of the disks was initially roughened by GC, Tokyo, Japan using sandblasting followed by acid etching, which is commonly used for commercially available implants. After degreasing with acetone followed by rinsing with ultrapure water, the disks were used as the experimental substrates. Surface modification without changing the macroscopic roughened structure was performed according to our previous report. For ozone (O₃) gas production, an ozone generator (ED-OG-R4, Eco Design, Saitama, Japan) was used. O₃ gas was generated from oxygen (O₂) gas by a silent discharge method under conditions of an output of 3.4 A and an oxygen flow rate of 2 nL/min. The generated O₃ gas was dissolved by bubbling in an aqueous solution of 100 mmol/L calcium chloride (FUJIFILM Wako Pure Chemical, Osaka, Japan) at 25°C. After 1 h, the concentration of ozone was maintained at approximately 20 ppm as long as bubbling was continued. The surface-roughened titanium disks were immersed in a calcium chloride solution (100 mM) containing 20 ppm of dissolved ozone at 25°C for 24 h and the resulting disks were denoted as Ca-O₃-Ti. For a control, surface-roughened titanium disks without treatment were also used to treat titanium disks and were denoted as Ti. For comparison, a calcium chloride solution without dissolved O₃ and an aqueous solution with dissolved O₃ without calcium chloride were prepared and denoted as Ca-Ti and O₃-Ti, respectively.

Morphological observations by scanning electron microscopy
The morphological surface structure of the roughened Ti disks before (Ti disk) and after treatment with different conditions (Ca-Ti, O₃-Ti, and Ca-O₃-Ti disks) was observed using a scanning electron microscope (SEM) (S-3400N, Hitachi High-Technologies, Tokyo, Japan). After gold-palladium coating by a magnetron sputtering device (MSP-1s, Vacuum Device, Ibaraki, Japan), the specimens were observed at an accelerating voltage of 10 kV.

Determination of the surface roughness by 3D laser microscopy
The surface roughness (Sa) of the roughened Ti disks before and after treatment with different conditions were evaluated using a 3D laser microscope (VK-9710, KEYENCE, Osaka, Japan). The Sa (μm) was calculated using VK analysis software (KEYENCE) associated with a 3D laser microscope. The mean and standard deviation (SD) were calculated from six different 50 μm squares at random.

Surface element analysis by X-ray photoelectron spectroscopy
Elemental analysis of the roughened Ti disks before and after treatment with different conditions was performed using a X-ray photoelectron microscope (XPS) (K-alpha, Thermo Fisher Scientific, East Grinstead, UK). Determinations were performed under a vacuum of 1.4×10⁻⁹ Pa using monochromatic X-ray (AlKα) as the radiation source with an irradiation spot size of 400 μm. The binding energy was corrected with the Cls peak detected at 285.0 eV.

Evaluation of the hydrophilicity
The static contact angle of distilled water was measured using an automatic contact angle measuring device (DM500, Kyowa Interface Science, Saitama, Japan). The test was performed using six specimens from each group and the data were presented as mean±SD.

Cell cultures
A mouse calvarium-derived osteoblast-like cell line, MC3T3-E1, was used for the cell culture study. Cells were cultured in Dulbecco's modified Eagle medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (Biowes, Nuaillé, France), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma) (referred to as cell growth medium) in 5% CO₂ at 37°C. For subculturing, 0.05% trypsin- ethylenediaminetetraacetic acid (Sigma) was used.

Initial cell adhesion and longitudinal cell growth
After sterilization with 70% ethanol and washing with pure water, the specimens were statically incubated in a 24-well cell culture plate (Becton Dickinson, Franklin Lakes, NJ, USA). MC3T3-E1 cells were scraped from the plate at room temperature to prepare cell suspensions of 50×10⁴ cells/mL and 1×10⁵ cells/mL. Cells were then transferred to the surface of each specimen at a surface density of 3.2×10²⁴ cells/cm² for evaluation of the initial cell adhesion or 6.4×10 cells/cm² for evaluation of cell growth, and cultured in the presence of 5% CO₂ at 37°C for 3 h, 1, 3, 5, or 7 days. Following 0.05% trypsin treatment, the cells were stained with 0.4% Trypan Blue solution (Sigma). The number of living cells was determined using a Bürker-Türk counting chamber (Erma, Tokyo, Japan). These tests were performed using five specimens from each group and the data were presented as mean±SD.

Morphological observations of the initial cell adhesion by SEM
After incubation for 3 h, the specimens were washed with PBS (−) and immediately prefixed in 2.5% glutaraldehyde (Fujifilm Wako) at 4°C for 2 h. Next, using 1% osmium tetroxide (Sigma), the specimens were fixed at 4°C for 1 h. The disks were subsequently dehydrated using t-butanol/distilled water mixtures (50/50, 70/30, 90/10, 95/5, 99/1, 100/0) for 10 min each.
Dehydrated disks were dried using critical point drying overnight (JCPD-5, JEOL, Tokyo, Japan). The disks were observed using SEM.

**Bacterial culture**
For determining the bacterial species, *Staphylococcus aureus* (*S. aureus*), a gram-positive coccus, and *Escherichia coli* (*E. coli*), a gram-negative bacillus, were selected as they are frequently used in the evaluation of bacterial adhesion on titanium\(^{16,17}\). After culturing in Brain Heart Infusion media aerobically for 18 h, the bacterial concentration was adjusted to 10\(^7\) CFU/mL.

**ATP determination of the adhered bacteria**
Each disk was sterilized with 70% ethanol and rinsed with super pure water, then placed statically in a 12-well cell culture plate (Becton Dickinson). One milliliter of bacterial suspension was transferred to each dish. After culturing for 3 h, the dishes were transferred to another plate and loosely adhered bacteria were removed with PBS (-). After adding 500 μL of the adenosine triphosphate (ATP) extraction reagent solution (ATP extraction reagent kit for microbe use, AF2K1, TOA DKK, Tokyo, Japan) to each specimen, ATP was extracted over a specific period of time. Finally, luminescent reagent (Luminescent reagent kit, AF-ZL1, TOA DKK) was added and the ATP value, an indicator of the number of bacteria, was determined using an ATP analyzer (Luminescensor, ATTO, Tokyo, Japan)\(^{18}\). These tests were performed using five specimens from each group and the data were presented as mean±SD.

**Statistical analysis**
Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by post hoc analysis by Tukey’s multiple comparison tests. For the calculation, BellCurve for Excel (Social Survey Research Information, Tokyo, Japan) was used. Differences were considered statistically significant at \(p<0.05\) and \(p<0.01\).

**RESULTS**

**Surface modification and characterization**
Figure 1 shows the SEM images of surface-roughened titanium disks before (Ti) and after treatment with different conditions (Ca-Ti, O\(_2\)-Ti, and Ca-O\(_2\)-Ti). The Ti disks have a microstructured rough surface because of the sandblasting and acid etching. A macroscopic topological change was not observed even after treatment with calcium chloride solution (Ca-Ti), the O\(_2\)-dissolved aqueous solution (O\(_2\)-Ti), or O\(_2\)-dissolved calcium chloride solution (Ca-O\(_2\)-Ti). The surface roughness (Ra) and water contact angle values on Ti, Ca-Ti, O\(_2\)-Ti, and Ca-O\(_2\)-Ti disks are summarized in Table 1. No statistically significant differences regarding Ra values were observed among the groups, regardless of treatment. Alternatively, the water contact angles on O\(_2\)-treated and Ca-O\(_2\)-treated Ti disks (O\(_2\)-Ti and Ca-O\(_2\)-Ti) were significantly less than those on untreated and Ca-treated Ti disks (Ti and Ca-Ti). Both O\(_2\)-Ti and Ca-O\(_2\)-Ti were notably super hydrophilic surfaces. Figure 2 shows an enlarged view (340–360 eV) of the XPS profiles of the surface-roughened titanium disks before (Ti disk) and after treatment with different conditions (Ca-Ti, O\(_2\)-Ti, and Ca-O\(_2\)-Ti disks). Ca2p doublets were only detected on Ca-O\(_2\)-treated Ti (Ca-O\(_2\)-Ti) disks. Ca was not detected on the Ca-treated Ti (Ca-Ti) disks even though Ca ions existed in the treatment solution.

![SEM images of the surface-roughened titanium disk before (Ti disk) and after treatment with different conditions (Ca-Ti, O\(_2\)-Ti, and Ca-O\(_2\)-Ti disks).](image)

| Sample code | Sa (μm) | Contact angle (º) |
|-------------|---------|-------------------|
| Ti          | 4.0±1.1 | 99±2              |
| Ca-Ti       | 3.1±0.6 | 101±3             |
| O\(_2\)-Ti  | 3.2±0.7 | 0±0**             |
| Ca-O\(_2\)-Ti | 3.3±0.5 | 0±0**             |

\(^{**p<0.01\ vs\ Ti\ and\ Ca-Ti\}
See text for the sample code.
Fig. 2 Enlarged view (340–360 eV) of the XPS profiles of surface-roughened titanium disk before (Ti disk) and after treatment with different conditions (Ca-Ti, O₃-Ti, and Ca-O₃-Ti disks).

Fig. 3 Number of MC3T3E1 cells adhered to Ti, Ca-Ti, O₃-Ti, and Ca-O₃-Ti disks after 3 h of incubation, **p<0.01.

Evaluation of the cell compatibility
Figure 3 shows the number of adhered MC3T3E1 cells on Ti, Ca-Ti, O₃-Ti, and Ca-O₃-Ti disks after incubation for 3 h. The number of initially adhered cells was significantly greater on the Ca-O₃-Ti disks compared with the Ti, Ca-Ti, and O₃-Ti disks (p<0.01). Figure 4 shows the SEM images of MC3T3E1 cells adhered to the Ti, Ca-Ti, O₃-Ti, and Ca-O₃-Ti disks after 3 h of incubation. There were more stretched cells observed on the Ca-O₃-Ti disks than the Ti, Ca-Ti, and O₃-Ti disks. Figure 5 shows the changes in the number of cells adhered to Ti, Ca-Ti, O₃-Ti, and Ca-O₃-Ti disks over 7 days. Regardless of the incubation period, the number of adhered cells on the Ca-O₃-Ti disks was significantly greater than that on the Ti, Ca-Ti, O₃-Ti, and Ca-O₃-Ti disks (p<0.01). On Day 7, the number of adhered cells on the O₃-Ti disks was significantly greater than that on the Ti and Ca-Ti disks (p<0.01). Furthermore, the number of adhered cells on the Ca-Ti disks was significantly greater than that on the Ti disks (p<0.05).

Evaluation of the antibacterial property
Figure 6 shows the ATP values of adhered bacteria (S. aureus) on Ti, Ca-Ti, O₃-Ti, and Ca-O₃-Ti disks after 3 h of incubation. The ATP values from cells on the Ca-O₃-Ti disks was significantly less than the Ti and Ca-Ti (p<0.05). There was no significant difference between the O₃-Ti and Ca-O₃-Ti disks. Figure 7 shows the ATP values of the adhered bacteria (E. coli) on Ti, Ca-Ti, O₃-Ti, and Ca-O₃-Ti disks after 3 h of incubation. The ATP level on the Ca-O₃-Ti disks was significantly less than on the Ti (p<0.01), Ca-Ti (p<0.01), and O₃-Ti (p<0.05) disks.
DISCUSSION

Results obtained from observations of the morphological surface structure and analysis of the surface roughness indicated that the Ca-O₃ treatment did not affect the roughened surface structure of the Ti disks (Fig. 1 and Table 1). As the roughened surface structure is important for acquiring strong fixation at an implanted site, the Ca-O₃ treatment could be a candidate for the surface treatment of commercial dental implants with macroscopic surface morphology.

XPS elemental analysis revealed that calcium was detected on the Ca-O₃-treated Ti disks whereas no calcium was detected on the Ca-treated Ti disks (Fig. 2), which indicates that the dissolved O₃ was effective for immobilization of calcium on the roughened Ti disks. Calcium ions interact with the negatively charged hydroxyl groups generated on Ti disks after Ca-O₃-treatment. However, there are no reports regarding the generation of hydroxyl groups on Ti by O₃-treatment. Although we attempted to examine the XPS O1s spectra of the Ti disks before and after Ca-O₃-treatment, no significant evidence of the generation of hydroxyl groups was observed (data not shown). Use of the surface-roughened Ti disks was considered to be one of the reasons for not obtaining a consistent O1s spectra. For quantitative analysis by XPS, a mirror polished surface is much better than a rough surface in principle. Therefore, another basic study regarding the generation of hydroxyl groups using a mirror polished Ti disk is necessary. Surface-roughened Ti became hydrophilic after treatment with O₃ (Table 1). Decomposed organic substances on the Ti surface, such as hydrocarbons, might contribute to the hydrophilicity because of the O₃-treatment. Although we tried to examine the XPS C1s spectra of the Ti disks before and after Ca-O₃-treatment, we could not obtain a consistent result because of the rough surface. Moreover, generation of hydroxyl groups on the Ti disks might also affect the hydrophilicity.

A method used to investigate the spontaneous precipitation of apatite in simulated body fluid has been reported for the in vitro evaluation of the osteoconductivity of materials. However, the previously reported results show that β-calcium phosphate (β-TCP), which does not precipitate apatite in simulated body fluid, promotes osteoconductivity. Accordingly, it is difficult to accurately evaluate osteoconductivity using only simulated body fluid. It is well known that calcium, employed in the present study in an attempt to modify the titanium surface, functions in association with proteins involved in cell adhesion. Accordingly, we evaluated the in vitro osteoconductivity using osteoblasts. On the surface of the Ca-O₃-treated titanium (Ca-O₃-Ti) disks, the initial cell adhesion and state of pseudopod stretching was significantly greater than cells on the other disks (Figs. 3 and 4). Furthermore, Ca-O₃-Ti disks had a significantly greater longitudinal cell count than cells on the other disks (Fig. 5). Thus, we concluded that calcium (Fig. 2), present only on the surface of the Ca-O₃-Ti disks, contributed to cell adhesion. Anselme et al. reported that calcium ions contributed to adhesion and proliferation of osteoblasts by involvement in the intercellular communication mediated by receptor activation on the surface of osteoblasts and cell conjugation. The present results demonstrate that Ca ions modified on a Ti surface effectively aided adhesion between the material and cells.

The antibacterial property of the titanium surface is an important issue for implant surface treatment. In our previous study, we found that bacteria was likely to be mixed in collected autologous bone, even when sufficient care was taken with an aseptic technique. As a result, attention must be paid to bacterial adhesion during implant surgery. Therefore, treatments that prevent a possible increase in bacterial adhesion while increasing osteoblast adhesion on a titanium surface are ideal. In the present examinations, the adhesion and
longitudinal cell growth of osteoblasts on the Ca-O₃-Ti disks increased, while the number of adhered bacteria on the Ca-O₃-Ti disks was significantly less than on Ti and Ca-Ti disks regardless of the gram-positive or negative bacteria (Figs. 6 and 7). Several reports regarding the mechanism of bacterial adhesion on a titanium surface have been presented[24,25] and the adhesion is inhibited by an increase in hydrophilicity of the material[26,27]. The present results indicate that the titanium surface becomes hydrophilic following calcium–ozone or ozone treatments (Table 1, Figs. 6, 7). Bacterial adhesion is suppressed more on a calcium–ozone treated surface compared with an untreated surface as ozone treatment makes the surface hydrophilic.

In the present study, Ca-O₃-treatment of the sand blasted and etched titanium surfaces, as typically applied to the surface of dental implants for clinical use, resulted in modification by Ca without changing the structure or roughness of the surface. Our findings indicate that Ca-O₃-treatment increases osteoblast adhesion while it suppresses bacterial adhesion, and is considered a suitable treatment for dental implants that preserves surface properties. We intend to perform further studies regarding the clinical application of titanium dental implants with a calcium-modified surface.

CONCLUSION

In the present study, calcium modification of surface-roughened titanium disks was successfully prepared by immersion in a calcium chloride solution containing ozone. The calcium-modified titanium disks showed no change in the surface roughness or super hydrophilicity compared with the untreated titanium disks. Furthermore, the modified disks had a significantly greater number of initial cell attachments and longitudinal cell growth compared with the untreated and calcium-treated specimens, while the initial bacterial adhesion on the calcium–ozone-treated titanium disks was statistically less than on the pure titanium disks and titanium disks treated without ozone. We concluded that the calcium–ozone treatment is applicable for titanium dental implants.

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