Original

Effect on Osteogenesis of Cleaning Titanium Implants with Ozonated Water

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Abstract: Carbon compounds accumulate over time on titanium surfaces. This is reported to reduce osseointegration of titanium implants in bone. Ozonated water was found to remove carbon compounds and to have a sufficient cleaning effect. The effects of the ozonated water on the titanium surface were evaluated by cell culture and mechanical tests. *In vitro* study, titanium disks (diameter 20 mm, thickness 1 mm, Grade 2) were treated with sulfuric acid. Disks in the control group were surface cleaned with distilled water, and disks in the O₃ group were surface cleaned for 10 min with ozonated water. The experimental animals were male Sprague-Dawley (SD) rats. We tested for cell proliferation activity, alkaline phosphatase (ALP) activity, calcification and measured calcium concentrations. *In vivo* study, titanium implants (diameter 1 mm, length 2 mm, Grade 2) were treated with sulfuric acid. Control group samples were washed for 10 min with distilled water immediately before implantation. O₃ group samples were washed for 10 min with ozonated water. We performed push-in testing using a mechanical testing machine. The femurs were removed at 2 and 4 weeks after implantation. Cell proliferation activity was higher in the O₃ group than in the control group at 24 h after cell seeding. Similarly, ALP activity was higher in the O₃ group than in the control group, indicating higher cell differentiation potential. Cell calcification was higher in the O₃ group than in the control group at all measurement times. Maximum compressive stress was significantly higher in the O₃ group compared with the control group at 2 weeks after implantation ($p < 0.05$). At 4 weeks after implantation, no difference was found between the groups. Osseointegration time was decreased by cleaning titanium implants with ozonated water.

Key words: Carbon compounds, Osseointegration, Ozonated water, Osteoblast, Titanium surface

Introduction

Titanium is a lightweight metal with high corrosion resistance and good biocompatibility¹. Therefore, it is widely used as a biomaterial in medicine and as a dental implant material in dentistry. The titanium surface and bone become fixed through the process of osseointegration, which results in close integration between the two at the light microscopic level^{2,3}. This process of integration requires approximately 6 months for the maxilla and 3 months for the mandibular bone⁴, which is a relatively long period of time when compared to prosthetic treatment such as dentures and bridges. Although improvement of the implant surface microstructure⁵⁻⁷ and techniques such as immediate loading⁸ and early loading⁹ have been attempted to improve the process, cell adhesion in the early stage remains important to clinical outcome. Cell adhesion is affected by the physicochemical properties of

the titanium surface such as its microstructure, wettability, and adhesion with carbon compounds¹⁰⁻¹². In addition, there is still room for improvement in the ratio of the titanium surface area that is in close contact with the bone, which is currently 45-65 %^{13,14}. In the titanium implants now used in implant treatment, cell adhesion, proliferation, and differentiation decrease over time due to adhesion of carbon compounds to the titanium surface^{15,16}.

Ozone is used in various fields such as medicine, industry, and dairy farming for sterilization, deodorization, and bleaching¹⁷ as well as in many areas of dentistry. When ozone dissolves in water, free radicals are formed due to the reaction with OH⁻ ions, imparting ozonated water with strong oxidizing action¹⁸. When Murakami et al.¹⁹, investigated the cleaning effect of ozonated water for removing carbon compounds from the titanium surface, the carbon compounds were found to be removed immediately after treatment.

In this study, to examine the influence of titanium treated with ozonated water on bone formation, we performed one *in vitro* and one *in vivo* study. *In vitro* study, osteoblast-like cells were applied

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Figure 1. Low and high magnification SEM images of the titanium surface (upper row: low magnification, bar=500 µm. lower row: high magnification, bar=20 µm). Left column: Machined titanium surface (A, B). Center column: Acid treated surface (C, D). Right column: Acid treated cylinder implant surface (E, F).

to pure titanium treated with ozonated water and cell proliferation, differentiation, and calcification were measured thereafter. *In vivo* study, pure titanium implants were placed in rat femurs, and mechanical strength was tested.

Materials and Methods

Experimental animals

The experimental animals were 5 male SD rats (Japan SLC, Hamamatsu, Japan) for *in vitro* study. Twenty-two 8-week old male SD rats were used for *in vivo* study. This study was approved by the Animal Research Committee of the Aichi Gakuin University School of Dentistry (approval number AGUD152, 217).

Ozonated water production equipment

An electrolytic ozonated water generator (Ozone Oral Irrigator, Ebara Jitsugyo Co, Tokyo, Japan) was used. The concentration of ozone was 5.0 mg/l (25 °C) and its production rate was 40 ml/min¹⁹⁾.

Production and surface treatment of experimental sample

The experimental samples were machined pure titanium disks (diameter 20 mm, thickness 1 mm, Grade 2, Nishimura Metal, Fukui, Japan) for *in vitro* study. Cylindrical titanium implants (diameter 1 mm, length 2 mm, Grade 2; Nishimura Metal, Fukui, Japan) were used as experimental samples for *in vivo* study. The surfaces of all samples were treated by thermal sulfuric acid processing for 75 s in a 67 % sulfuric acid solution at 125 °C, after which ultrasonic cleansing was performed 3 times in pure water. Scanning electron microscopy images of the machined titanium surface and the titanium surface after acid processing

are shown in Fig. 1.

After drying, all samples were kept in a dark room at normal temperature, and high-pressure steam sterilization was performed before the experiment. Samples were pre-treated for 10 min in either ozonated water (O₃ group) or distilled water (control group).

Cell culture

Marrow cells extracted from the femur of SD rat were cultured in medium containing 15% FBS (Equitech-Bio, Kerrville, TX), 10⁻⁸ M dexamethasone (Sigma, St. Louis, MO), 50 mg/mL L-ascorbic acid 2 phosphoric acid (Sigma), 10 mM β-glycerophosphate (Sigma), antimicrobial and antifungal agents (100 U/ml penicillin G sodium, 100 mg/ml streptomycin sulfate, 250 ng/ml amphotericin B; Life Technologies, Carlsbad, CA), and α-MEM (Life Technologies) with osteoblast inducer reagent. Cell culture took place in a 5 % CO₂, 37 °C environment. As an initial culture, a 10-cm diameter polystyrene culture plate (BD, Franklin Lakes, NJ) was seeded, and floating cells were removed on day 2 of culture and washed with phosphate-buffered saline (PBS). The culture medium was changed once every 3 days. On day 7 of culture, cells were collected for passaging after washing with PBS and incubating for 5 min with 0.25 % trypsin-EDTA (Life Technologies). Centrifugal separation was performed in a 15-ml tube (BD) at 1500 rpm for 5 min and the supernatant solution was removed. The precipitate was then suspended in osteoblast inducer reagent and seeded into a stationary 12-well culture plate (Corning, Corning, NY) (3×10⁴ cells/well).

Cell proliferation test

For the cell proliferation test, a WST-8 kit (Cell Counting

Figure 2. Schematic of implant placement in the rat femur.
a: Cylinder titanium implant. b: Cortical. c: Medullary cavity.
d: Resin. e: Metal frame.

Kit-8, Dojin Kagaku, Kumamoto, Japan) was used and cell numbers were measured. The titanium disks were moved to a new 12-well culture plate and washed twice with PBS. WST-8 reagent was added to 1000 μ L culture solution, incubated for 60 min, and color reaction was performed. After that, the reagent was moved to a 96-well culture plate (BD), 450 nm absorbency was measured with an absorption spectrometer (Microplate Reader 680, BIORAD, Hercules, CA), and cell number change was measured as a relative value.

Measurement of ALP activity

ALP activity was measured on days 5 and 7 of culture using LabAssay™ALP (Wako Pure Medicine Industries, Osaka, Japan). Titanium disks were moved to a new 12-well culture plate and washed twice with PBS. Then, 100 μ L buffer substrate was added and the plates were incubated for 10 min before color reaction was performed. Subsequently, 100 μ L of reaction liquid was moved to each well of a 96-well culture plate, and 405 nm absorbency was measured with an absorption spectrometer.

Evaluation of calcification

Alizarin Red staining was performed on days 7, 14, and 21 of culture to evaluate calcification. Titanium disks were moved to a new 12-well culture plate and fixed for 10 min in neutral buffered formalin solution (Muto Pure Chemicals, Tokyo, Japan). After washing in pure water, the titanium disks were stained with pH 4.1–4.3 adjusted Alizarin Red S stain and washed 4 times with pure water. The titanium disk was photographed with a digital camera, and the positive reaction area was quantified with image processing software (ImageJ, NIH, Bethesda, MD).

Calcium determination test

The calcium determination test was performed on days 14 and 21 of culture (Calcium Assay Kit, Cayman Chemical, Ann Arbor, MI). The calcium concentration on the titanium disks was quantified with observed data by absorption spectroscopy. The

Figure 3. cell proliferation (n=5)
Absorption evaluated by the WST-8 method. *P<0.05.

titanium disks were moved to a new 12-well culture plate and soaked for 24 h in 0.5 M hydrochloric acid to melt the surface calcium, and then 10 μ L samples were inserted into a 96-well culture plate. Calcium Detector R 1 (100 μ L) and Calcium Detector R2 (100 μ L) mixture was then added and calcium concentration measured at 570 nm with an absorption spectrometer.

Placement of cylindrical titanium implants

After shaving, an incision was made in the femoral area of an anesthetized SD rat (2 % isoflurane), and muscle and periosteum were avulsed and femoral bone exposed. A stoma was formed 10 mm from the kneecap with a 0.8 mm dental round bar (E0123, Dentsply Maillefer, Ballaigues, Switzerland), which was widened with a #80 K file (06026, Ormco, Orange, CA) to form the bone fossa. A control group implant was placed in the right femur and an O₃ group implant was placed in the left femur. The muscle and dermis were then sutured closed.

Mechanical testing of osseointegration

Push-in test²⁰⁾ was used for mechanical testing. On days 14 and 28 after placement of the cylindrical titanium implant, 11 rats were humanely killed and the femur was extracted with ophthalmological scissors and fixed to a metal frame with autopolymerizing resin (GC, Tokyo, Japan) (Fig. 2). After that, a universal/tensile testing machine (EZ-S, Shimadzu Manufacturing, Kyoto, Japan) was used with a load applied in the long axis direction of the implant and compressive load (N) and pressure displacement (μ m) were measured.

Statistical analysis

F-test was used in all experiments. After verifying homoscedasticity, T-test analysis was performed. Statistical significance was set at 5 %.

Results

Cell proliferation test

Figure 4: ALP activity (n=5)
Protein mass calculated from absorption measured by the ALP activity test. *P<0.05.

Figure 6: Evaluation of calcification area (n=5)
Arizarin Red S staining image analysis results. *P<0.05.

The results of the cell proliferation test are shown in Fig. 3. The graph in Fig. 2 shows absorption spectrometer value on the vertical axis and culture days on the horizontal axis. Values obtained from the absorption spectrometer after 24 and 48 h are expressed as increments. In the 24 h culture, the number of cells in the O₃ group was significantly greater than in the control group (*P<0.05). However, there was no significant difference in the 48 h culture.

Measurement of ALP activity

The results of ALP activity are shown in Fig. 4. In the graph in Fig. 4, the protein quantity calculated from absorption spectrometry is shown on the vertical axis and culture day is shown on the horizontal axis. Values obtained from the absorption spectrometer after 5 and 7 days are expressed as ALP activity increments. On culture days 5 and 7, ALP activity was significantly higher in the O₃ group than in the Control group (*P<0.05).

Evaluation of calcification

Fig. 5 shows the results of Alizarin Red S staining (left: Control

Figure 5: Arizarin Red S staining. (bar=5 mm)

Figure 7: Calcium determination (n=5)
Calcium concentration calculated from absorption spectra in the calcium determination test. *P<0.05.

group, right: O₃ group). On culture days 7, 14, and 21, the Alizarin Red S-stained positive reaction area was measured by image processing software (Fig. 6). The graph in Figure 6 shows the staining area ratio on the vertical axis and culture days on the horizontal axis. On culture days 7, 14, and 21, the staining area ratio was significantly higher in the O₃ group compared to the control group. (*P<0.05)

Calcium determination test

The results of calcium determination are shown in Fig. 7. The calcium quantity calculated from absorption spectrum is shown on the vertical axis and culture days on the horizontal axis. Values

Figure 8. Example 1 of push-in test (O_3 group at day 28).

obtained from the absorption spectrometer after 14 and 21 days are expressed as increases in calcium quantity. On culture days 14 and 21, calcium quantity was significantly higher in the O_3 group than in the control group (* $P < 0.05$).

Push-in test

Fig. 8 shows an example of the mechanical testing of osseointegration. Compressive load is shown on the vertical axis and pressure displacement on the horizontal axis. In addition, the result is shown in Fig. 9. The vertical axis shows maximum compressive load value, and the horizontal axis shows duration in days since implantation. The compressive load on the cylindrical titanium implant increased gradually and decreased suddenly after the maximum load value was reached. On day 14 after implantation, the maximum compressive load value was significantly higher in the O_3 group compared with the control group (* $P < 0.05$). However, at 28 days after implantation this value was higher in the O_3 group but not significantly so.

Discussion

Ozonated water

Methods of generating ozonated water include bubbling or mixing ozone through distilled or purified water, as well as electrolysis²¹⁾. Here, following Murakami *et al.*¹⁹⁾, we used an electrolytic ozonated water generator (Ozone Oral Irrigator) that uses diamond electrodes. This method has a high efficiency of ozone generation and low production of ozone gas. When ozone molecules dissolve in water, they can remain as residual O_3 or decompose into OH radicals, which are a more powerful oxidant than molecular ozone. There are two types of reactions involving soluble organic matter and ozone in the water: a direct reaction with ozone, and a free radical reaction with the formed OH radicals. Although lower temperature results in slower ozone autolysis and longer ozone half-life, in this study, the temperature was set at 25 °C for highest stability at room temperature. As 1 mg/l is sufficient for sterilization, and 4 mg/l is sufficient for virus inactivation^{18,22)}, concentration was set at 5 mg/L to take the short half-life into consideration. In a preliminary experiment, treatment was

Figure 9. Push-in test ($n=11$)

Maximum value of compressive load measured by mechanical testing. * $P < 0.05$.

performed for 1, 3, 5, 7 and 10 min. At 10 min a significant difference was observed in cell proliferation. So for this study, treatment time was set at 10 min.

Sample production

The as-produced titanium surface is hydrophilic, but with time carbon compounds adhere to the titanium surface, reducing the contact ratio and strength of the titanium/bone contact^{23,24)}. The carbon compounds on the titanium surface increase sharply over 4 weeks after production, and then gradually over the next 3 months, while absorption of the proteins necessary for cell adhesion and osteogenesis falls notably. In light of this, the titanium disks in this study were used 3 months after initial processing.

Cell culture of osteoblast-like cells

Bone marrow contains mesenchymal stem cells, which have high differentiation potential. The osteoblast inducer reagent added to the culture solution functions to differentiate the mesenchymal stem cells into osteoblast-like cells. Mesenchymal cells can also differentiate into cartilage cells and adipose cells²⁵⁻²⁷⁾ but for this study, 10^{-8} M dexamethasone, 50 mg/ml L-ascorbic acid, diphosphoric acid, and 10 mM β -glycerophosphate were added to the culture solution to specifically differentiate the bone marrow cells into osteoblast-like cells.

Cell proliferation test

In the 24-h culture, the number of cells was significantly higher in the O_3 group than in the control group, but there was no significant difference in the 48-h culture. This is likely due to the remarkable cell proliferation seen in the 24-h culture's proliferation curve during the logarithmic growth phase. It is expected that no difference would be seen at 48 h, as the O_3 group, in which the cells had reached confluence first, had stopped proliferating and the control group caught up to this level.

ALP activity

ALP promotes the growth of hydroxyapatite crystals by breaking down pyrophosphoric acid and increasing local phosphoric acid concentration, which is characteristic of differentiation to osteoblast cells. On culture days 5, 7 ALP activity in the O₃ group was significantly higher, as compared with the Control group.

Evaluation of calcification

Alizarin Red S staining confirmed that on culture days 7, 14 and 21, the calcification area had increased significantly more in the O₃ group compared with the control group. In the calcium determination test on culture days 14 and 21 in particular, the O₃ group had a significantly higher value in comparison with the control group, at 1.5 times greater on day 14 and 1.9 times greater on day 21. However, the Alizarin Red S staining area ratio was 1.2 times greater on day 14 and 1.2 times greater on day 21. The significant difference in area ratio was not large, but it suggests that the calcification node formed in the O₃ group was sterically large. Carbon compounds were deposited on the titanium disks, which inhibits osteoblast activity¹⁶⁾ but by cleaning the disk surface with ozonated water, the carbon compounds were removed, as indicated by the promotion, proliferation, and differentiation of osteoblast-like cells.

Push-in test

Several studies have investigated the mechanical properties of the cylindrical titanium implants after placement in the rat femur^{29,30)}. Ogawa et al.'s push-in test has been proposed as one method of mechanical testing for verifying implant osseointegration²⁰⁾. Using the same method as Ogawa et al, this study tested implants of 1 mm in diameter and 2 mm in length, that were placed so as not to penetrate the opposite side (non-penetration type). The mechanical test was performed on days 14 and 28 after implantation.

The compressive load of all samples was higher after placement of the cylindrical titanium implant and fell immediately after reaching the maximum value, which indicates total failure of osseointegration. The results of mechanical testing confirmed osseointegration in both groups. In both groups, there was a trend for the maximum value of compressive load to be greater after 28 days than after 14 days. On day 14 after placement of the cylindrical titanium implant, the maximum value of compressive load was significantly higher in the O₃ group than in the control group. But on day 28, no significant difference was observed between the O₃ and control group. Furthermore, the maximum value of compressive load for the O₃ group on day 14 was close to the maximum value of the control group on day 28. This is thought to demonstrate that the O₃ group was able to achieve osseointegration at an early stage. From the results above, ozonated

water treatment allows early osseointegration, which suggests the possibility of a shorter treatment period.

From *in vitro* and *in vivo* study, it is thought that carbon compounds adhere to most titanium implants currently used in implant treatment^{13,14,31)}. The carbon compounds adhering to the titanium surface at the time of placement of the implant are an important factor in determining early adhesion of osteoblast cells to the titanium surface¹⁶⁾. However, it is not possible to control the accumulation of organic matter during production of titanium implants^{10,32)}.

Other reported methods of managing carbon compound adhesion are ultraviolet treatment³³⁾, storage in saline solution³⁴⁾, and gamma-ray irradiation³⁵⁾. Ultraviolet treatment is reported to cause physicochemical changes such as changing the titanium surface from hydrophobic to hydrophilic³⁶⁾, causing the carbon atom ratio to decrease^{37,38)}. However, this method has drawbacks in that decomposed carbon compounds remain on the surface, high-concentration ozone gas generated by the ultraviolet rays must be removed, and the apparatus is expensive. Gamma-ray irradiation of the titanium surface³⁵⁾ also decreases titanium surface carbon compounds and promotes osteoblast proliferation and differentiation, enabling early osseointegration. However, similar to ultraviolet treatment, the apparatus is expensive. Storage in saline solution immediately after manufacture of the titanium implant is reported to protect the pure titanium surface from contamination with organic matter and atmospheric carbon compounds and organic elements by keeping the implant hermetically sealed in saline³⁴⁾. However, most titanium implants produced today are kept in sterile cases after being exposed to the air. The cleaning effect of the ozonated water used in this study allows the by-products from decomposed carbon compound to be washed away, and the ozonated water not only promotes the decomposition reaction but also quickly returns to water after treatment, so it is non-toxic.

In conclusion, in this study, to examine cell adhesion and osseointegration in ozonated water-treated titanium surface, the proliferation, differentiation, and calcification potential of osteoblast-like cells was assessed by titanium surface cell culture testing and mechanical testing performed on cylindrical titanium implants after placement. The results showed that ozonated water treatment of the titanium surface promoted osteoblast-like cell proliferation and differentiation, and the improvement of mechanical strength suggested promotion of osteogenesis.

Conflict of Interest

The authors have declared that no COI exists.

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