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Antibacterial effect and cytocompatibility of nano-structured TiO_2 film containing Cl

Min-Kyung KANG, Seung-Kyun MOON, Kwang-Mahn KIM and Kyoung-Nam KIM

BK21, Department and Research Institute of Dental Biomaterials and Bioengineering, and Research Center for Orofacial Hard Tissue Regeneration, College of Dentistry, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-752, Republic of Korea
Corresponding author, Kyoung-Nam Kim; E-mail: kimkn@yuhs.ac

The aim of this study was to investigate the antibacterial effect and cytocompatibility of a nano-structured TiO_2 film that contained Cl and had been coated onto commercially pure titanium. First, we prepared nano-structured TiO_2 by anodization with hydrofluoric acid. Then, to confer an antibacterial effect, we performed a second anodization with NaCl solutions of different concentrations (0.5 M, 1 M, 2 M). The morphology, composition, and wettability of the surface were investigated by SEM, EDS, and a video contact angle measuring system. The antibacterial effect was evaluated by film adhesion method. And cytotoxicity was determined by the viability of MG-63 cells in a MTT assay. The SEM and EDS results showed that the TiO_2 nano-structure containing Cl had successfully formed after the second anodization. The contact angle analysis showed that the anodized titanium had a hydrophilic character. The results of this *in vitro* investigation demonstrated that the TiO_2 nano-structure film anodized in 1 M NaCl had an antibacterial effect and good cell compatibility.

Keywords: Antibacterial effect, Cl, Nano, TiO_2

INTRODUCTION

The success of a dental implant depends on the surface characteristics of the implant, including surface morphology, composition, and surface energy. In particular, commercially pure titanium (cp-Ti) has been used as an implant material, due to its high biocompatibility. In addition, the oxidation of titanium can affect its biocompatibility, because titanium oxide offers an osteoconductive surface for osteointegration[1]. The best technique for oxidization is anodic oxidation, because a thick, porous oxide surface can be formed. Many researchers have attempted to prepare anodic oxide surfaces with various characteristics. Previously, Ishizawa and Ogino developed an oxide layer on titanium that contained Ca and P. That oxide layer could precipitate hydroxyapatite in a hydrothermal treatment[1-7]. Recently, another approach was reported for preparing titanium oxide that formed a nano-structured titanium oxide layer that could enhance apatite formation. In addition, the nano-structured titanium oxide surface facilitated cell adhesion, proliferation, and migration, because cells could directly interact with nano-structured extracellular matrices[8].

Although developing these surface coating methods was successful, implant failure occurred due to peri-implantitis. Peri-implantitis is an inflammatory reaction that destroys the alveolar bone and connective tissues, and eventually causes implant failure. The frequency of peri-implantitis was reported to be 5 to 8%. In addition, some implant patients had previously had periodontitis. A history of periodontitis and the presence of bacteria are risk factors that can influence peri-implant infections, because the periodontopathic bacteria of natural teeth can migrate to the periphery of the implant[9,10]. Peri-implantitis can be treated with a systemic approach. There are various antibiotics, dosages, and durations for peri-implantitis treatments. However, this treatment can also cause side effects, which increases the resistance of the bacteria to antibiotics. In contrast, the local delivery of antibiotics to the implant surface can treat peri-implantitis effectively without side effects. Therefore, many studies have attempted to develop antibacterial coatings for implants[11-14]. One study showed that titanium anodized in sodium chloride solution had a significant antibacterial effect and good cell compatibility[15,16]. Therefore, the aim of this study was to prepare this nano-structured titanium coating with Cl and antibacterial effect and cell compatibility were evaluated.

MATERIAL AND METHODS

Preparation of specimens

1. Polishing

The present study was performed with specimens made of cp-Ti, grade 3 (10×10×1 mm). The titanium surfaces were polished mechanically (Buehler, Lake Bluff, IL, U.S.A.) with SiC paper with grits of 100, 600, and 1,200. The polished surfaces were then cleaned ultrasonically in acetone, ethanol, and distilled water for 15 min (Group A).

2. The first anodization for nano-structured titanium formation

The titanium surface was anodized in two steps with a DC power supply (Genesys 600-2.6, Densi-Lambda, Japan). First, a nano-structured titanium film was formed at 20 V in 0.5% hydrofluoric acid (HF) for 40 min

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(Group B).
3. The second anodization was performed to add Cl to the titanium coating
After the first anodization, the specimens were subjected to anodic-oxidization at 20 V for 2 min with NaCl solutions of different concentrations (0.5 M, 1 M, and 2 M for Groups C-1 to C-3, respectively).

Surface morphology
The morphology of the anodic oxide film was investigated by field-emission scanning electron microscopy (FE-SEM, JSM-6700F, Jeol, Tokyo, Japan).

Elemental analysis of Cl
The elemental analysis was performed by Energy dispersive spectroscopy (EDS, Inca program, Oxford, U.K.). Furthermore, Electron probe microanalysis (EPMA, JXA-8900 R, Japan) was used to analyze the distribution of Cl. Three samples from each group were prepared, and three measurements were performed on each sample to evaluate the elemental analysis.

Surface wettability
The wettability of a surface was examined with contact angle measurements (DSA10, Kerus, Germany). Ten samples from each group were prepared, and two measurements were performed on each sample to evaluate the average contact angle.

Evaluation of antibacterial activity
Antibacterial activity was evaluated with a film adhesion method. This method modified ISO 22196; Plastics —Measurement of antibacterial activity on plastic surfaces and JIS Z 2901; Antimicrobial products— Test for antimicrobial activity and efficacy. First, *Streptococcus mutans* (*S. mutans*; IFO 13955) was cultured aerobically in a conical tube that contained 100 mL Brain Heart Infusion (BHI) medium at 37°C. The initial concentration of bacteria was adjusted to 10^6–10^7 colony-forming units (CFU)/mL by dilution with phosphate-buffered saline (PBS, pH 7.2, GIBCO). To test the antibacterial activity of the prepared surfaces, 4 μL of bacterial solution was pipetted onto each specimen. A polyethylene film was placed over the specimen and bacterial solution. After 3 h, the specimens and polyethylene films were rinsed in 1 mL PBS and bacteria cells were detached by sonication from the specimen. With a spiral plater, 100 μL of harvested bacteria cells of each specimen were plated onto Bacto-Agar plates. Plates were incubated for 48 h at 37°C to determine the number of adherent, viable *S. mutans* in terms of colony-forming units (CFU). For antibacterial activity test, fifteen samples of each group were used.

Evaluation of cytotoxicity
The methylthiazol tetrazolium (MTT) cell proliferation assay is a simple colorimetric assay that measures cell cytotoxicity, proliferation, and viability. MTT is yellow and water-soluble. MG-63 human osteosarcoma cells (KCLB 21427, Korean Cell Line Bank, Korea) were used in the MTT evaluation. Cells were suspended in Dulbecco's modified Eagle's medium at a density of 10^4 cells/mL. Aliquots of 0.18 mL were placed into each well of a 96-well plate, and cells were incubated in a humidified, 5% CO_2 incubator at 37°C for 24 h. The specimens were extracted in distilled water at 121°C for 1 h. The extraction volumes were determined by the surface areas of the specimens. The extraction ratio was 1 mL of distilled water per 3 cm^2 of surface area. Extraction liquid (0.02 mL) was added to the cells in each of the 96 wells. Control cells received 0.02 mL of distilled water. After incubations of 24 h, 48 h, and 72 h, 0.05 mL MTT solution was added to each well and allowed to incubate for 4 h. MTT solution was removed, and insoluble formazan crystals were dissolved in dimethyl sulfoxide. The absorbance of each well was measured at 570 nm with an ELISA plate reader, and the percentage viability was calculated. Fifteen samples were from each group were used to evaluate cytotoxicity.

Statistical analysis
Results of surface wettability, the antibacterial activity test, and the cytotoxicity test were analyzed with the one-way ANOVA test to compare significant differences. The significance level was set at 95%.

RESULTS AND DISCUSSION

Surface morphology
The results of the FE-SEM imaging are shown in Fig. 1. All of the anodized titanium was similar (Fig. 1a–h). After the second anodization, the morphology did not change (Fig. 1c–h). The nano-structured surface was consistent at less than 100 nm. The outer diameter of nano-structure was 100 nm and inner diameter was 80 nm. Electrochemical conditions and electrolytes can affect the morphology and structure of titanium oxide[17]. In most electrolytes, nanotubes with diameters of 15–200 nm can be grown by applying anodic potentials in the range of 10–20 V[18,19]. Titanium oxide nano-structures can be fabricated easily with precisely controlled diameters and lengths[20].

Elemental analysis of Cl
Elements were identified by EDS and EPMA. Cl was detected on all titanium surfaces that were anodized in NaCl solutions (Figs. 2 and 3). In titanium anodized in 0.5, 1, and 2 M NaCl, the percentages of Cl atoms were (0.85±0.4), (3.85±1.2), and (5.4±0.9)% respectively. As the concentration of Cl in the electrolyte was increased, the percentage of Cl atoms incorporated into the coating was also increased. The intense peak of about 2.1 keV corresponds to Pt element which was coated on the sample for analysis SEM and EDS. The Pt coating was carried out before the SEM analysis to improve the conductivity of the specimen and hence cause more emission of secondary electrons (Fig. 2a–c).

Figure 3 shows color mapping images of Cl distribution. The average Cl levels were founded by considering Cl levels for each different color and

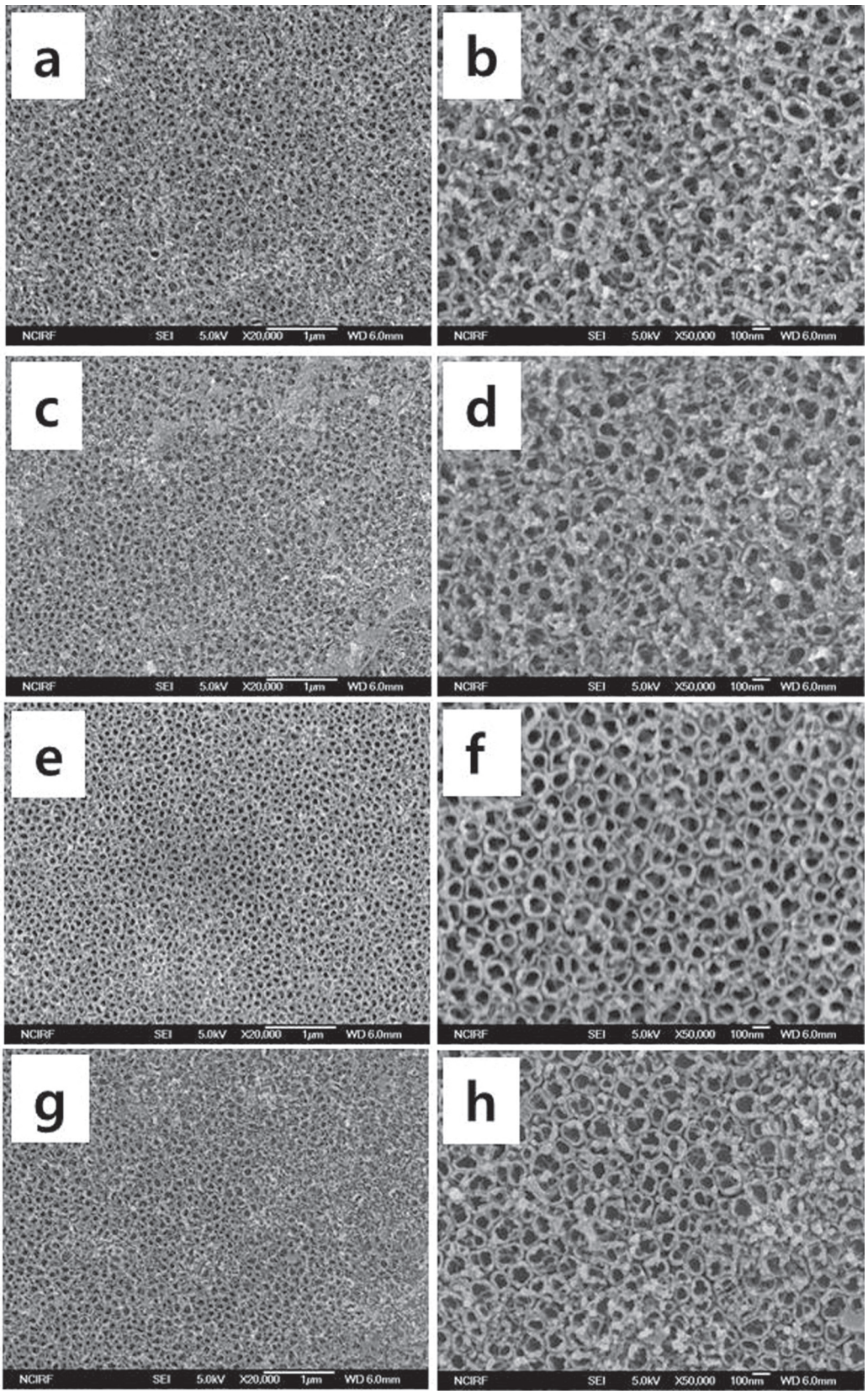

Fig. 1 Scanning electron microscope image of (a) Group B (×20,000), (b) Group B (×50,000), (c) Group C-1 (×20,000), (d) Group C-1 (×50,000), (e) Group C-2 (×20,000), (f) Group C-2 (×50,000), (g) Group C-3 (×20,000) and (h) Group C-3 (×50,000).

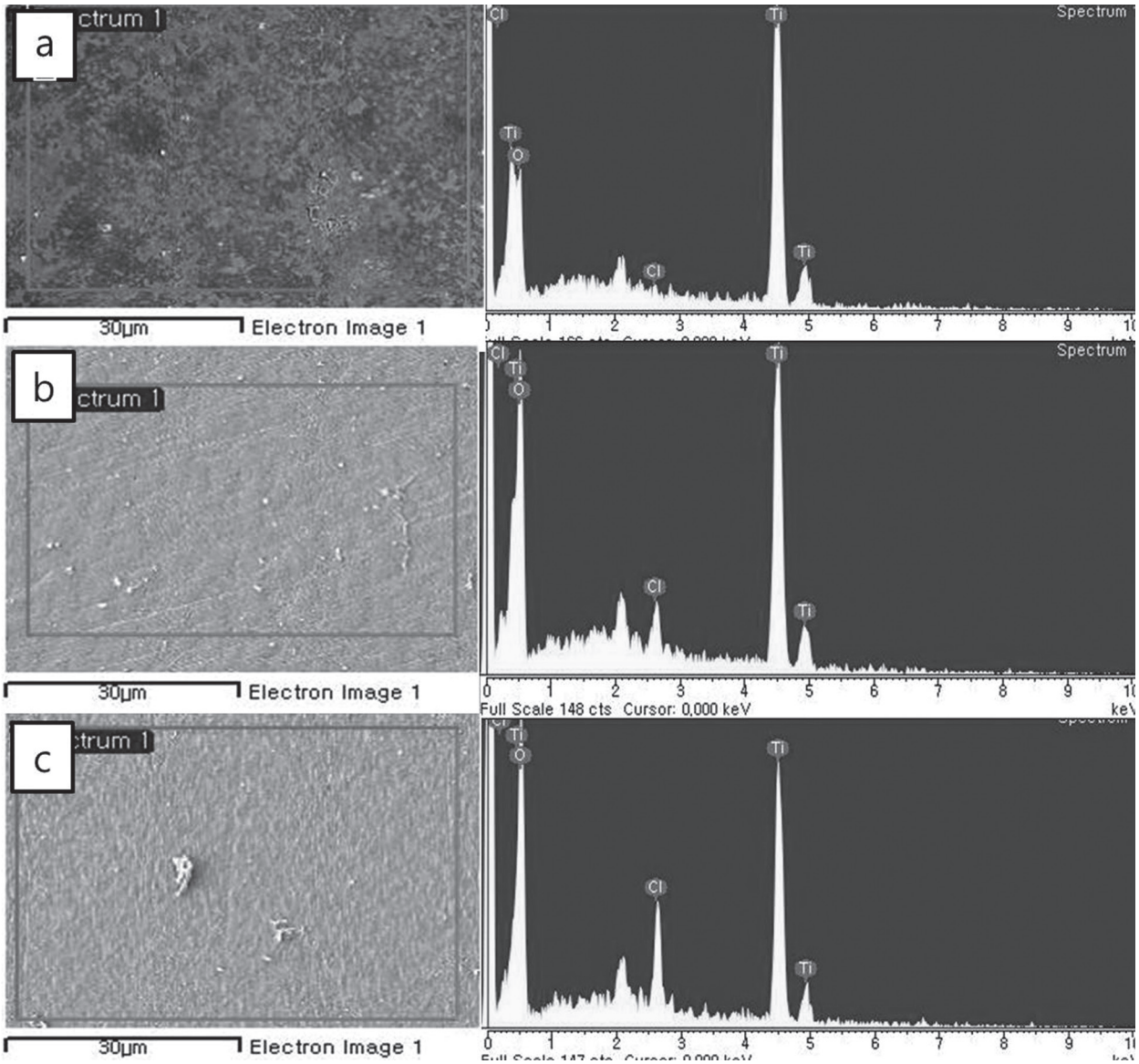

Fig. 2 Surface elements of titanium anodized in hydrofluoric acid followed by NaCl. (a) Group C-1, (b) Group C-2, and (c) Group C-3.

percentage of that color exist on the surface. (*e.g.* Color mapping of group C-1 surface showed that 16.9% of the surface was covered with color corresponding level 3). However, the average level of mapping image is regardless of numeric data of EDS results. So call, this color mapping image is not quantitative analysis. That could indicate distribution of elements. Therefore, as shown mapping image, the Cl with high concentration was aggregated as the added Cl was increased. The C-3 showed more diverse color; Red, Yellow, Green, Blue image compared to C-1; Green and Blue (Fig. 3a–c).

Surface wettability

The titanium surfaces acquired hydrophobic and hydrophilic properties with different surface treatments. Anodized specimens had a hydrophilic character compared to polished titanium (Fig. 4). Figure 5 shows an optical image of a water drop on the different specimens. The wettability could be affected by surface oxygen component. In this study oxygen component may present on the surface with the anodization process[21]. And porous surface of nano-structure TiO_2 layer, which is sufficiently hydrophilic, would result in improved wettability as the capillary forces causing the filling of the entire pore[22]. The surface wettability of an implant influences cell behavior in the initial osseointegration process[3]. Hydrophilic surfaces facilitate cell proliferation. The initial osseointegration process starts when the implant makes contact with blood. Thus, on hydrophilic surfaces, signs of thrombin and prothrombin are

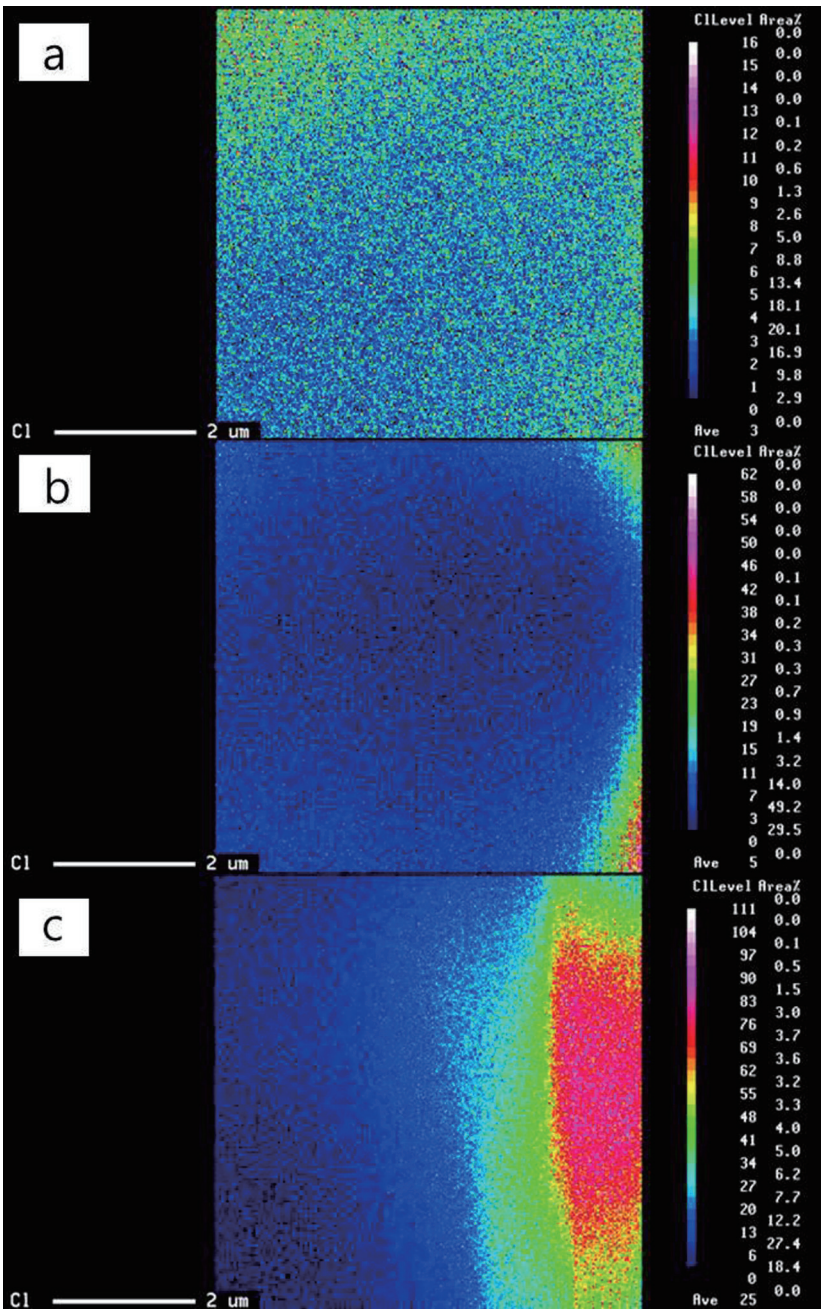

Fig. 3 Color mapping images of Cl distribution. (a) Group C-1, (b) Group C-2 and (c) Group C-3.

Table 1 Experimental groups used in this study

Group	Method	Electrolyte
A	Procedure (1)	–
B	Procedure (1) + (2)	0.5 wt% HF
C-1	Procedure (1) + (2) + (3)	0.5 wt% HF → 0.5 M NaCl
C-2	Procedure (1) + (2) + (3)	0.5 wt% HF → 1 M NaCl
C-3	Procedure (1) + (2) + (3)	0.5 wt% HF → 2 M NaCl

Procedure (1): Polishing and rinsing the titanium surface; Procedure (2): anodization with hydrofluoric acid (HF). Procedure (3): second anodization with NaCl to incorporate Cl into the surface coating.

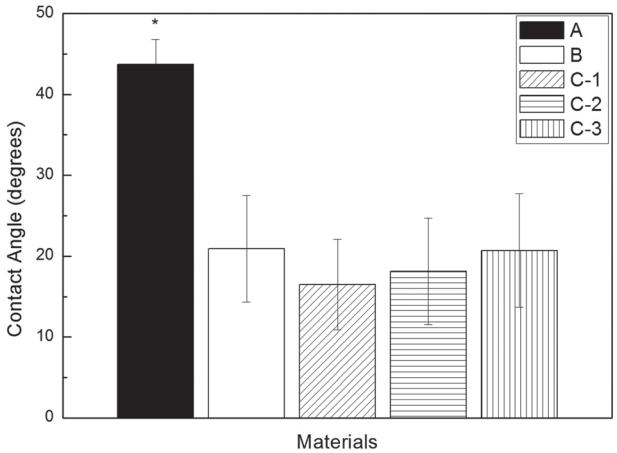

Fig. 4 Contact angle of specimens.
*: indicates significant difference between contact angles in each group.

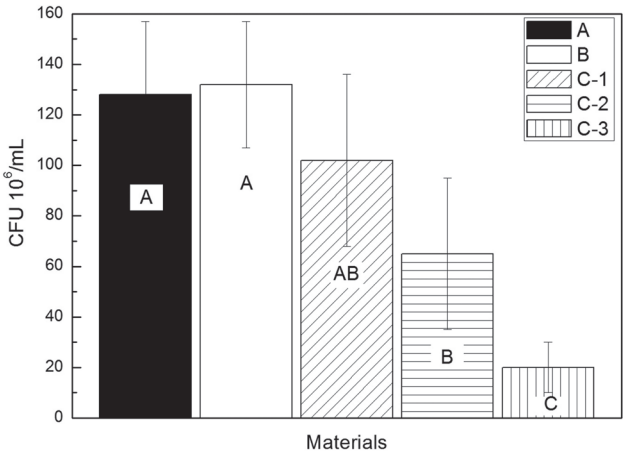

Fig. 6 CFU of *S. mutans* in the antibacterial activity test.
A, B, C: bars with the same letters showed no significant differences ($p>0.05$).

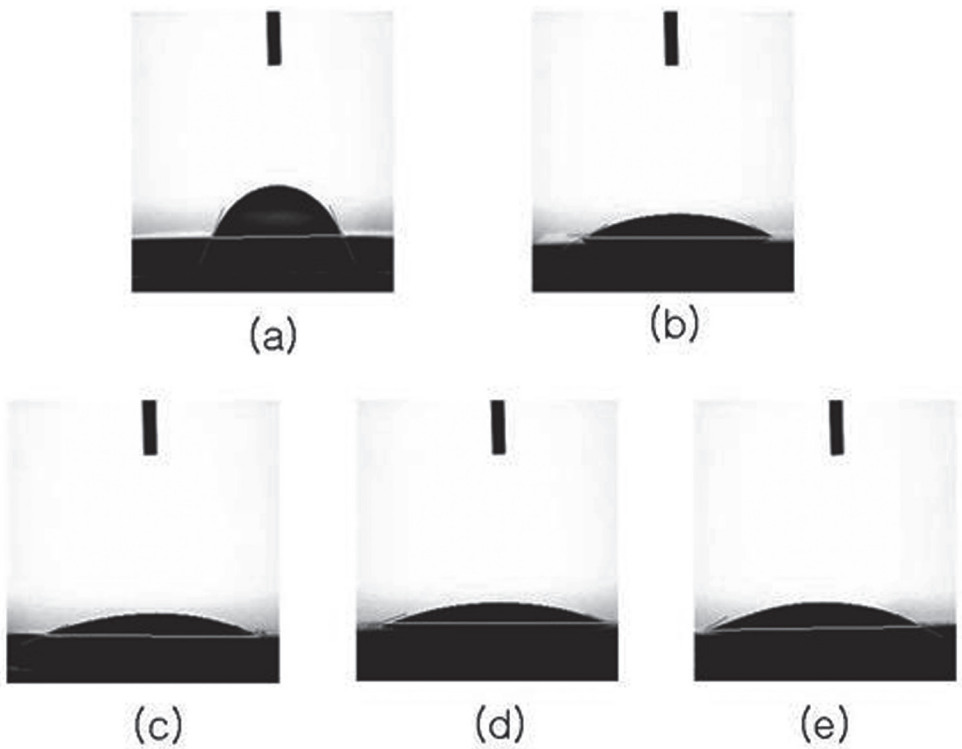

Fig. 5 Optical images of a water drop on different experimental specimens. (a) Group A, (b) Group B, (c) Group C-1, (d) Group C-2, and (e) Group C-3.

predominant, and adsorption is stimulated[17]. Therefore, anodized specimens with hydrophilic characteristics would have good cell compatibility.

Evaluation of antibacterial activity

Results of the antibacterial activity evaluation are shown in Fig. 6. The CFUs of *S. mutans* decreased in the following order: Group B> Group A> Group C-1> Group C-2> Group C-3. The group C-3 showed significantly fewer *S. mutans* CFUs compared to any other experimental group ($p<0.05$). Group C-2 showed significantly more antibacterial activity compared to group B or group A. However, there were no significant differences between the group C-1 and group C-2

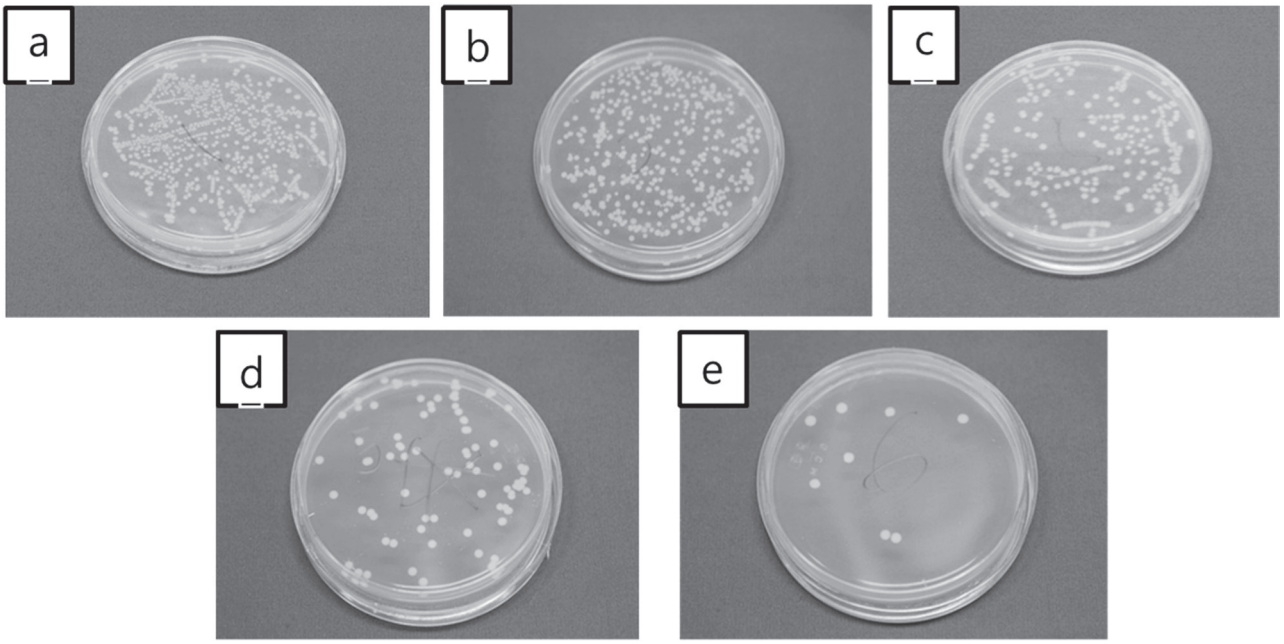

Fig. 7 Agar plates with *S. mutans* colonies to determine the antibacterial effects of extracts from (a) Group A, (b) Group B, (c) Group C-1, (d) Group C-2, and (e) Group C-3.

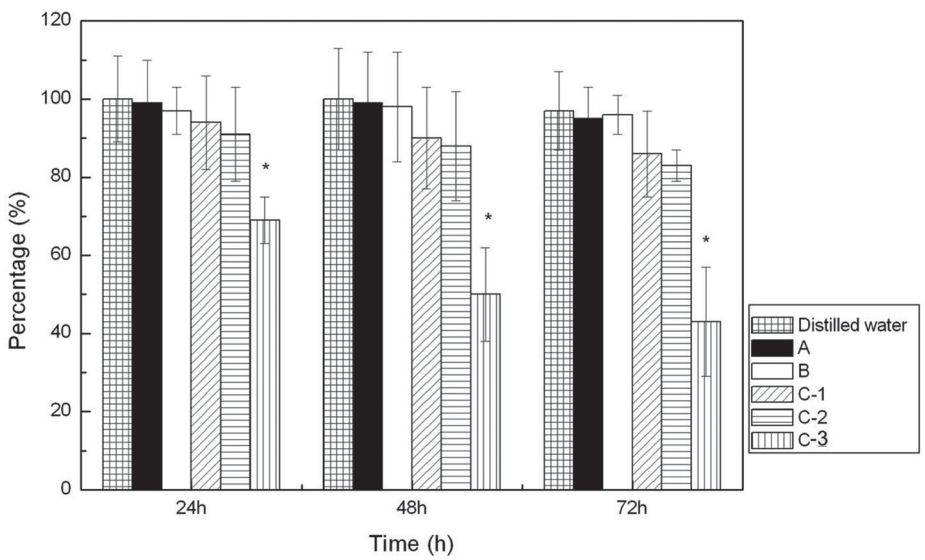

Fig. 8 The percentage viability of MG-63 cells exposed to the different materials in the MTT assay.
* indicates significant difference between the percentage viability of MG-63 cells.

(p>0.05). The group B, group A, group C-1 showed no significant differences in antibacterial activity (p>0.05).

Figure 7 shows agar plates with *S. mutans* CFUs. The Cl observed by elemental analysis (Fig. 2 and 3) is known to be gradually released from the specimen into the culture medium, and then it is hydrolyzed to HCl, HOCl, and TiOH[15,16]. HOCl is a highly reactive component that is thought to be involved in the oxidation of microbial membranes; it increases cell permeability and results in macromolecule leakage and cell death[16,23]. Nakagawara *et al.* reported that antibacterial activity was correlated to the concentration of HOCl released into the medium[24]. The present study also demonstrated that increases in the Cl in the TiO_2 coating caused increases in the antibacterial activity (Figs. 6 and 7). Nakajima *et al.* reported that this antibacterial effect of

free Cl increased with time and current in electrolyzed water[25]. Recent studies have shown that more subtle events are also involved in the bactericidal mechanism of chlorine; for example, uncoupling of the electron transport chain and inactivation of enzymes in the cell membrane and cell interior [16,23,26].

Evaluation of cytotoxicity

The viability of MG-63 cells in the presence of the titanium samples was determined with an MTT assay (Fig. 8). There were no significant differences in viability among the experimental groups (group A, B, C-1, and C-2) and the control group (distilled water) ($p>0.05$), except for group C-3, which comprised titanium specimens anodized in HF followed by 2 M NaCl ($p<0.05$). The group C-3 specimens showed the lowest cell viability during all incubation times. These results suggested that the titanium anodized in HF followed by 2 M NaCl (group C-3) was cytotoxic. Although the surface wettability is known to influence the cell behavior of the implant, we believe that the discrepancy was occurred in this experiment due to high concentration of Cl that resulted in cell cytotoxicity even with high wettability. Of note, titanium anodized in HF followed by 1 M NaCl had some antibacterial effect, but no cytotoxicity. There is reason about that susceptibility of Cl to antibacterial effect and cytotoxicity. That is difference of cell structure between eukaryotic cell (MG-63 cell) and prokaryotic cell (*S.mutans*). Especially, cell wall of prokaryotic cell is thicker than eukaryotic cell wall which could restrict diffusion of HOCl through its cell wall[27]. Therefore, prokaryotic cell might be less susceptible to the action of Cl. This difference was applied in this experiment for the selective toxicity of antimicrobial agent. Furthermore, this nano-structured surface may provide a suitable surface for cell functions, because it mimics the structure of the natural extracellular matrix[20].

CONCLUSION

These results suggested that titanium anodized in HF followed by 1 M NaCl had an enhanced antibacterial effect compared with cp-Ti, and it was more cytocompatible than titanium anodized in HF followed by 2 M NaCl. Therefore, this antibacterial, cytocompatible titanium showed promise as a material for dental implant systems. However, further study on the biocompatibility and surface characteristics are necessary to ensure that this material can be safely used in dental and medical fields. Finally, a porous implant with nano-morphology might be a useful carrier for the local release of bone-stimulating or other types of drugs.

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