Effects of acidic sodium fluoride-treated, commercially pure titanium on periodontal pathogens and rat bone marrow cells

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The purpose of the present study was to clarify the potentiality of acidic fluoride solution in treating peri-implantitis. We examined bactericidal activity of fluoride solution against periodontal pathogens; and evaluated the effects of fluoride on titanium, and the effects on cell proliferation and differentiation of rat bone marrow cells on the fluoride-treated titanium. Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis were used to determine minimal inhibitory concentration (MIC) and short-time exposure. The cells were seeded on the titanium surface with or without fluoride treatment. Then, cellular proliferation, differentiation and mineral deposition were analyzed. The MIC values for A. actinomycetemcomitans and P. gingivalis were 225 and 900 ppm F⁻, respectively. In short-time exposure test, both bacterial strains exhibited a significant decrease in a concentration-dependent manner. Cell proliferation and mineral deposition were significantly increased on the fluoride-treated surface. Within the limitation of this study, acidic sodium fluoride solution has the potentiality in treating peri-implantitis.

Keywords: Fluoride, Peri-implantitis, Titanium

INTRODUCTION

In the last several decades, osseointegrated titanium implant treatment has proved to be a reliable treatment to rehabilitate partially or fully edentulous patients. A review of the literature demonstrated survival rates of the implants supporing fixed prosthesis1,2 were 94.5–97% after 5 years. Additionally, a study showed that long-term survival rates of the implants were 89% and 82% after 10 and 16 years, respectively3.

Nevertheless, the occurrence of implant failure or loss caused by peri-implant disease has been observed to increase during long-term prognoses. Zitzmann et al. reported the occurrence of peri-implantitis in 28–82% after 10 and 16 years, respectively3. Peri-implantitis was defined as inflammatory reactions associated with loss of supporting bone around an implant4, and caused by anaerobic periodontal pathogens. Periodontitis and peri-implantitis exhibit many similarities in etiology and pathogenesis, but peri-implantitis elicits more pronounced inflammatory response and inflammatory cell infiltration because of the structural difference in the surrounding soft tissue5,6. Thus, treatment for peri-implantitis must include anti-infective measures to reduce inflammation and preserve supporting bone.

Another issue with peri-implantitis pertains to osseointegration following treatment. During the progression of active peri-implantitis, acute bone resorption occurs around an implant owing to inflammatory response. Numerous bone regenerative materials and methods have been developed, and several studies have shown re-osseointegration around previously infected implants8–10. However, in most clinical situations, thin connective tissues are observed to surround the infected implants, resulting in separation from the peri-implant bone.

The surface topography and chemical properties of a titanium implant affects cell attachment, proliferation and differentiation11,12. Previous research has demonstrated that implants with moderately rough surfaces enhance the ratio of bone-to-implant contact, and on that several surface modification techniques, such as plasma spray, grit blasting, chemical etching, and anodic oxidation, have been developed to improve the osteoconductive properties of the titanium implant13. Titanium has long been considered a stable material for chemical agents owing to its passive state and on that several surface modification techniques, such as plasma spray, grit blasting, chemical etching, and anodic oxidation, have been developed to improve the osteoconductive properties of the titanium implant13. Titanium has long been considered a stable material for chemical agents owing to its passive state film, but it is possible to change the oxidized titanium surface through exposure to high temperature and strong acid or alkali solutions14,15. Recent studies that showed the use of hydrofluoric acid at low concentrations to modify titanium dioxide surfaces revealed increased bone-to-implant contact, enhanced removal-torque, pull-out forces, and differentiation of mesenchymal stem cells16–18. Acidic fluoride solutions have also been observed to corrode commercially pure titanium and its alloys19,20.

Previous clinical studies have shown the effective use of fluorides in the prevention of dental caries22,23, whereas more recent studies have demonstrated both the bactericidal activity of fluoride against periodontal pathogens and its efficacy in the reduction of periodontitis24,25. With respect to bone tissue, fluoride appears to affect bone metabolism in a dose-dependent manner. Numerous studies have demonstrated that...
fluoride at physiological levels promotes osteoblast proliferation and differentiation\(^{16,26,27}\), whereas excess fluoride suppresses osteoblast proliferation through apoptosis\(^{28,29}\).

Thus, it is likely that fluoride is a candidate molecule for treating peri-implantitis. The purpose of the present study was to determine the potentiality of acidic fluoride solution in treating peri-implantitis. We evaluated acidic sodium fluoride solution for bactericidal activity against periodontal pathogens, and short-term efficacy against microorganisms simulating in vivo conditions. In addition, we determined the effects of acidic sodium fluoride solution on commercially pure titanium, comparing surface properties between fluoride-treated and non-treated titanium. Furthermore, we examined cell proliferation and osteoblastic differentiation of rat bone marrow cells culture on fluoride-treated or non-treated surface and evaluated the effect of remained fluoride ion to osteoblastic cell behavior.

MATERIALS AND METHODS

**Bacterial strains**
For the microbiological tests, 2 strains of periodontal pathogen were obtained: *Aggregatibacter actinomycetemcomitans* (ATCC 43718) from the Department of Periodontics at Tokyo Medical and Dental University (Tokyo, Japan) and *Porphyromonas gingivalis* (GAI 7802) from the Life Science Center, Gifu University (Gifu, Japan). Both strains were cultured in growth medium (ABCM broth, Eiken Chemical, Tokyo, Japan) at 37°C under anaerobic condition.

**Preparation of titanium materials**
Titanium disks (8 mm in diameter) were produced from commercially pure grade 2 titanium bars (Rare Metallic Co., Tokyo, Japan). Specimens were cut and machined, followed by sonication treatments in ultrapure water, and additional sonication treatments in 70% ethanol, prior to drying under the hood. Half of the titanium disks were immersed in 9,000 ppm F\(^-\) sodium fluoride solution adjusted to pH 5.0 with phosphoric acid, for 30 min at 37°C, followed by washing with ultrapure water for 1 min. Both of specimens were observed using an atomic force microscope (SPM-9600, Shimadzu Corporation, Kyoto, Japan) and roughness average (Ra) measurement was done (Fig. 1). Ten specimens were used for every test condition.

**Culture of bone marrow cell**
As established previously\(^{30}\), rat bone marrow cells were isolated from the femurs of 4-week-old male Wistar rats. Bone marrow cells were cultured in alpha-modified Eagle’s medium (alpha-MEM, Gibco Laboratories, CA, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were incubated at 37°C in a humidified atmosphere consisting of 95% air and 5% CO\(_2\). The medium was changed every 2 days.

**Determination of MIC**
A broth-dilution technique was used to determine the susceptibility of the *P. gingivalis* and *A. actinomycetemcomitans* to sodium fluoride solution. The initial density of microorganisms was approximately 10\(^6\) colony-forming units (CFU)/mL. Brain heart infusion broth (BHI broth, Becton Dickinson, NJ, USA) was supplemented with 5 g/L.
yeast extract, 5 mg/L hemin, 0.5 mg/L vitamin K1, and sodium fluoride to an adjusted concentration range of 112.5–1800 ppm F. To determine the bactericidal effect of sodium fluoride without any additives, all tested media were adjusted to pH 7.4. The bacterial growth was monitored visually after 24-h incubation in the test media. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of fluoride ion observed to repress visible growth.

Short-time exposure of microorganisms to fluoride
Short-time exposure test was performed according to the method of Eick et al.31 with some modification. Microorganisms at 10⁶ CFU/mL were exposed to 1125, 2250, 4500, 9000 ppm F⁻ acidic sodium fluoride solution, or saline as the control group, for 30 min. All test solutions were adjusted to pH 5.0 using phosphoric acid. After centrifugation at 4,000g, the supernatant was removed, and 10 mL of BHI broth was added. The bacterial suspensions were incubated under anaerobic conditions for 24 h, followed by the determination of CFU. The experiment was replicated 5 times for each concentration.

X-ray photoelectron spectroscopy (XPS)
To characterize the surface passive film of commercially pure titanium specimens, which were untreated or treated with acidic sodium fluoride solution, the surface chemical analyses of the specimens were assessed using an X-ray photoelectron spectrometer (XPS, JPS-9010MC, JEOL, Tokyo, Japan). All binding energies given in the present study were relative to the Fermi level, and all spectra were excited with the Mg Ka line (1253.6 eV). The spectrometer was calibrated against the Au 4f peak of pure gold and the Cu 2p peak of pure copper. The binding energies were calibrated using the energy of the C 1s peak of contaminant carbon at 285.0 eV. The take-off angle of the photoelectron was 90° to the surface of the specimen.

Cell proliferation
Cell proliferation was assessed by measuring the viability of cells by using WST-8 based colorimetry (Cell Counting Kit-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). This procedure determines the mitochondrial dehydrogenase activity in living cells by measuring the conversion of WST-8 into a water-soluble disulfonated tetrazolium salt. Cells were plated onto titanium specimens in a 24-well plate (5×10⁴ cells/well) and cultured with alpha-MEM supplemented with 10% FBS. After 48 and 96 h of incubation, the culture media were removed, culture layers were rinsed twice with PBS, and cells were cultured for 4 h in alpha-MEM containing 10% WST-8 reagent and 5% CO₂ at 37°C. The amount of formazan produced was measured using a microplate reader at 450 nm.

Gene expression
Gene expression was analyzed using reverse transcriptase-polymerase chain reaction (RT-PCR) for examination of alkaline phosphatase (ALP), type I collagen (COL1), osteocalcin (OCN), and GAPDH. In total, 5×10⁴ cells/well were seeded onto the titanium specimens and cultured in osteogenic medium: alpha-MEM supplemented with 10% FBS, 50 mg/mL ascorbic acid, 10 mM b-glycerophosphate, and 10⁻⁶ M dexamethasone. After 7 and 21 days of culturing, total RNA was extracted from cells with TRIzol Reagent (Life Technologies, CA, USA). cDNA was synthesized from 1 μg of RNA with the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies, CA, USA). Quantitative PCR was performed using the Power SYBR Green PCR master Mix (Life Technologies, CA, USA) in the Applied Biosystems 7300 Real Time PCR System (Life Technologies, CA, USA). Forty amplification cycles were performed (denaturing step of 15 s at 95°C, annealing and extension step of 60 s at 60°C). All results were calculated using 2-ΔΔCt method and normalized to GAPDH expression level measurements.

Mineral deposition
Mineralized matrix formation activity was assessed according to the method of Uchimura et al.32. Bone marrow cells were grown on titanium specimens and cultured as previously described. Briefly, 5×10⁴ cells/well were seeded onto the titanium specimens in a 24-well plate, and then cultured with the osteogenic medium containing calcine (1 μg/mL). After culturing for 21 days, the titanium specimens were washed with ultrapure water and dried at room temperature. The surfaces of the specimens were observed using a 20× objective lens (Plan Apo, Nikon, Tokyo, Japan) under a fluorescence microscope (BIO ZERO BZ-8000, Keyence, Osaka, Japan) at 495 nm. Captured images of the bone-like mineralized area (staining area) were analyzed by Image-J 1.45S software (National Institutes of Health, MD, USA). The amount of bone formation was expressed as a percentage (staining area/statistics vision area ×100%).

Statistical analyses
PASW statistics 18 software was used for all statistical analyses. The data were expressed as mean±SE. Student’s t test was used for statistical analysis, and multigroup comparisons were evaluated using two-way ANOVA followed by Tukey’s test as a post hoc test.

RESULTS

Determination of MIC
In the microbiological assays, sodium fluoride solution exhibited bactericidal activity against anaerobic species. The inhibitory effects of fluoride solution against A. actinomycetemcomitans and P. gingivalis were observed at concentrations just above 225 and 900 ppm F⁻ in the determination of MIC, respectively.
**Short-term exposure of microorganisms to the fluoride solution**

The majority of the tested fluoride solutions significantly reduced the number of periodontal pathogens compared to saline 30 min after initiation of the fluoride exposure assay. The reduction of both *P. gingivalis* and *A. actinomycetemcomitans* occurred in a concentration-dependent manner. Both of strains exhibited significant decreases in higher than 2250 ppm F\(^-\) when comparing to saline and 1125 ppm F\(^-\) (p<0.05). 1125 ppm F\(^-\) solution was not sufficient for bacterial reduction. No difference in the reduction of periodontal pathogens was detected between 4500 and 9000 ppm F\(^-\). Comparison of the reduction between bacterial strains revealed that *A. actinomycetemcomitans* was more susceptible to reduction than *P. gingivalis* at concentrations exceeding 4500 ppm F\(^-\) (p<0.05), whereas *P. gingivalis* was more susceptible (p<0.05) at 1125 ppm F\(^-\) (Fig. 2).

**X-ray photoelectron spectroscopy (XPS)**

The surface property of fluoride-treated and non-treated specimens were analyzed by AFM and the chemical compositions of the modified surfaces treated with acid sodium fluoride solution and non-treated surfaces were compared by X-ray photoelectron spectroscopy (XPS) analysis. AFM analysis revealed that acidic fluoride treatment did not alter the titanium surface significantly (Fig. 1). Fluoride (F 1s) and phosphate (P 2p) were both detected on the fluoride-modified surface, whereas neither was observed on the non-treated surface. Concentrations of other trace elements, including carbon and nitrogen, were equivalent on the 2 surfaces (Table 1). XPS element analyze detected F 1s peak (685.0 eV), but sodium fluoride (684.0 eV) was not found on fluoride-treated surface. This finding indicated that acidic sodium fluoride solution was eliminated completely by washing in ultrapure water, therefore fluoride-treated specimen was modified by the tested solution. The residue of fluoride-treated surface was estimated to be TiF\(_4\) or Na\(_2\)TiF\(_6\) (Fig. 3), but sodium peak was not detected in this experiment.

**Cell proliferation**

Proliferation of rat bone marrow cells was examined on the 2 types of titanium surfaces. The cell viability of adherent cells was measured by WST-8 assay. At 48 h, comparable values of cell proliferation were observed on both surface types; however, cells adhered to the fluoride-modified surface showed greater proliferation (p<0.05) than did those adhered to the untreated surface after 96 h of culturing (Fig. 4).

**Gene expression**

Osteoblastic differentiation of rat bone marrow cells cultured on the 2 types of titanium surfaces, in the presence of osteogenic supplements, was examined by RT-PCR assay. The mRNA levels of the early and late osteoblastic markers were not significantly affected by the surface type. Cell differentiation on both fluoride-modified and non-treated surfaces exhibited comparable values in terms of ALP, type I collagen, and osteocalcin expression at 7 and 21 days. In all cases, although a trend toward higher values of type I collagen, and osteocalcin seemed to exist on the fluoride-modified surface compared with the non-treated surface, no significant difference was found in any markers (Fig. 5).

<table>
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<th>Table 1</th>
<th>XPS element percentage of fluoride-treated and non-treated titanium (at%). XPS analyze detected fluorine and phosphorus signals on the fluoride-treated surface.</th>
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<tr>
<td>Element</td>
<td>Non-treated</td>
</tr>
<tr>
<td>Ti</td>
<td>27.7</td>
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<tr>
<td>O</td>
<td>72.3</td>
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<tr>
<td>F</td>
<td>0</td>
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Fig. 3 Comparison of chemical compositions between machined and fluoride-modified surfaces. F 1s and P 2p signals were observed on both the fluoride-modified titanium surface (a) and the non-treated surface (b). XPS analyze revealed the existence of fluoride compound (685.0 eV) which was different from sodium fluoride (684.0 eV).

Mineral deposition
Measurements to quantify the area of mineral deposition were performed using the image analyzer under a fluorescence microscope. Extracellular mineral deposition exhibited a significant difference (p<0.05) between the fluoride-modified surface and the non-treated surface after 21 days of culturing (Figs. 6, 7).

DISCUSSION
Several clinical trials have demonstrated that fluoride agents effect the reduction of periodontitis, and exhibit bactericidal activity against P. gingivalis in vitro studies. In the present study, the bactericidal activity of fluoride agents against anaerobic bacterial strains was confirmed based on analysis with the periodontal pathogens P. gingivalis and A. actinomycetemcomitans. Interestingly, P. gingivalis exhibited lower survival rates at lower fluoride concentrations and, conversely, higher survival rates at higher concentrations, than A. actinomycetemcomitans did, in short-time exposure tests. In MIC determination tests, P. gingivalis required higher concentrations of fluoride for growth inhibition in MIC determination tests than did A. actinomycetemcomitans, indicating that P. gingivalis is less susceptible to fluoride agents. In general, the optimal pH for bacterial growth is close to neutral, and microorganisms lose viability in acidic environments; however, some bacterial strains exhibit acid-neutralizing activity through amino acid fermentation and may thereby acquire resistance to acidity. Because neither P. gingivalis nor A. actinomycetemcomitans are able to grow even in mildly acidic pH, the acidic sodium fluoride solution reduced the cell viability of the tested microorganisms.

In this present study, XPS analysis revealed
fluoride and phosphate ions on the surface of fluoride-modified titanium. Previous studies detected fluoride atoms on surfaces treated with fluoride solutions\textsuperscript{19,38}. Ellingsen et al. reported significant increases in bone retention around sodium fluoride-treated titanium implants in rabbits\textsuperscript{39}, hypothesized that the fluoride forms covalent bonds with the titanium surface and is later replaced by oxygen from phosphate. Such a mechanism would be useful, because phosphate ions on a titanium surface are known to exert a beneficial effect on bone formation\textsuperscript{40,41}. The detection of fluoride and phosphate ions on titanium surfaces are consistent with the results presented in this study.

Previous studies have shown that osteoblastic

cell proliferation on titanium surfaces is enhanced following treatment with hydrofluoric acid\textsuperscript{16,42}, and that sodium fluoride stimulates proliferation of bone marrow stem cells and osteoprogenitor cells\textsuperscript{26,27,29}. In the present study, results of a WST-8 assay indicated a significant increase in cell proliferation on the fluoride-modified titanium surface after 96 h of culturing, but no difference at 48 h. These results suggest that titanium surface modification by sodium fluoride treatment only
enhances osteoblast lineage cell proliferation.

Additionally, the nano-topographical and chemical modification of fluoride-treated surfaces modulate osteoprogenitor cell differentiation and increase expression of osteogenic markers. Type I collagen, the most abundant protein component of the bone extracellular matrix, is secreted by stromal cells during early stage of differentiation into osteoblasts, and subsequently forms a scaffold for the bone matrix. ALP is considered a marker of osteoblastic differentiation and commitment of the osteoblast to form mineralizing bone tissue. Osteocalcin is a non-collagenic protein of the bone matrix, and is expressed in late stages of osteoblastic cell differentiation.

In the present study, real-time PCR measurements were performed to determine the expression levels of these cell differentiation and osteogenic markers. No significant differences were observed in the expression levels of type I collagen and osteocalcin, or ALP activity, between fluoride-modified surfaces and the untreated machined surfaces.

Osseointegration involves processes related to bone formation, adaptation to function, and repair as a consequence of continuous cellular events. In the early stage of bone formation around titanium implants, neovascularization occurs, which involves recruitment of osteoblasts from bone marrow stem cells, thereby leading to osteogenesis through the proliferation and differentiation of osteoblastic cells. Surface topography and chemical modification of titanium implants may positively influence bone formation during the early stages of osseointegration. In the current study, the area of calcified bone-like tissue on the titanium surface was measured as the volume of mineralized matrix formation. Significant bone formation was observed on the fluoride-modified titanium surface, suggesting that acidic sodium fluoride treatment may enhance bone retention. Recent study reported that sodium fluoride affects proliferation and apoptosis of murine osteoblastic cell through Insulin-like growth factor I receptor. In this study, sodium fluoride enhanced rat bone marrow cell and osteoblast-like cell growth and consequent mineralization.

The main objectives of the present study were to determine bactericidal activity against periodontal pathogens and chemical modifications to the commercially pure titanium surface, as these represent potentially important clinical aspects associated with implantation. The results indicate that an acidic sodium fluoride solution exhibits bactericidal activity against anaerobic bacterial strains as observed in peri-implantitis. Additionally, this solution can modify the surface of commercially pure titanium and enhance bone formation. The difficulty of inducing re-osseointegration in a previously infected implant site is likely associated with conditions resulting from efforts to treat infection and the bone regenerative therapy itself. Fluoride appears to both reduce the levels of pathogenic bacteria and create a favorable surface for re-osseointegration. In clinical situation, these findings are preferable to recover the health of peri-implant tissue. However, excessive concentration of fluoride may cause cell death in the surrounding epithelial and bone tissues. Therefore, separation technique from the surrounding tissue must be considered in fluoride application.

In the present study, pristine machined titanium specimens were used in the cell biology tests. However, the surface topography and design of currently available titanium implants are often diversified, and may not facilitate osseointegration as effectively as the moderately rough surfaces. In this study, surface roughness measurement by using AFM was done. Ra value was not changed by treating with acidic sodium fluoride solution, but the enhancement of rat bone marrow cell proliferation and mineralization were found in vitro tests. These findings indicated that fluoride treatment can affect to chemical character on the titanium surface, but not alter the surface topography. Base metals of titanium implants also differ by vendors. Commercially pure titanium and its alloys (e.g., Ti-6Al-4V, Ti-6Al-7Nb) exhibit different corrosion behaviors in fluoride solutions. Therefore, further studies are needed to demonstrate the efficacy of treating rough titanium surfaces and various titanium alloys with acidic sodium fluoride solution, and also on the contaminated titanium surface.

In conclusion, these results demonstrate that acidic sodium fluoride solution exhibits bactericidal activity against P. gingivalis and A. actinomycetemcomitans. Furthermore, bone marrow cell proliferation and
bone formation were both observed to increase on the commercially pure titanium samples treated with acid sodium fluoride solution. These findings suggest that treatment with acidic sodium fluoride solution could eliminate anaerobic bacteria around infected implant sites and modify commercially pure titanium implants, which would be favorable in re-osseointegration although further studies are absolutely required to conclude this.

REFERENCES