Effect of silver ion coating of fixed orthodontic retainers on the growth of oral pathogenic bacteria

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INTRODUCTION

Retention after active orthodontic treatment is one of the most important procedures in orthodontics. It prevents the relapse of teeth in dental arches; orthodontic retainers are typically used to retain teeth in the arches. In particular, fixed retainers are often applied to lower anterior teeth (canine-to-canine fixed retainer) to prevent relapse of the teeth (Fig. 1). The retainer ensures a steady and stable retention effect in the treatment and it causes little discomfort to patients, because it consists of a piece of thin wire attached to the lingual surfaces of the lower anterior teeth with orthodontic resin. However, fixed retainers have some side effects; the wire is a gathering site for bacteria, biofilm, and dental calculus that cause caries and periodontal disease.

The initial bacterial adhesion to the wire is a key step that induces a biofilm. The subsequent growth of these initially adherent bacteria is difficult to remove completely by tooth brushing, and consequently can lead to the formation of pathogenic oral biofilms, which are key factors in dental caries and periodontal disease.

Thus, it is important to prevent pathogenic bacteria from adhering to the wire in the first place.

Silver is recognized as a strong disinfectant, having a broad bactericidal spectrum. An important characteristic is that bacteria do not build up strong resistance against Ag. Even at low concentrations, silver ions are effective against bacteria and fungi in aqueous solution. Recently, Ag ions and silver nanoparticles have been used to prevent the initial bacterial adherence to the dental materials by incorporating them into the materials. This has reduced biofilm formation. However, to the best of our knowledge there is no study report that describes antibacterial activities of surface coated fixed orthodontic retainers. Thus, we sought to examine the effects of silver ion coating of the orthodontic wires on the inhibition of growth of oral pathogenic bacteria.

MATERIALS AND METHODS

Orthodontic wires

Commercially available stainless steel (SS) and titanium (Ti) wires, typically used for orthodontic fixed retainers (canine-to-canine fixed retainers), were used. For all experiments, 0.016 inch (0.04 cm) diameter wires were cut into different lengths according to the needs of the experiments before applying the coat as described below. Briefly, the surfaces of SS and Ti wires were cleaned with alcohol and dried before applying the coat. A modified microwave plasma process was employed to coat the wires that doesn't require air-vacuum, can be done at a low temperature (below 50°C) in short period of time. A microwave machine; EM-1900 (SANYO Electric Co., Ltd., Osaka, Japan) at 570 w was used to generate plasma. The cleaned surfaces of the wires were coated with silver ions (Ag ions) by immediately dipping into a solution containing Ag ion (5,400 ppm) and generating plasma inside the microwave machine for 90 s limiting the risk of any damage that may occur. After 90 s, the wires were washed with distilled water and dried to obtain a uniform coat. With this method an average of 14.817±2.163 μm coating ranging from 10.672 μm to 19.01 μm was obtained (Pika Power Technology Ltd. Co., Hyogo, Japan). Equal numbers of uncoated
plates were then cultured under anaerobic conditions at 37°C for 48 h in an anaerobic box (Anaeropack System, Mitsubishi Gas Chemical Co. Inc, Tokyo, Japan) for the cariogenic group or in a complete anaerobic box (10% H₂, 10% CO₂ and 80% N₂) for the periodontopathic group.

For the growth of periodontopathic bacteria hemin (Wako Pure Chemical Industries Ltd., Osaka, Japan) and vitamin K (Wako Pure Chemical Industries Ltd.) were added to all culture media. After incubation for 48 h, the diameters of the clear zones around the wires were measured with a slide caliper.

**Effect on biofilm formation**

The inhibitory effect on biofilm formation was examined on the surfaces of the wires using a *S. sobrinus* suspension. Five pieces of 50 mm long wire from each group were incubated in TSs medium with a suspension of *S. sobrinus* (TS without dextrose but containing 1% sucrose) for 8, 12, or 24 h. Wires were then gently shaken in chilled PBS to remove loose biofilm. Biofilm retained attached on the wires was then stained with BacLight. A LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Invitrogen Detection Technologies, Carlsbad, CA, USA) was used to evaluate the bactericidal effects of the Ag ion coat. *S. sobrinus* biofilms attached to the wires were stained with 0.5 μL BacLight stain (a mixture of SYTO 9 and propidium iodide) in separate microtubes. In this staining system, viable bacterial cells exhibit green fluorescence, whereas nonviable bacterial cells show red fluorescence. The selective dye uptake depends upon cell membrane integrity, allowing dead bacteria to be distinguished readily from viable bacteria. The excitation/emission wavelengths of the dyes were approximately 480/530 nm for SYTO 9 (green signals) and 520/580 nm for propidium iodide (red signals). Bacteria cells were evaluated using a fluorescence microscope (CKX41, Olympus, Tokyo, Japan).

**Water-insoluble glucan measurement**

The bacterial cell suspension of *S. sobrinus* (OD₅₄₀=0.7) was cultured in 7.5 mL of TSs medium with 50-mm long pieces of wires under anaerobic condition to form biofilms on the surfaces of wires for 8 or 16 h. The water-insoluble glucan (WIG) in the retained biofilms was measured after separating the bacterial cells from WIG on the wire. Each piece of wire was gently washed in PBS to wash away culture medium and then transferred carefully in 1 mL of 0.5 M sodium hydroxide solution, incubated for 15 min on ice, vortexed, and centrifuged (5,000 rpm, 10 min) to separate the WIG from the bacterial cells in the biofilms. The amount of dissolved WIG was measured colorimetrically using the phenol-H₂SO₄ method using a Bio-Rad iMark Microplate reader (Bio-Rad, Tokyo, Japan) at 492 nm. The 500 μL of WIG solution from each sample was reacted with 5% phenol and H₂SO₄, and then 200 μL of each reaction mixture was used to measure the amount of WIG (μg/mL). To obtain a standard curve, 0, 25, 50, 75, 100, 150, and 200 μg/mL of glucose were also measured using the same method. The experiments were repeated.
three times for reproducibility.

**Gas-chromatography after inoculation with Porphyromonas gingivalis**

A gas chromatography method (Shimadzu GC-2010; Shimadzu Corporation, Kyoto, Japan) was used to evaluate the effect of Ag ion-coated fixed orthodontic appliance by measuring the volumes of two major volatile sulfur compounds (VSCs); hydrogen sulfide (H$_2$S) and methyl mercaptan (MMC) gases from overnight cultures of periodontopathic bacteria; Porphyromonas gingivalis (P. gingivalis) in this study. These gases are responsible for the offensive odor commonly known as bad breath or oral malodor, which is most commonly attributed to periodontal pathogens, including P. gingivalis, as the chemical end products of bacterial putrefaction\(^\text{16-18}\). Ti and Ti$^+$ wires used in this experiment; two pieces of 50-mm long wire were incubated for 0, 8, or 16 h under anaerobic conditions in P. gingivalis (ATCC33277) suspensions (OD$_{540}$=1.0). Each time, the total gas from each culture tube was collected using a 2.5-cc disposable syringe and a 23-G needle. Then, VSCs was measured using a gas chromatography unit (Shimadzu GC-2010 GC, Kyoto, Japan). After 24 h, the concentrations of the gases were high and thus were diluted 500-fold with pure O$_2$ and then measured. Bacterial OD also was measured by the method mentioned above.

**Measurement of released Ag ions**

The release of Ag ion from the coating Ti wire was measured with inductively coupled plasma atomic emission spectrometer (ICP-AES, system, CIROS-120, Rigaku, Japan). The quantity of Ag ion released after 24 h from the specimens (50 mm long wire) was expressed as the amounts (ppm) of Ag ion in PBS solution (10 mL). Ti wire without Ag ion coating were also prepared as controls. Three specimens from each group were measured.

**Cell cytotoxicity**

To test the effect of Ag ion released from specimens on human gingival fibroblast cell (HGF Gin-1, DS Pharma Biomedical, Japan) were used. The cytotoxicity of Ag ion was tested by using a standard MTT assay described elsewhere with a minor modification\(^\text{19}\). Briefly, HGF cells were plated in 96-well plates at a density of approximately 7.0×10$^4$ cells/mL in medium DMEM with 10% FBS, 24 h after plating above mentioned solutions (PBS with Ag ion and without Ag ion) were added. The Ag ion solution added to the cell culture medium at 10%, 1%, 0.1% concentrations separately. After 48 h of incubation, the medium was replaced with MTT solution \([3-(4,5\text{-dimethylthiazol-2-yl})-2,5\text{-diphenylterazolium bromide}]\) (Roche Diagnostics Corporation, Indianapolis, IN) dissolved at a final concentration of 1 mg/mL in serum-free, Phenol red-free RPMI (Biochrom KG, Germany), for a further 4 h incubation. Then, the MTT formazan reaction was measured at a wave length of 540 nm and a reference wave length of 655 nm using a Bio-Rad iMark Microplate reader (Bio-Rad, Tokyo, Japan).

**Statistical analysis**

All numerical data were analyzed using the SPSS software (ver. 11 for Windows; SPSS Inc., Chicago, IL, USA). In all experiments minimum of three samples (e.g., for bacterial OD 6 sample-data, total WIG 4 sample-data, for VSC 4 sample-data, for MTT assay 6 sample-data, for coat thickness 10 measurements, for others 3 sample-data) were used for each group and the bacteriological experiments were repeated three times under the same conditions to ensure reproducibility. For comparisons, the Mann-Whitney U-test was used. The levels of WIG/mm$^2$ were analyzed by one-way analysis of variance (ANOVA) and Tukey’s HSD with a confidence level of 95%.

**RESULTS**

**Effects on bacterial growth**

The radial diffusion test demonstrated that most of the Ag ion-coated samples (Ti$^+$ and SS$^+$ wires) showed a clear zone of more than 2 mm diameter around them with both the cariogenic and periodontopathic bacteria (Figs. 3, 4). In contrast, almost no detectable clear zone

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**Fig. 3** A photograph of culture plates showing the results of radial diffusion tests (a). All coated samples demonstrated bacterial growth inhibition zones around the wires. Two cariogenic bacteria used in this study showed similar result (b, c). The asterisks indicate statistically significant differences ($p<0.05$).
Fig. 4 Photographs indicating the clear zone in radial diffusion test in Fn (a). Antibacterial activities of SS and Ti wires against Fn (b), Pg (c), and Pi (d) are shown. The asterisks indicate statistically significant differences ($p<0.05$).

Fig. 5 Fluorescence photomicrographs of *S. sobrinus* biofilms stained with the BacLight Bacterial Viability Kit. Clusters of *S. sobrinus* biofilm adhered on the surfaces of the different wires: SS (a), SS+ (b), Ti (c), and Ti+ (d). Viable bacterial cells exhibit green fluorescence, whereas nonviable bacterial cells exhibit red fluorescence.
was observed around uncoated SS or Ti wires with the cariogenic (Fig. 3) or periodontopathic (Fig. 4) bacteria. The diameters of the growth-resistant zones of the Ag ion-coated groups were significantly larger than those of the uncoated groups. The effect on the cariogenic bacteria was stronger than on the periodontopathic bacteria.

Effects on formation of S. sobrinus biofilms
Ag ion-coated wires showed bactericidal effects using the BacLight (Fig. 5). Live S. sobrinus cells are visualized with green fluorescence and dead cells with red in the same microscopic location on a slab surface by changing only the emission filters. In the uncoated groups, only live cells were observed on the slab surface. However, in the coated group, both dead and live cells were present. When bacterial cells were incubated with uncoated wires, large numbers of S. sobrinus cells remained alive in the biofilm clusters (Figs. 5a, 5c). There was no marked difference between the control groups. In reverse, more dead than live cells were observed in all the coated groups. Biofilm clusters were not formed in the normal way; most bacteria appeared to have suffered damage and some died even before secreting glucan (Figs. 5b, 5d). The growth of biofilms was apparently inhibited by the presence of Ag ions.

The amounts of bacteria and WIG in the retained biofilm on the wire surfaces are summarized in Fig. 6. From both results, both the coated wires (SS+ and Ti+) showed significantly lower susceptibility to biofilm formation compared with the uncoated wire samples (p<0.05) after 24 h specifically. It appeared that a maximum amount of biofilms was formed on SS and a minimum on Ti+.

Effects on the production of oral malodor gases
The cell growth of Pg was reduced in the presence of Ti+ compared with Ti and control (Fig. 7a). Gas chromatography showed that production of hydrogen sulfide (H,S) and methylmercaptan (CH₃SH) gases by Pg were reduced significantly in the coated groups compared with uncoated groups in the 8-h culture. Lower amounts of H₂S and CH₃SH gases were produced in the 16-h culture in the presence of Ag ion coated on Ti wire surfaces (Figs. 7b, 7c).

Amount of released Ag ion
The Ag ions released from the specimens were measured by ICP-AES. After 24 h of incubation the

![Fig. 6](image)

The amount of bacterial cells (a) and WIG (b) after S. sobrinus biofilm formation with different types of wires for 5, 10, or 24 h are shown. The asterisks indicate statistically significant differences (p<0.05).

![Fig. 7](image)

Bacterial cell viability of Pi after 8 and 16 h cultures with Ti wires (a). The amounts of H₂S (b) and CH₃SH (c) are shown. The asterisks indicate statistically significant differences (p<0.05).
Fig. 8 Activity of human gingival fibroblast (HGF) cells acquired from MTT assay shows that addition of Ag ion didn’t cause any change compared with the control (grown without Ag ion) at any stages. Growth activity was same with the control even when the solution (released Ag ion from Ti+ in PBS) was added at 10% concentration in the cell culture medium (DMEM).

Table 1 Silver ion release from the titanium wire

<table>
<thead>
<tr>
<th>Wire immersion (24 h)</th>
<th>Silver ion release (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Ti)</td>
<td>0</td>
</tr>
<tr>
<td>Ti+</td>
<td>0.043±0.005</td>
</tr>
</tbody>
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average concentrations of detected Ag ion were as follows: 0 ppm for Ti wire, 0.043±0.005 ppm for Ti+ wire; shown in Table 1.

Cell cytotoxicity

Results of MTT assay show that there were no significant differences in detection of formazan concentrations between the experimental group and the control group (Fig. 8). That indicates that cellular activities were kept at almost the same levels at all these experimental conditions. Even cell activity was same when Ag ion concentration was 10% and also when cells were cultured for 24 h.

DISCUSSION

The surfaces of dental appliances act as passive surfaces and are prone to bacterial adhesion that can eventually develop into material-associated infection. To prevent the adhesion of bacteria on biomaterials surfaces several surface coating materials and surface coating techniques are being tested. Among them, the antibacterial and antifungal effects of silver ions have long been known. For centuries silver has been known to have bactericidal properties. As early as 1000 B.C., the antimicrobial properties of silver in rendering water potable were appreciated. Silver compounds have been exploited for their medicinal properties for centuries as well. Avicenna, in 980 AD, used and prescribed silver as a blood purifier for heart palpitations and for offensive breath. Silver was combined with arsephenamine, because of its antimicrobial properties, and was used to treat syphilis during the early part of the 20th century. From this historical background, we sought to inhibit bacterial growth and activities on the surfaces of coated wires.

In this study, Ag ion coating on the wire showed anti-bacterial effects against the most common species of oral pathogenic bacteria. In radial diffusion tests, clear zones were obviously produced around the coated wires, indicating that Ag ions on the coated wires were diffusing into the gel and inhibited bacterial growth. Clear zones caused by the coated wire were significantly larger than those of the uncoated samples. There were some differences in the susceptibility to Ag ions between cariogenic and periodontopathic pathogens. The size of clear zone in the gel with S. mutans was larger than that with S. sorbinus. The effect of Ag ions on cariogenic pathogens was stronger than on the periodontopathic pathogens. This may be because anaerobic bacteria absorb a lower amount of the ions during metabolic activity.

Microscopy with the LIVE/DEAD BacLight Bacterial Viability Kit revealed that Ag ions have bactericidal activity on cariogenic cells as well as antibacterial activity. Although not investigated in this study, these antibacterial and bactericidal behaviors of Ag ions may result from the following mechanisms: inhibition of DNA replication and mitosis, effects of the permeability of the cell membrane, and control of the oxidation of glucose as reported by other researchers.

The antibacterial and bactericidal effects of the Ag ion-coated wires were confirmed in measuring water-insoluble glucan (WIG), the main component of biofilm formed by cariogenic bacteria. The coated wires showed a significant reduction of WIG on the wire after 24 h. This indicates that the Ag ion coat inhibited the bacteria from producing the biofilm through its antibacterial and bactericidal effects. The same effects of the Ag ion-coated wires with the cariogenic bacteria were confirmed in the measurements of H2S and CH3SH, two major components of the VSCs produced by periodontopathic bacteria. VSCs were significantly reduced after 8 h culture with the Ag ion-coated wire. Although that effect was not significant at 16 h but reduced production of VSCs was observed. In the present culture protocols the antibacterial effect of Ag ion on periodontopathic bacteria appeared to be weaker than that on the cariogenic bacteria. More specifically, small amount of S. sobrinus produced biofilms were collected directly from the surfaces of the wires for analysis. While, proportionally large volume of gases produced by Pg were collected for VSCs analysis; might be due to the availability of culture medium for excessive Pg growth even after 8 h. That may have made the difference—needs further investigations with an improved experimental
The level of released Ag ion in PBS was 0.043 (±0.005) ppm in this study, which is well below the mark that WHO allows for drinking water disinfection (up to 0.1 mg/L)\(^\text{[27]}\). Furthermore, it seemed that this level of Ag ion didn’t cause detectable cytotoxic effect on HGF cells. Therefore, it can be suggested the Ag ion coated orthodontic wires would serve better by maintaining low toxicity for mammalian tissue when used clinically.

In summary the results of this study suggest that Ag ion coating on pure titanium and stainless steel wires inhibited the growth and pathogenic activities of oral cariogenic and periodontopathic bacteria, important evidence for the future application of simple Ag ion-coated dental materials, including orthodontic appliances. Remarkable resemblance could also be observed with the data reported in some recently published articles, most of them showing enough promises needing little more improvement\(^\text{[19-25]}\). In contrast, the effect of Ag ion coating on two types of orthodontic wires (stainless steel and titanium) was investigated by analyzing antibacterial potency against five species of oral pathogenic bacteria including biofilms and VSC gases produced by Pg in this study. In addition, this study opens a wide range of possibility for coating the surfaces of various types of materials without limiting on the orthodontic wires only that also may serve better while antibacterial Ag-ion is applied. However, further studies on the long-term anti-bacterial effects of the Ag ion coat are required, along with clinical trials.

REFERENCES