Effect of microbubbled water on the removal of a biofilm attached to orthodontic appliances — An in vitro study —

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Orthodontic appliances often cause oral diseases such as dental caries and gingivitis due to the attachment of an oral biofilm. However, there are few reliable methods to remove the biofilm from the orthodontic appliances. The aim of this study was to investigate the effects of microbubbled water on the removal of biofilms made with Streptococcus mutans or Candida albicans on orthodontic appliances. The orthodontic appliances with biofilm were immersed with microbubbled water and the remaining biofilm on the appliances was detected and measured using a micro-plate reader and an absorbance meter. The microbubbled water had a sufficient effect on the removal of biofilm from orthodontic appliances. The effects of microbubbled water were significantly higher than those of tap water (S. mutans: p<0.05, C. albicans: p<0.01). The results of this study suggest that microbubbled water is effective in the removal of biofilm from the mouth of orthodontic patients.

Keywords: Microbubbled water, Removal of biofilm, Orthodontic appliance

INTRODUCTION

In orthodontics, fixed and removable appliances are essential tools in the treatment of patients. However, the placement of orthodontic appliances often causes oral biofilm, which contains a number of specific microorganisms associated with oral diseases1-3). A typical cariogenic bacterium, S. mutans, increases in the mouth of orthodontic patients with fixed appliances4). Hibino et al also showed that C. albicans an opportunistic pathogen, was commonly isolated from the mouth of orthodontic patients with removable appliances5). These microorganisms in the biofilm sometimes enter into blood stream and cause bacteremia. Therefore, it is critical to remove the biofilm on the orthodontic appliances.

There are various methods to remove the biofilm attached to the orthodontic appliances. An ionic toothbrush was examined for the removal of biofilms attached to the appliances in the mouth, but the results did not show substantial effects on the removal of biofilm or the reduction of gingivitis6). A water-irrigating device was also applied for the removal of the biofilm7). However, the device was no more effective than an ordinary tooth-brushing or flossing. Furthermore, mouth washing with chlorhexidine and tooth brushing with antimicrobial tooth-paste shows no statistically significant effect in comparison to ordinary methods8). Removal of biofilm remains a major clinical problem for the maintenance of oral hygiene in dentistry.

Microbubbled water has been utilized in different fields: Strict cleaning of vegetables, decomposition of organic chemicals, waste-water treatment, noninvasive assessment of inflammation, and food processing9-18). There are only a few reports on the removal of microorganisms in dentistry. Sharma et al.19-20) reported that Streptococcus oralis was completely removed from salivary pellicles with microbubbled water but Actinomyces naeslundii was not completely removed. No report has so far examined the effect of microbubbled water on the removal of major oral pathogens such as S. mutans and C. albicans, which are among the major components of biofilms attached to orthodontic appliances. The former is the major bacterium on the fixed orthodontic appliances9), and the latter is that of removable orthodontic appliances10).

The aim of this study was to investigate whether microbubbled water had a substantial effect on the removal of a biofilm consisting of two pathogens, on orthodontic appliances.

MATERIALS AND METHODS

Production of microbubbled water

Microbubbled water (MB) was produced in a water tank, with a device (Fig.1, Shinwa Co., Ltd. Tokyo, Japan). The bubbles became small during production and then the water became transparent after 10 min, indicating the production of microbubbled water. Therefore, water processed for at least after 10 min was used in this study. The diameter of microbubbles was measured to confirm the production of microbubbles in the water. The samples were obtained from the upper and lower parts of the water tank three times. The size distribution of the microbubbles was examined with the dynamic light scattering method in a particle characterizer (Zeta sizer nano-ZS, Syamex Co., Ltd., Hyogo, Japan).

Experiment 1: Effect of microbubbled water on the removal of biofilm from S. mutans on bracket of fixed appliance

Metal or plastic brackets (Metal brackets, Plastic
brackets, Tomy Co., Ltd., Tokyo, Japan) were put in each well of 96 well plates. The bacterial number of *S. mutans* ATCC 25175 was adjusted at a range from $10^6$ to $10^7$ CFU/mL by Brain Heart Infusion (BHI) broth with 5% sucrose. An aliquot of 100 μL of *S. mutans* was poured into each well and cultivated for 24 h at 37°C. The culture supernatant was removed from the wells, and the microbubbled water was poured into the wells, and the pouring and discarding with micropipettes was repeated for 3 min in the same wells. Tap water (TW) was also used as a control. The supernatant was discarded form each well and 100 μL of culture media (Beckton Dickinson&Co., MD, USA) with a 10% redox indicator (Alamar blue®, TREK Diagnostic Systems, Cleveland, OH, USA) was poured into the wells and incubated for 24 h at 37°C. Subsequently the optical density at 540 nm (OD540) was measured on a micro-plate reader (Multiskan Multisoft, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). The data for OD540 in the MB group were compared with that in the TW group.

Experiment II: Effect of microbubbled water on the removal of biofilm from *S. mutans* on the fixed appliance

Six intact upper first premolars from six orthodontic patients that provided informed consent were used in this study. The study was approved by the Ethics Committee at Tsurumi University (Approval number; 802). A plastic bracket was bonded on the buccal surface of each tooth with adhesive resin cement (Fig. 2, Super-Bond, SUN MEDICAL Co., Ltd., Shiga, Japan) after sterilization of the teeth with the autoclave. The tooth with bracket was fixed with paraffin wax (Paraffin wax, FEED Co., Ltd., Kanagawa, Japan) on the bottom of the cap of a clear case, and the case containing tooth and cap was disinfected with 70% ethanol. The bracket and teeth were dipped in 1.5 mL of filtrated human saliva in a 24-well plate at 4°C overnight in order to acquire a pellicle on the surface. The tooth and bracket with the acquired pellicle were placed in the bottom of a clear case. *S. mutans* was cultivated in the same procedure described above, and 5 mL of *S. mutans* culture was poured into the clear case and cultivated for two days at 37°C. The tooth with the bracket on the cap was transferred to a new sterile plastic container with two vents for injection and discharge, connected with two silicone tubes. A sterile syringe (Terumo syringe, TERUMO Co., Ltd., Tokyo, Japan) was inserted into the injection tube, and 50–100 mL of microbubbled water or tap water were then injected into the container through the syringe at a speed of 1 mL/s. The water was immediately drained through the other tube after the injection in some containers (Group a). The water was stored for 3 min in the other containers and then drained (Group b). The residual biofilm was detected with dental plaque staining (Dent liquid plaque tester, LION Co., Ltd, Tokyo, Japan). The stained biofilm on the bracket and tooth was extracted with 70% ethanol after separating.
the tooth and bracket using bracket remover (Bracket remover, Tomy Co., Ltd., Tokyo, Japan), and the optical density was measured for the extracts from both surfaces using an absorbance meter (UV-1200, SHIMADZU Co., Ltd., Kyoto, Japan) at OD540 to determine the quantity of residual biofilm. The data was compared between the teeth that were immediately drained and those drained after immersion for 3 min.

**Experiment III: Effect of microbubbled water on the removal of biofilm from C. albicans on polystyrene plate representing a removable appliance**

A 96-well polystyrene micro-titer plate was used as a material of representing a removable orthodontic appliance. The microbial number of C. albicans ATCC 18804 was adjusted by a Tryptic Soy Broth (TSB) with 4% glucose at a range from 10^6 to 10^7. An aliquot of 100 μL of C. albicans was poured into each well and cultivated for 24 h at 37°C in order to make a biofilm on the plate. The culture supernatant was discarded and the biofilm on the plate of the well were treated with the same procedure described in experiment I. C. albicans and the amount of residual biofilm was measured with the same procedure in the experiment I. The data of the MB was compared with TW groups.

**Experiment IV: Effect of microbubbled water on the removal of biofilm from S. mutans on the bracket of a fixed appliance**

The effect of microbubbled tap water for the S. mutans biofilms was compared with that of ordinary tap water using metal or plastic brackets in 96 well plates. The remaining amount of S. mutans biofilms was measured by a redox indicator at an absorbance of 540 nm on the metal bracket, and was significantly lower in the MB group than that in the TW group (Fig. 3a, **p<0.01). The S. mutans biofilms remaining on the plastic bracket was also significantly lower in the MB group than the TW group (Fig. 3b, *p<0.05). Therefore, microbubbled water (MB group) had a significantly stronger effect than that of ordinary tap water (TW group) in both the metal and plastic brackets.

**RESULTS**

**The size of microbubble**

The size distribution of the microbubbles was examined using the dynamic light scattering method in a particle characterizer. The particle size of the microbubbles obtained from the upper and lower parts of water tank was measured with a liquid particle counter 6 times. The results showed that the average diameter of microbubble was 531.1±39.46 nm in the lower part of water tank, and that was 613.6±210.41 nm in the upper part of water tank (Table 1). Therefore, microbubbled water from the lower part of tank was used in this study.

**Statistical analysis**

Each experiment was independently repeated out 3 times. The data from the each experiment was compared between the microbubbled water and the tap water groups, using the Mann-Whitney test. Statistical significance was defined as p less than 0.05. The statistical analysis was carried out with SPSS 11.0 statistical software package (SPSS, Chicago, USA).

**Table 1 The size of the microbubbles**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak number</th>
<th>Diameter (nm)</th>
<th>Intensity(%)</th>
<th>Z-Average (nm)*</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower faucet</td>
<td>1</td>
<td>502.9</td>
<td>100</td>
<td>531.1</td>
<td>±39.46</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper faucet</td>
<td>1</td>
<td>363.9</td>
<td>89.7</td>
<td>613.6</td>
<td>±210.41</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4914</td>
<td>10.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Z-Average(nm) Average particle diameter of scattered light intensity criteria

**Effect of microbubbled water on the removal of biofilm from S. mutans on the bracket of a fixed appliance**

The effect of microbubbled tap water for the S. mutans biofilms was compared with that of ordinary tap water using metal or plastic brackets in 96 well plates. The remaining amount of S. mutans biofilms was measured by a redox indicator at an absorbance of 540 nm on the metal bracket, and was significantly lower in the MB group than that in the TW group (Fig. 3a, **p<0.01). The S. mutans biofilms remaining on the plastic bracket was also significantly lower in the MB group than the TW group (Fig. 3b, *p<0.05). Therefore, the microbubbled water (MB group) had a significantly stronger effect than that of ordinary tap water (TW group) in both the metal and plastic brackets.

**Effect of microbubbled water on the removal of biofilm from S. mutans on a the fixed appliance**

The effect was also observed on the removal of the biofilm on the brackets attached to the tooth surface. Dental plaque staining clearly showed that the amount of biofilm remaining in the MB group was less than that in the TW group, both when the samples were drained immediately (Fig. 4a) and when they were drained...
Fig. 3  Effect of microbubbled water on the removal of biofilm from *S. mutans* on the bracket of fixed appliance.

- **a**: The remaining amount of *S. mutans* on the metal bracket
- **b**: The remaining amount of *S. mutans* on the plastic bracket

The vertical axis of the graph showed quantity of remaining microbes which measured by a redox indicator at an absorbance of 540 nm.

Fig. 4  Effect of microbubbled water on the removal of biofilm from *S. mutans* on the fixed appliance.

- **a**: Draining immediately
- **b**: Draining after three minutes soaking
Fig. 5  Effect of microbubbled water on the removal of biofilm from *S. mutans* on the fixed appliance.

a-1: The residual level of biofilm on the surface of the tooth with immediate draining  
a-2: The residual level of biofilm on the surface of the bracket with immediate draining  
b-1: The residual level of biofilm on the surface of the tooth with draining after 3 min of soaking  
b-2: The residual level of biofilm on the surface of the bracket with draining after 3 min of soaking  
*: A significant difference was observed, \( p < 0.05 \).
The vertical axis of the graph showed quantity of remaining microbes in a 70% ethanol extract of dental plaque stain at an absorbance of 540 nm.

Fig. 6  Effect of microbubbled water on the removal of biofilm from *C. albicans*.

a: The polystyrene plate  
The vertical axis of the graph showed quantity of remaining microbes which measured by a redox indicator at an absorbance of 540 nm.  
b: The removable appliance  
The vertical axis of the graph showed quantity of remaining microbes in a 70% ethanol extract of dental plaque stain at an absorbance of 540 nm.
after three min of soaking (Fig. 4b). The amount of residual biofilm in a 70% ethanol extract of dental plaque stain at an absorbance of 540 nm on both the tooth (Figs. 5a-1, b-1) and bracket (Figs. 5a-2, b-2) in the MB group was significantly smaller than that in the TW group, both after draining immediately (Figs. 5a-1, a-2 \( p < 0.05 \)) and after draining following 3 min of soaking (Figs. 5b-1, b-2, \( p < 0.05 \)). No significant difference was observed in the results between the immediate draining and the draining after immersion for 3 min.

**Effect of microbubbled water on the removal of biofilm from C. albicans on a polystyrene plate representing a removable appliance**

A 96-microtiter well plate was used in the examination of the effect of microbubbled water. The remaining amount of *C. albicans* biofilms was assessed using a redox indicator at an absorbance of 540 nm. The absorbance on the 96 well plate surface was significantly lower in the MB group than the TW group (Fig. 6a, \( **p < 0.01 \)). Therefore, the results showed that the effect of microbubbled water was significantly higher than that of tap water.

**Effect of microbubbled water on the removal of biofilm from C. albicans on the removable appliance**

The effect on polystyrene plate was also confirmed on the removable orthodontic appliance. The remaining amount of *C. albicans* biofilms was assessed using a redox indicator at an absorbance of 540 nm. The absorbance on the 96 well plate surface was significantly lower in the MB group than the TW group (Fig. 6a, \( **p < 0.01 \)). Therefore, the results showed that the effect of microbubbled water was significantly higher than that of tap water.

**DISCUSSION**

Although ordinary bubbles rapidly move upward and burst at the water surface, the microbubbles in this study were very fine particles and drift quite slowly in the water, and they shrink and finally disappear during ascending in the water. This unique property is called “nanobubbles”\(^{21}\). The microbubbled water used in this study was negatively charged due to an excess of OH- ions in comparison to H+ ions\(^{10,19}\), and this imbalanced charge distribution thus affects either the bacterial adsorption or opposition on the bubble surface, which causes the detachment of the biofilm from the orthodontic bracket.

In addition, the gas-water interface of microbubble is negatively charged due to an excess of OH- ions in comparison to H+ ions\(^{10}\), and this imbalanced charge distribution thus affects either the bacterial adsorption or opposition on the bubble surface, which may assist the detachment of the biofilm.

The microbubbled water used in this study was produced from ordinary water. It is safe and causes no harm in the living body. Thus it is easily applied during tooth-brushing, rinsing after brushing. In orthodontics microbubbled water makes it much easier to remove the biofilm on the areas such as surfaces of the fixed appliance and teeth where it is difficult for tooth brushing to reach, and consequently might be reduce the probability of incidence of dental caries and periodontal disease. It is also very convenient in the removal of biofilm from the removable appliances, when combined with ultrasonic bath sonicator. In addition, oral rinsing with microbubbled water might be effective in the cleaning of mouth at pre and post operation in oral surgery.

Further study will therefore be necessary to
reconfirm these effects in clinical situations.

CONCLUSION

This study, examined the effect of microbubbled water on the biofilms produced by the caries bacteria, *S. mutans* and the opportunistic pathogenic fungus, *C. albicans*. The microbubbled water showed an excellent ability to remove biofilms from orthodontic appliances. The results of this study suggest that application of microbubbles water has a potential to remove biofilms in the mouth of patients with orthodontic appliances.

ACKNOWLEDGMENTS

The microbubble generator was provided by Shinwa Co., Ltd., Tokyo, Japan and the particle characterizer was obtained from Sysmex Co., Ltd., Hyogo, Japan.

REFERENCES