INTRODUCTION

The use of fixed appliances, such as multi-bracket appliances, is essential in orthodontic treatment. However, plaque tends to develop around the fixed orthodontic appliances, which results in poor oral hygiene. This may increase the risk of dental caries and periodontal diseases during the course of the orthodontic treatment.1-3)

There are several methods available to remove dental plaque, which is a kind of biofilm. The main method involves mechanical cleaning using a toothbrush. An ionic toothbrush was developed for a more effective removal of biofilm 4). However, the ionic toothbrush is insufficient in both removal of biofilm and improvement of gingivitis 4). After auxiliary cleaning with mouthwash, chlorhexidine and antibacterial toothpaste, the bacteremia status was evaluated 5). The occurrence rate of bacteremia in these groups was not significantly different from that of patients using the normal method 5). Moreover, the existing disinfectants and antibacterial agents have major disadvantages. For example, long-term use of chlorhexidine damages the mucous membrane, which may lead to dysgeusia or anaphylactic shock 6). Regular use of antibacterial drugs has been associated with the risk of developing resistant bacteria 6,7). Thus, an ultrasonic scaler can be used in addition to brushing during the orthodontic treatment; however, ultrasonic scalers are known to damage both the tooth surface and the soft tissue 8-11). In addition, this method can only be employed in dental clinics and cannot be used at home on a daily basis. Therefore, there is a need to develop an effective method that patients can independently use at home.

Fine bubbles (FBs) with a diameter of approximately 10 to 30 micrometers are generally called microbubbles (MBs), while finer bubbles with a diameter ≤1 μm are called ultrafine bubbles (UFBs). They were previously referred to as nano-bubbles 12). FBs have different characteristics compared to normal bubbles, such as a longer residence time. A longer residence time results in a negatively charged surface, which removes positively charged organic matter through adsorption and negatively charged organic matter through repulsion 13). UFBs have various usages, such as cleaning of vegetables, decomposition of organic chemicals, wastewater treatment and food processing 13-22). Furthermore, UFBs are harmless to the human body and are used in the medical field 23,24). Bubbles scatter in MB water (MBW), and hence, their presence can be confirmed by the cloudy appearance of the liquid. MBs have the characteristic property of self-contraction. They rise slowly and remain in the water for a long time owing to their minute surface area. For example, bubbles with a diameter of 10 μm may rise at a rate of approximately three mm per minute 19,25,26). However, MBs do not have long-term stability in a range from days to months. Thus, the MBW loses its cloudiness within several seconds to
several minutes, thereby offering a visual confirmation for the disappearance of MBs. The diameter of bubbles in UFBW (Ultrafine Bubble Water) is around 20–30 nm, which is smaller than the wavelength of the visible light. Thus, the UFBW appears transparent. Moreover, it has been confirmed that the bubbles remain in the liquid for an extremely long time and carry an electrical charge \(^{27,28}\).

In our previous in vitro study\(^{29}\), we used MBW to clean orthodontic appliances, including a multi-bracket appliance. We found that the plaque-removal effect of MBW was dramatically higher than that of tap water\(^{29,30}\). However, the bubbles in MBW lack a long-term stability and disappear within 30 s to several minutes\(^{26}\).

The aim of this study was to examine the cleaning effect of UFBW, which is more stable than MBW, and to establish a new method for prevention of dental caries in patients undergoing orthodontic treatment. We evaluated the cleaning effect of UFBW in vitro, and conducted a clinical study.

**MATERIALS AND METHODS**

**Production of fine bubble water and measurement of bubble size and density**

We used a UFB generator (Ultrafine Galf, IDEC, Osaka, Japan) to create UFBs. In this device, the flow rate of the pumped liquid is increased until the Venturi tube narrows which, in turn, lowers the static pressure and mediates the negative-pressure suction of the gas. Once the liquid and the gas are in the gas-liquid mixed phase, the tube is widened again to lower the flow rate, thereby increasing the static pressure and causing dissolution. Finally, a rapid discharge under atmospheric pressure makes the liquid supersaturated, which generates a large number of fine air bubbles. We generated the UFBs using ultrapure water (distilled water, DW; Milli-Q Integral 3, Merck Millipore, Darmstadt, Germany) to minimize the number of particles that can be detected as background. We used air as the gas to generate high-concentration UFBW (HUFBW) with a density of 1.1–1.5 \(\times 10^9\) air bubbles/mL. We used a nanoparticle tracking analyzer (NanoSight NS500, Malvern Panalytical, Malvern, UK) to measure density and diameter of the UFBs. HUFBW was diluted two and four times with DW to prepare medium-concentrated UFBW (MUFBW) and low-concentrated UFBW (LUFBW).

**In vitro study: Biofilm-removal effect**

Figure 1 shows the study method/process. We examined the biofilm-removal effect of different concentrations of UFBW in comparison to the widely used mouthwashes Listerine mouthwash (L; Listerine, Johnson and Johnson, NJ, USA) and Neostelin Green 0.2% mouthwash solution (N; Nihon Shika Yakuhin, Yamaguchi, Japan). Table 1 shows the ingredients of each product.

Disks coated with biofilm were inserted into 24-well plates with 1 mL each of HUFBW, MUFBW, LUFBW, the mouthwashes L, N or DW. For cleaning, the plates were shaken at maximum speed with a mixer (Tray mixer, SAKURA, Tokyo, Japan) for 30 s. After cleaning, the residual biofilm on the plastic disk was stained with a plaque-staining solution (PROSPEC, GC, Tokyo, Japan), and subsequently washed with water. All disks were measured as indicated in Fig. 1A–C (n=10). After photo graphing for staining values, the stain was extracted with 99.9% ethanol, followed by measurement of the absorbance at 540 nm with a microplate reader (Varioskan, Thermo Fisher Scientific, MA, USA) (Fig. 1A). We used a confocal laser microscope (OLS3000, OLYMPUS, Tokyo, Japan) to measure the thickness of the residual biofilm (Fig. 1B). The film staining values were measured with image processing\(^{31}\) (Fig. 1C).

**Table 1** Product name and ingredients of mouth washes

<table>
<thead>
<tr>
<th>Product name</th>
<th>Ingredients</th>
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<tbody>
<tr>
<td>Ultrafine bubble water</td>
<td>density of 1.1–1.5 (\times 10^9) air bubbles/mL of DW</td>
</tr>
<tr>
<td>High-concentration (HUFBW)</td>
<td>two-fold dilution of HUFBW</td>
</tr>
<tr>
<td>Medium-concentration (MUFBW)</td>
<td>four-fold dilution of HUFBW</td>
</tr>
<tr>
<td>Low-concentration (LUFBW)</td>
<td></td>
</tr>
<tr>
<td>Listerine (L)</td>
<td>1,8-cineol, thymol, methyl salicylate, i-menthol, ethanol</td>
</tr>
<tr>
<td>Neostelin Green (N)</td>
<td>0.2%benzethonium chloride</td>
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Fig. 1 Method of the in vitro study.

We compared the cleaning effect of various solutions on the biofilm formed on a plastic disk.
1. Biofilm formation
A plastic disk of 13.5 mm diameter and 0.1 mm thickness, (Cell Disk LF1, Sumitomo Bakelite, Tokyo, Japan) was immersed in human saliva (filtered with Milex 0.22 μm; Merck Millipore) overnight at 4°C. 1×10⁸ CFU/mL of pre-cultured Streptococcus mutans (ATCC25175) were suspended and inoculated in the BHI medium containing 5% sucrose under anaerobic conditions. The inoculated medium was shaken and cultured for two days at 37°C under aerobic conditions to facilitate the formation of a biofilm.

2. Statistical analyses
We used the Kruskal-Wallis test for comparison of the test data between multiple groups, and the Mann-Whitney U test for comparison between two groups. Statistical significance was set at \( p < 0.05 \) in the sum of comparison. SPSS version 11.0 statistical software (SPSS, Chicago, IL, USA) was used for the analyses.

Clinical study
1. Selection of subjects
This study was performed with the approval of the ethics committee of Tsurumi University (approval number: 1145) and informed consent was obtained from all the subjects beforehand. Moreover, the study was in accordance with the World Medical Association Declaration of Helsinki.

Twelve subjects (three men, nine women) with the mean age of 24.2 years (range, 17–46 years) were enrolled in the study. The inclusion criteria for the subjects included the use of a multi-bracket appliance as part of an orthodontic treatment for more than six months; two or more visits to the facility during the test period; absence of any systemic disease; absence of antibiotic administration in the last three months; absence of dental caries and periodontal disease; and absence of smoking history. Conversely, exclusion criteria included a history of periodontal disease; extensive restoration of maxillary anterior teeth; use of professional mechanical tooth cleaning and antibacterial mouthwash within one week of the study; and antibiotic administration within three months prior to the study.

2. Study design
Each subject was examined on two separate days at monthly intervals. The experiment was performed using HUFBW, while DW was used as control. The subjects brushed their teeth in their usual manner before arriving at our clinic. Their oral cavities were stained using a plaque-staining solution to evaluate the plaque before rinsing (B value). Subsequently, the subjects rinsed their mouths with HUFBW or DW for 30 s (20 mL per 10 s of rinsing, three times), and the plaque was evaluated after rinsing. The flow chart of the procedure is shown in Fig. 2.

Plaque was evaluated using the plaque control record (PCR) and photographic image data. PCR was used to evaluate all teeth, except those with extensive restoration, such as a metal inlay, or those fitted with metal bands for orthodontic correction. The photographic image data were used to evaluate the plaque on the labial surfaces of the upper central incisors, including the area around the multi-bracket appliance. We fixed the subject’s head with ear rods for standard facial photography and fixed the camera onto a tripod to standardize the photography conditions. To correct variations between the photographs and to define the range of the stained area to be evaluated, we added a color chart (PANTONE LLC, NJ, USA) during photography. On the digital images, we trimmed each upper central incisor and unified the overall color of the teeth. Subsequently, we presented a pseudo-color display of the staining density. The stain-extracted areas were synthesized with the original image to calculate the plaque volume ratio (PVR) of the upper central incisors.

3. Statistical analysis
The Wilcoxon test (SPSS version 11.0) was used for analysis of significant differences between volumes of HUFBW and DW (\( p < 0.01 \)) for rinsing.

RESULTS

Stability of UFBW
The diameter of the UFBs immediately after generation was ≤100 nm. The UFBs showed no change in terms of number and diameter after a month of refrigerated storage (Table 2).

In vitro determination of biofilm-removal effect
Mean values and standard deviations of the absorbance at 540 nm for HUFBW, MUFBW, LUFBW, L, N and...
Table 2  Measurement results of HUFB density and diameter

<table>
<thead>
<tr>
<th>Concentration (×10⁹/mL)</th>
<th>Mean diameter (nm)</th>
<th>Mode diameter (nm)</th>
</tr>
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<tbody>
<tr>
<td>Immediately after synthesis</td>
<td>1.058</td>
<td>88</td>
</tr>
<tr>
<td>One month later</td>
<td>1.355</td>
<td>99</td>
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Fig. 3  Biofilm-removal effect in the in vitro study.
(a) Measurement of residual biofilm by spectrometry. The decrease in residual biofilm is dependent on the bubble concentration. B value was measured prior to cleaning with each solution. (b) Biofilm thickness was measured using confocal laser microscopy. The decrease in the amount of residual biofilm is dependent on the bubble concentration. B value was measured prior to cleaning with each solution. (c) The amount of residual biofilm is measured as the staining value. The lowest high-concentration ultra-fine bubble water (HUFBW) value was set as zero. The decrease in the amount of residual biofilm is dependent on the bubble concentration, as seen in the results obtained by spectrometry and biofilm thickness measurement. B value was measured prior to cleaning with each solution.

There was a significant difference among groups (Kruskal-Wallis p<0.05), and also between each group marked with asterisk (Mann-Whitney p<0.0024), but not between distilled water (DW) and Listerine (L) or Neostelin-Green (N) treatment groups.

B were 0.07±0.03, 0.12±0.07, 0.34±0.11, 0.77±0.11, 0.78±0.11, 0.82±0.11, and B was 16±0.16, respectively.

The spectrometry results revealed that the amount of the residual biofilm decreased as the bubble concentration increased, and there were significant differences in the amount of residual biofilm in the DW and LUFBW, LUFBW and MUFBW, and MUFBW and HUFBW groups (p<0.01; Fig. 3a). However, there was no significant difference in the amount of residual biofilm between the DW, L, and N treatment groups. Findings of the confocal laser microscopy were similar to that of spectrometry and revealed that the biofilm thickness decreased as the bubble concentration increased. Moreover, they showed significant differences between the cleaning effect in the DW and LUFBW, LUFBW and MUFBW, and MUFBW and HUFBW groups (p<0.01) (Fig. 3b). Mean values and standard deviations of the Biofilm thickness for HUFBW, MUFBW, LUFBW, L, N and B were 13.50±3.33, 32.83±9.66, 58.50±6.41, 84.25±2.46, 87.74±6.57, 87.00±3.04, and B was 86.55±13.05, respectively. Mean values and standard deviations of the staining values of for HUFBW,
MUFBW, LUFBW, L, N and B were 0±4.12, 8.98±4.0, 17.60±4.11, 27.69±4.11, 24.89±4.11, 28.40±4.11, and B was 31.44±4.16, respectively.

The treatment with HUFBW showed a six-fold decrease in the thickness of the residual biofilm compared with the treatment with DW. Similar to the spectrometry results, confocal laser microscopy revealed no significant difference in the cleaning effect between the DW, L, and N treatment groups. The staining value also showed results similar to those of spectrometry wherein the biofilm thickness depended on the bubble concentration ($p<0.01$) (Fig. 3c). The HUFBW group demonstrated the lowest staining value.

**Clinical study to determine the biofilm-removal effect**

As HUFBW demonstrated the highest cleaning effect in the *in vitro* study, we used HUFBW in the clinical study to examine the biofilm-removal effect. HUFBW was used for rinsing, and the residual plaque was evaluated with PCR and PVR. The PCR (Fig. 4a) showed a decrease of 2.7% in the DW group and a decrease of 9.8% in the HUFBW group. The PVR (Fig. 4b) changed by 13.3% in the DW group and by 35.0% in the HUFBW group. In addition, photographic image analysis for PVR calculation (Fig. 4c) confirmed the difference in PVR between the DW and HUFBW groups. The HUFBW group showed a significantly higher plaque-removal effect compared to that of the DW group when the mean change was compared before and after rinsing for all the subjects.

![Fig. 4](image-url)

Comparison of change in plaque control record (PCR) and plaque volume ratio (PVR) before and after rinsing with high-concentration ultra-fine bubble water (HUFBW) in the clinical study. (a) The changes in PCR are quantified. The amount of change in the HUFBW group is significantly larger than that in the distilled-water (DW) group. Decrease in PCR of DW groups was 2.7±1.9, and of HUFBW groups 9.8±2.1. (b) The changes in PVR are quantified. The amount of change in the HUFBW group is significantly larger than that in the DW group. Decrease in PCR of DW groups was 13.3±5.2, and HUFBW groups 35.0±5.3. (c) Example of PVR calculation. Upper row is DW and bottom row is HUFBW. (c-1) Photograph of anterior teeth with a color code (PANTONE). (c-2) Each individual upper central incisor was trimmed digitally. (c-3) Stain density is displayed using pseudo colors. (c-4) Stain-extracted area is overlaid onto the original image to calculate PVR. (c)
DISCUSSION

Characteristics of UFBs
Normal bubbles rise rapidly through water and burst upon reaching the surface, whereas FBs with microscopic bubble volume rise slowly through the water and remain in the water for a longer time. Our study confirmed that UFBs with diameter of 1 μm remained in water for up to one month, which is indicative of their long-term stability. These results are consistent with an earlier report. Although the reason has not been clarified yet, it can be considered that the bubbles have been preserved for at least one month, because due to the small diameter and buoyancy of the bubbles as well as the decreasing kinetic energy of the molecule at low temperature, a reduced motility can be assumed.

Reason for the evaluation method using the staining value
The amount of biofilm was evaluated using spectrometry, confocal laser microscopy and staining value. It is known that the three tests are highly correlated, however each test has its drawbacks. It appears to be impossible to extract 100 percent of the dye by absorbance measurement. In case of the confocal laser microscope representative measurements are 10 measurement values per sample, but there are actually infinite measurement points. And for the staining value it is difficult to perfectly match the photographing conditions. The staining value was calculated on the basis of the image analysis of the stained biofilm. The biofilm thickness and the staining value showed a strong correlation. Therefore, the staining value was considered as an index for the plaque amount.

In vitro study
S. mutans is a typical pathogenic bacterium that causes dental caries. Various other microorganisms exist in the natural oral plaque, but we selected S. mutans as it produces the strongest matrix involved in plaque formation. Thus, the S. mutans model was considered suitable to compare the irrigation effect. In this study a model of plaque, adhering to a multi-bracket appliance and tooth surfaces was formed by culturing S. mutans for 3 days to produce the most robust insoluble glucan as a biofilm matrix.

HUFB biofilm-removal effect of HUFB
This study showed that the commercially available mouthwashes did not remove the biofilm. Although the mouthwashes are reported to have bactericidal effect, it has been well known that they cannot penetrate the biofilm. As a result, these disinfectants are not expected to be effective against the biofilm. On the other hand, UFB might have a removal ability by activating the hydrodynamic shear and surface tension forces, which cause the detachment of the biofilm. When comparing the concentrations of UFBW, the removal effect of HUFBW was the highest (Figs. 3a, b, and c) according to the concentration-response relationship. It is possible that if the concentration exceeds a certain threshold, a higher concentration may show a higher removal effect. In addition, it is considered that the concentration we measured this time was above this threshold. Therefore, even LUFBW showed a higher biofilm-removal effect than DW and the commercially available mouthwashes. Based on these findings, we conducted a clinical study using HUFBW.

Clinical study
PCR is generally used to evaluate plaque on the tooth cervix and the risk of periodontal disease in patients. PCR can only evaluate the presence or absence of plaque, but not the thickness of plaque. However, in our study, PCR was able to reveal a significant decrease in the plaque amount of the whole tooth, but not in the area around the bracket despite its low sensitivity (Fig. 4a). The changes in PCR are not detected unless the plaque is completely removed from the tooth cervix. As shown in the PVR example image in Fig. 4c, PVR enables evaluation of the plaque around a multi-bracket, while the difference could not be identified with PCR. Thus, in this study, we used photographic image data for calculating PVR and adopted a method for evaluating the amount of plaque around the multi-bracket appliance through image processing (Figs. 4b and c). PVR evaluation utilizes a novel algorithm that considers the shade of the stain to accurately evaluate the amount of plaque. It was reported that the amount of stained plaque correlates with the G channel by focusing not only on the area of the plaque, as in the past, but also on the three-dimensional amount of plaque (volume). This approach is based on the new concept of volumetric ratio of plaque to evaluate the teeth.

In this clinical study, HUFBW showed higher plaque-removal compared to DW in all subjects. However, the cleaning effect was clinically weakened compared to the result of the in vitro study. The reason may be the simple rinsing in the clinical condition not providing a sufficient amount of HUFBW on the labial surface of the upper central incisors. The effect is expected to improve by a prolonged duration of use. The results suggest that rinsing with HUFBW is effective in cleaning the areas that are difficult to brush during orthodontic treatment, such as around the multi-bracket appliances and beneath the wires. HUFBW is expected to be an effective rinsing agent and its use should be followed by thorough brushing to minimize plaque. Rinsing with HUFBW may also aid supplementary cleaning for those who face brushing difficulties, such as the elderly population and patients before and after oral surgery. HUFBW rinsing might become economically feasible and effective if patients take the generated UFBW to their homes and use it as part of their home-care routine.

CONCLUSION
HUFBW has a long-term stability in terms of bubble diameter and concentration. Its biofilm-removal effect is dependent on the bubble concentration. Therefore,
HUFBW can be effective for removing plaque in the areas that are difficult to clean with mechanical brushing alone, such as the area around multi-bracket appliances. Rinsing with HUFBW as a routine home care is expected to reduce the incidence of dental caries during orthodontic treatment.

ACKNOWLEDGMENTS

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REFERENCES


