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ORIGINAL ARTICLE

Effect of water containing organic acids on aspiration pneumonia-causative bacteria in the biofilm on the tooth surface

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KEYWORDS
mouthwash;
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Abstract *Background/purpose:* The tooth surface is a source of oral microbes in dentulous individuals, it is difficult for elderly people requiring nursing care to perform mechanical tooth cleaning by themselves. The objective of this study was to investigate the antimicrobial effect of water containing organic acids (WOA) made by some organic acids as food additives on chemical cleaning for elderly people on aspiration pneumonia-causative bacteria in the biofilm on the tooth surface.
Materials and methods: Ninety-six specimens made from bovine incisors were divided into four groups and incubated with one of four aspiration pneumonia-causative bacteria. Each group was further divided into six subgroups according to treatment as follows: control group (DW), chlorhexidine gluconate solution group (CHX), WOA group (WOA), ultrasonic treatment in distilled water group (DW-U), ultrasonic treatment in chlorhexidine gluconate solution group (CHX-U) or ultrasonic treatment in WOA group (WOA-U). After treatment, the levels of viable microbes in the biofilm were evaluated by quantitative adenosine triphosphate analysis and compared among the six groups.
Results: For every evaluated microbe, there were significant differences between DW and WOA, and DW and WOA-U. However, there was no significant difference among the WOA, DW-U, CHX-U and WOA-U groups. These results suggested that the antimicrobial effect of WOA on microbes attached to the tooth surface was similar to that of ultrasonic cleaning.
Conclusion: WOA has an antimicrobial effect on microbes in the biofilm on the tooth surface.

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Introduction

Aspiration pneumonia is caused by silent aspiration of oral microbes present in the mouth and pharynx.[1–3] The control of oral microbial flora by oral health care is an effective preventive measure.[4] Ryu et al.[5] found that the level of adherence of tongue coating and denture plaque was related to the total number of salivary anaerobic bacteria in edentulous subjects. Yasui et al.[6] reported that periodontal pathogens were detected at a high rate on the dorsum of the tongue, denture base and artificial teeth. The tooth surface is also a source of salivary microbes in dentulous individuals.[4,7] These reports confirm that the tooth surface, tongue and dentures are foci for oral microbes, and that controlling the number of microbes in these areas is important for effectively reducing the aspiration of oral microbes. Currently available antimicrobial mouthwashes such as chlorhexidine gluconate and povidone-iodine pose a risk of causing anaphylactic shock depending on the concentration and application method.[8,9] Because of these shortcomings, the development of an antimicrobial mouthwash that is suitable for elderly people requiring nursing care is required.

In this study, we focused on water containing organic acids (WOA). The main components of WOA are organic acids such as citric acid and lactic acid, commonly used as food additives for humans.[10]

WOA has been shown to exhibit an antimicrobial effect against planktonic and resin-attached aspiration pneumonia-causative microbes as well as against total anaerobic bacteria attached to dentures in use.[10] This report suggested that its antimicrobial effect may extend to oral microbes attached to tooth surfaces. However, the drug sensitivity of oral microbes attached to tooth surfaces differs from that of planktonic microbes, and adherent form of oral microbes attached to tooth surface differ from to dentures.[11,12] So it is necessary to investigate the effect of WOA on microbes forming a biofilm on the tooth surface.

Streptococcus sanguinis (*S. sanguinis*) initially adheres to the pellicle as a dental plaque bacterium.[13] *Porphyromonas gingivalis* (*P. gingivalis*), *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pneumoniae* (*S. pneumoniae*) are bacteria that cause aspiration pneumonia.[14–16] The objective of this study was to investigate the antimicrobial effect of WOA on aspiration pneumonia-causative bacteria in the biofilm on the tooth surface.

Materials and methods

Preparation of WOA and specimens

WOA was prepared using the BIOioNURSE (Separator System Kogyo, Nara, Japan). Water was ultrapurified using a reverse osmosis membrane via an ion-exchange resin. A total of 10,000 ppm of citric acid, 2,000 ppm of lactic acid, and trace amounts of oxalic acid and tartaric acid were added to 1 L of ultrapure water. The concentration of total organic acids was adjusted to 3% by volume by adjusting the quantity of acids. The water was colorless and transparent, with a pH of 2.19 and an oxidation-reduction potential of 250 mV.[10]

Ninety-six bovine incisors were used to prepare specimens for this study. The incisors were polished and washed by ultrasonic cleaning. In the preliminary experiment, we confirmed that there was no significant difference on number of bacterial adhesion between specimens after washing by ATP assay, so we considered that the specimens were enough clean. The incisors were cut into 5-mm lengths from the incisal edge perpendicular to their major axis using a diamond disc under running water. The cut surfaces of the specimens were polished using silicon carbide abrasive paper up to #1000 under running water to standardize the surface roughness. The surface roughness of the enamel of the specimens was analyzed before treatment within a single group using a scanning electron microscope (SEM) with a three-dimensional shape analysis function (3DSEM: Era-8900Fe, Elionix, Tokyo, Japan). The mean surface roughness (Ra) was determined using SEM images that were taken at a magnification of $\times$2000. Each specimen was measured at three randomly selected points. It was confirmed that there were no significant differences in the ATP levels of bacteria attached to the cut surface among the specimens under the initial polishing conditions as a preliminary experiment (data not shown). The specimens were divided randomly into four groups for incubation with the following microbes: 1) *S. sanguinis*, 2) *P. gingivalis*, 3) *S. aureus*, and 4) *S. pneumoniae*. Each group was divided randomly into six subgroups ($n = 4$) that were immersed in three solutions with or without ultrasonic treatment. Each specimen was weighed using an electronic scale (LA230S Sartorius, Tokyo, Japan) to infer the surface area of the specimens.

Whole saliva without stimulation was collected from four healthy adult volunteers (2 men, 2 women; mean age 29 $\pm$ 1 years) as described previously.[17,18] The Ethics Committee of Tokyo Dental College approved collection of saliva (#554). The tooth specimens were treated with the saliva for 10 min at room temperature to form a pellicle on the surface.

Antimicrobial effect of WOA on microbes in the biofilm on the tooth surface

S. sanguinis ATCC 10556, *P. gingivalis* ATCC 33277 and *S. aureus* 209P, obtained from the Department of Microbiology, Tokyo Dental College, and *S. pneumoniae* GTC 261 obtained from the Department of Microbiology, Gifu University Graduate School of Medicine, were used in this study. *S. sanguinis* and *S. pneumoniae* were maintained on Todd Hewitt agar plates (Becton Dickinson, Franklin Lakes, NJ, USA), *S. aureus* was maintained on trypticase soy agar plates (Becton Dickinson), and *P. gingivalis* was maintained on trypticase soy agar plates (Becton Dickinson) supplemented with hemin (5 μg/mL), menadione (0.5 μg/mL), and 10% defibrinated horse blood. To obtain cells in the late log phase, *S. sanguinis* was precultured in brain heart infusion broth at 37 °C for 24 hours under anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2). *P. gingivalis* was first cultured in trypticase soy broth with 1 mL of hemin and menadione per liter at 37 °C for 6 days under anaerobic conditions. *S. aureus* and *S. pneumoniae* were first cultured in trypticase soy broth at 37 °C for 12 hours for

the former and 24 hours for the latter under aerobic conditions.

Twenty-four specimens of each group were incubated in 2 mL of microbial media as follows: 1) *S. sanguinis*, 37 °C for 2 days under anaerobic conditions; 2) *P. gingivalis*, 37 °C for 3 days under anaerobic conditions; 3) *S. aureus*, 37 °C for 24 hours under aerobic conditions; and 4) *S. pneumoniae*, 37 °C for 3 days under aerobic conditions. After incubation, the media were removed and treated with each condition as described below. In the control group (DW), specimens were immersed in 2 mL of distilled water. In the chlorhexidine gluconate group (CHX), specimens were immersed in 2 mL of 0.0006% chlorhexidine gluconate solution, which was prepared by dissolving 0.28 mL of mouthwash (ConCool F, Weltec, Osaka, Japan) including 0.05% chlorhexidine gluconate in 25 mL of distilled water. The concentration was decided by direction for use of the mouthwash (Concool F, Weltec, Osaka, Japan). In the water containing organic acids group (WOA), specimens were immersed in 2 mL of WOA. In the ultrasonic groups, specimens were subjected to ultrasonic treatment in 2 mL of distilled water (DW-U), 0.05% chlorhexidine gluconate solution (CHX-U) or WOA (WOA-U). Mechanical cleaning of the tooth surface was standardized in this study by using ultrasonic cleaning (US CLEANER US-1R, AS ONE Corporation, Osaka, Japan, frequency of ultrasonic cleaning; 28 kHz) that instead of tooth cleaning with a toothbrush.

After treatment under each condition for 5 min at room temperature, the specimens were removed. The level of viable *S. sanguinis*, *P. gingivalis*, *S. aureus* and *S. pneumoniae* in the biofilm was evaluated by quantitative adenosine triphosphate (ATP) analysis. Briefly, specimens were immersed in 2 mL of ATP assay buffer (BactTiter-Glo, Promega Corporation, Madison, WI, USA) for 15 min at room temperature, and the bioluminescence of each sample was measured using a Lumicounter (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA).

Surface roughness of specimens after treatment with WOA

To evaluate Ra of the specimens after treatment, six specimens were prepared as described above and divided randomly into two groups. In the control group (DW), three specimens were immersed in 2 mL of distilled water, and three specimens in the water containing organic acids group (WOA), specimens were immersed in 2 mL of WOA. After immersion for 30 min, the surface roughness of the specimens was measured using a scanning electron microscope with 3DSEM.

Statistical analysis

The mean weight of the specimens among the six groups was analyzed using one-way ANOVA followed by the Bonferroni test. The mean Ra value of the specimens which selected randomly in six groups was analyzed using the Bonferroni test followed by one-way ANOVA. The Kruskal–Wallis test followed by the Scheffe test was used to compare groups according to luminescence levels associated with specimens in the six groups. Student's *t*-test was used to compare the roughness of the tooth surface after treatment of DW and WOA.

Statistical analysis was performed using SPSS software for Windows version 21 (IBM Corp., Armonk, NY, USA). A level of $p < 0.05$ was considered significant.

Results

The mean weight of the specimens before treatment was 804.1 ± 50.2 mg and there were no significant differences in the mean weights among the six groups when analyzed using one-way ANOVA ($p = 0.998$ for *S. sanguinis*, $p = 0.510$ for *P. gingivalis*, $p = 0.969$ for *S. aureus*, and $p = 0.781$ for *S. pneumoniae*).

The mean Ra value of the specimens in the representative group was 0.29 ± 0.32 μm and there were no significant differences in the mean Ra value among the six groups ($p = 0.662$).

The ATP levels of *S. sanguinis*, *P. gingivalis*, *S. aureus* and *S. pneumoniae* attached to the tooth specimens when analyzed using ATP analysis after treatment under each immersion condition are shown in Figs. 1–4. For every evaluated microbe, there were significant differences between DW and WOA (*S. sanguinis*: $p = 0.005$, *P. gingivalis*: $p = 0.046$, *S. aureus*: $p = 0.019$, *S. pneumoniae*: $p = 0.036$), and between DW and WOA-U (*S. sanguinis*: $p = 0.024$, *P. gingivalis*: $p = 0.048$, *S. aureus*: $p = 0.009$, *S. pneumoniae*: $p = 0.047$). Compared with DW, the mean ATP levels of *S. sanguinis* after treatment with WOA and WOA-U were 18.8% and 30.1%, respectively. Compared with DW, the mean ATP levels of *P. gingivalis* after treatment with WOA and WOA-U were 14.6% and 15.6%, respectively. Compared with DW, the mean ATP levels of *S. aureus* after treatment with WOA and WOA-U were 21.3% and 12.9%, respectively. Compared with DW, the mean ATP levels of *S. pneumoniae* after treatment with WOA and WOA-U were 23.7% and 38.7%, respectively.

The Ra of the enamel of the specimens after treatment with DW and WOA was 0.23 ± 0.17 μm and 0.34 ± 0.15 μm, respectively. There was no significant difference in the roughness of the tooth surfaces between these groups ($p = 0.662$).

Discussion

On *S. sanguinis*, *P. gingivalis*, *S. aureus* and *S. pneumoniae*, there were significant differences in the levels of microbes attached to the specimens between DW and WOA, and between DW and WOA-U. On *S. sanguinis*, there were also significant differences in the levels of microbes attached to the specimens between CHX and WOA, DW-U, and WOA-U. The levels in the WOA group were more than 60% lower than the DW group for every investigated microbe (Figs. 1–4). However, there was no significant difference in the level of any investigated microbe among the WOA, DW-U, CHX-U and WOA-U groups. These results suggest that the antimicrobial effects of WOA on *S. sanguinis*, *P. gingivalis*, *S. aureus* and *S. pneumoniae* attached to the tooth surface were similar to those of ultrasonic cleaning. It also suggests that applying WOA to the tooth surfaces affects oral microbes similarly to

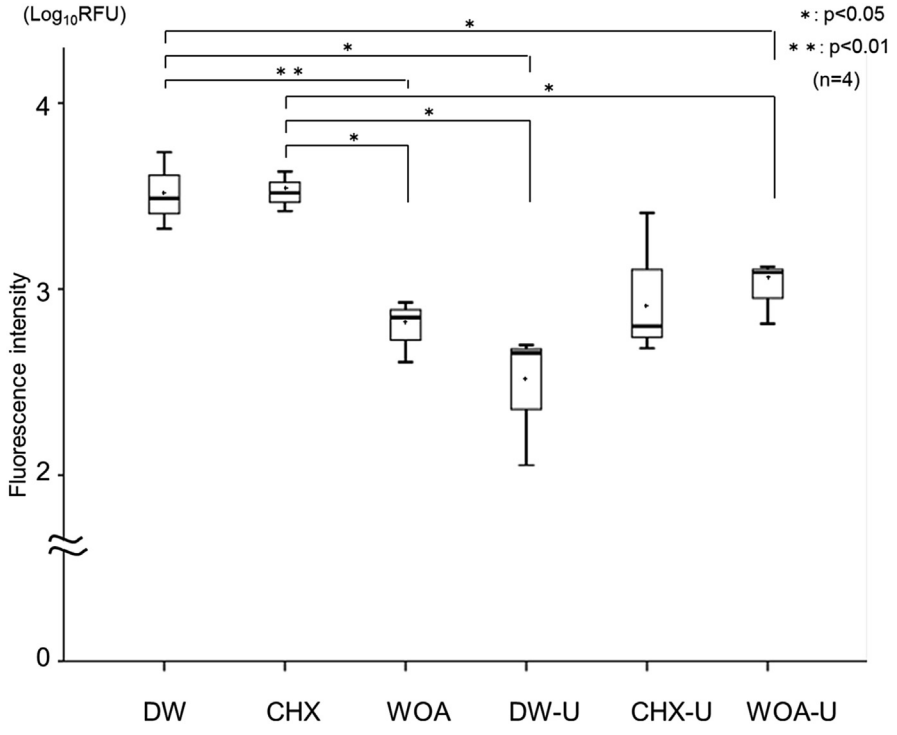

Figure 1 Luminescence levels of *S. sanguinis* attached to specimens after treatment under each condition. The plus forming in the center of the box is the mean values. Immersion in distilled water (DW), immersion in chlorhexidine gluconate (CHX), immersion in WOA (WOA), ultrasonic treatment in distilled water (DW-U), ultrasonic treatment in chlorhexidine gluconate (CHX-U), ultrasonic treatment in WOA (WOA-U). The Kruskal—Wallis test followed by the Scheffe test was used to compare groups according to the luminescence levels associated with specimens in the six groups. The horizontal line forming the top of the box is the 75th percentile and the line forming the base of the box is the 25th percentile. The horizontal line that intersects the box is the median. Horizontal lines above and below the box, called whiskers, represent maximum and minimum values respectively.

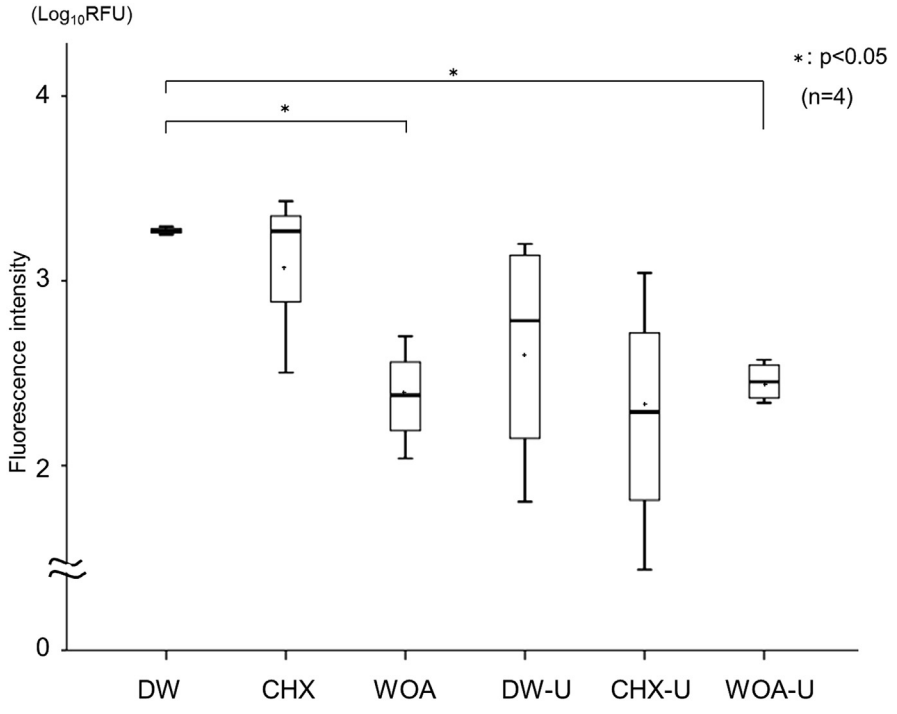

Figure 2 Luminescence levels of *P. gingivalis* attached to specimens after treatment under each condition.

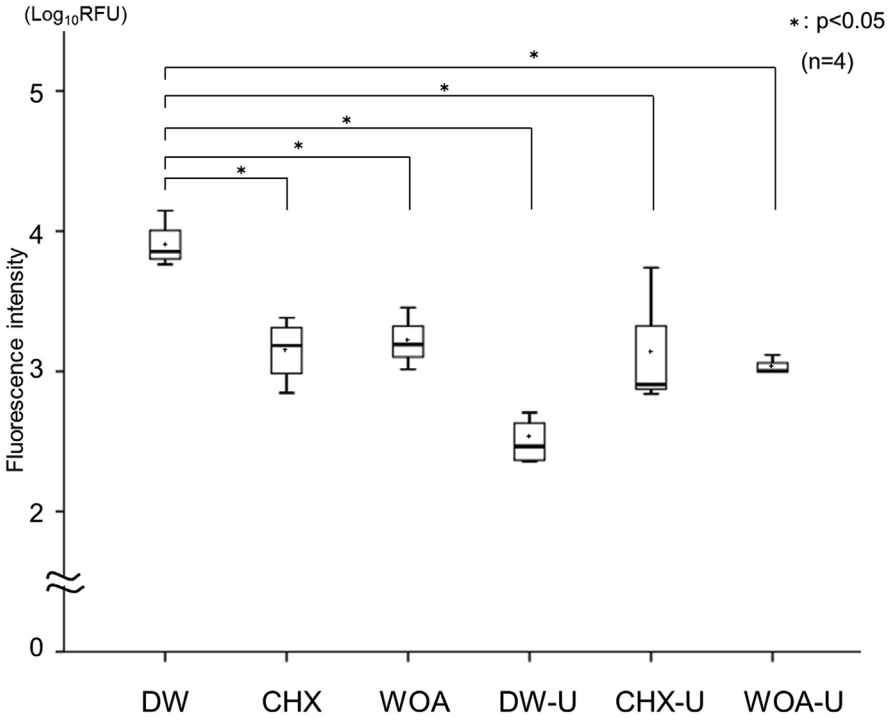

Figure 3 Luminescence levels of *S. aureus* attached to specimens after treatment under each condition.

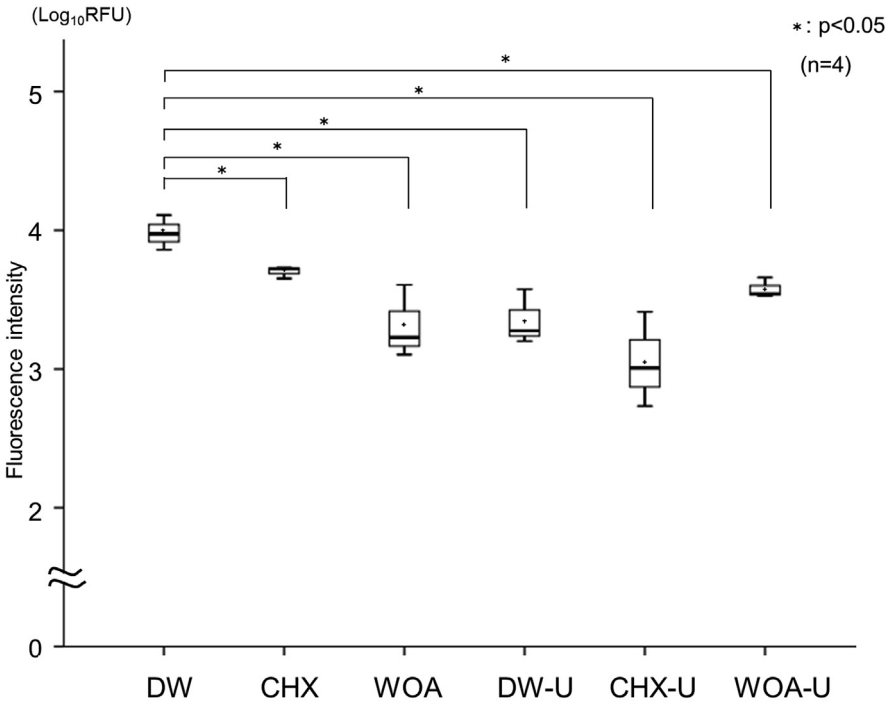

Figure 4 Luminescence levels of *S. pneumoniae* attached to specimens after treatment under each condition.

mechanical tooth cleaning, which may be of benefit in aged care facilities where nursing staff is limited.

S. sanguinis initially adheres to the pellicle as a dental plaque bacterium.[13,19] *P. gingivalis* is one of the bacteria that causes aspiration pneumonia,[16] and it forms a biofilm through co-aggregation with many other plaque bacteria and becomes the framework of dental plaque.[20] The number of oral microbes forming a biofilm increases with an increase in *S. sanguinis* and *P. gingivalis*. *S. aureus* is detected in the mouths of many people,[21] and its involvement in aspiration pneumonia.[14,22] *S. pneumoniae* is also a key pathogen that causes aspiration pneumonia.[15,22]

According to our finding that WOA reduces the number of these microbes in the biofilm on the tooth surface, it is expected that WOA may decrease the colonization of aspiration pneumonia-causative microbes.

The observed antimicrobial effect may have been due to the effect of the organic acids contained in WOA. Undissociated-state molecules in the organic acids penetrate the cell membrane and release hydrogen ions into cells, exerting an antimicrobial effect by inhibiting microbial metabolism.[23] When undissociated-state molecules in the organic acids increase in number with a decrease in the pH, more molecules readily penetrate through the cell membrane.[24] Because the pH of WOA is set at a low level (pH 2.19), many undissociated-state molecules may have penetrated the cell membrane, dissociated in the cells, and released hydrogen ions, inhibiting microbial metabolism and exerting a strong antimicrobial effect. Additionally, microbial growth is interfered with at a low pH,[25] which may also be related to the antimicrobial effect of WOA. Although the antimicrobial effect on biofilm consisting of a single microbe was investigated in this study, it has been reported that WOA has an antimicrobial effect on biofilm consisting of multiple microbes on denture surfaces.[10] Therefore, the findings raise the possibility that WOA also has an antimicrobial effect on biofilm consisting of multiple microbes on tooth surfaces.

There are several kinds of mouthwashes, including chlorhexidine, povidone-iodine. We used chlorhexidine, a commercial mouthwash, because it is particularly effective against dental plaque.[26] However, instances in which anaphylactic shock caused by application of chlorhexidine to the oral mucosa have been reported.[8] Additionally, chlorhexidine at a concentration of 0.2% has been shown to cause staining of the teeth.[27,28] Povidone-iodine may cause anaphylactic shock depending on the concentration and application method.[9] Commercial mouthwashes currently used for oral health care have these disadvantages, whereas WOA may be safe for the body because it is composed of organic acids used as food additives, such as citric acid and lactic acid, and its biological safety has been verified by an acute oral toxicity study in mice.[29] It has been reported that damage of cell by applying strong acid was reversible.[30] It has also been reported that citric acid at 4% to 10% concentrations did not yield cytotoxicity to the osteoblastic cells.[31] Also, there is epithelium with oral mucosa, so we conceived that WOA has little effect to the cell. Therefore, we considered that WOA may be safe for the body, and it may be enough to confirm about toxicity even if not using other toxicity test as MTT assay.

There was no significant difference in the effects of WOA on the tooth surface between the groups immersed in WOA and those immersed in distilled water. If WOA cause decalcification, Ra of specimens may be decreased. However, Ra did not change in the WOA group in this study. Additionally, it has been reported that not only the pH, but also the composition of the organic acids affect decalcification of the tooth surface.[32] The application of WOA may not cause decalcification of the teeth because the oxidation-reduction potential of WOA is not within the range at which electrons are exchanged. Therefore, WOA may not cause decalcification of the teeth less 30 min. Because application of WOA for 30 min has little effect on the tooth surface roughness, application for 5 min as in this study may have little effect.

The number of elderly people requiring nursing care will increase as society ages, which will lead to an increased number of elderly people with poor oral hygiene due to the difficulty of self-care. We believe that applying WOA combined safety and antimicrobial effect may facilitate effective self-care and improve oral hygiene in elderly people in nursing care who can't perform mechanical cleaning. Within the limitation of this study, we concluded that WOA has an antimicrobial effect on microbes attached to the tooth surface, and its effect is equivalent to that of ultrasonic cleaning, which was used as a substitute for standardized mechanical cleaning.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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