Bactericidal effect of acid-electrolyzed functional water and its effect on host cells

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Contents

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Abstract

Background/Aim: Acid-electrolyzed functional water (FW) is an efficient bactericide and gargling with FW might be an effective method of oral care. I investigated the possible use of FW as a mouth wash by an *in vitro* study.

Materials and Methods: The bactericidal effect of FW against different species of bacteria (*Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Candida albicans, and Klebsiella pneumoniae*) was evaluated using the numbers of colony-forming units (CFU). The experiment was conducted using PBS, LISTERINE, and ConCool F (undiluted, and the optimal concentration indicated). To investigate the bactericidal mechanism of FW, the activity of superoxide dismutase (SOD), an indicator of oxidative action, was measured in *S. aureus*. FW was diluted with purified water to concentrations of 10, 30, 50, and 70%. The numbers of CFU were measured for each concentration. XTT assays were performed using two types of cell lines (HSC-3 and HeLa), to examine the viability of the cells following treatment with FW. The same experiment was conducted with phosphate buffered saline (PBS), LISTERINE, and undiluted ConCool F.

Results: No bacteria treated with FW formed colonies. SOD activity peaked at a 50% concentration of FW and was more than twice that of the control. A significant decrease in the number of CFU was observed following 50% treatment. Since the peaks of the SOD activity and the starting concentrations of the bactericidal effects coincided, the bactericidal effect of FW might be related to its oxidative effects. Bacteria treated with FW had the same survival rate as the other mouth washes.

Conclusion: FW might be clinically applicable as a mouth wash.

Keywords: acid-electrolyzed functional water, aspiration pneumonia, elderly, mouth wash, oral care.

Introduction

The average life expectancy continues to increase worldwide (1). However, healthy life expectancy is generally more important than lifespan itself for the elderly. Healthy life expectancy is the period in which the elderly does not need significant care, and the gap between healthy life expectancy and average life expectancy is currently a matter of global concern. This gap creates a considerable financial and social burden (2). The increasing number of elderly individuals, and the length of time for which they need nursing care, have led to increases in medical costs. Extending healthy life expectancy is therefore an urgent issue (3). Among the various diseases that shorten healthy life expectancy, aspiration pneumonia is particularly relevant to the field of dentistry (4). Aspiration pneumonia is a major concern in the elderly (5). It is widely recognized that aspiration pneumonia is related to poor oral hygiene, and that oral care is vital for its prevention (6, 7).

There are various approaches to oral care that can prevent aspiration pneumonia, but the central method is tooth brushing by the individual to physically remove bacteria (8). However, in clinical practice, it is often observed that the oral cavity of the elderly is unhygienic. The main factor is disability. Elderly individuals may not be able to perform

fine hand movements, due to functional decline (8). It is not uncommon for caregivers to brush the teeth of elderly individuals because they cannot do it themselves.

Given this situation, it is important to develop a practical and straightforward method of oral care for the elderly. Gargling is one way to achieve this cleansing, and various methods involving gargling have been proposed. There have been many reports on the effects of chlorhexidine gluconate (CHG) (9-12). However, the use of chlorhexidine gluconate carries a risk of anaphylactic shock (13), and the elderly need safer mouth washes. I focused on using acid-electrolyzed functional water (FW) which is produced by electrolyzing low concentrations of salt water and collected the product from the anode chambers (14, 15). FW has been used in a variety of situations in the dental field. However, there have been no reports on its usefulness as a mouth wash for the elderly. Laboratory evidence has not demonstrated a strong antimicrobial effect of FW against the oral microorganisms, which are related to aspiration pneumonia. I hypothesized that FW could be an efficient mouth wash, and in this study, I investigated the bactericidal effects of FW, and its mechanism of action on bacteria associated with the development of aspiration pneumonia. I also investigated the impact of FW on the host cells. This study is a preliminary step toward the practical application of FW for oral care.

Materials and Methods

Purification of FW. FW (30 ppm actual chloride concentration (ACC), pH 2.2-2.7, and oxidation-reduction potential of more than 1,100 mV) was obtained using the Oxilyzer (Miura Denshi, Akita, Japan). The ACC was measured using an ACC measuring kit (Sibata, Soka, Japan). pH values were measured with a pH measuring device (ASONE, Osaka, Japan).

Culture of microorganisms. There are many microorganisms that can cause aspiration pneumonia (16, 17). In this study, I chose five frequently encountered species: the bacteria *Staphylococcus aureus* FDA209P, *Streptococcus pneumoniae* ATCC6305, *Pseudomonas aeruginosa* JCM2776, the yeast *Candida albicans* NUD202, and *Klebsiella pneumoniae* JCM1662. All organisms were maintained on brain-heart infusion (BHI; BD Biosciences, Rockville, MD, USA) agar. The subcultures were freshly prepared before use. Each strain was cultured for 24 to 48 h in nutrient broth and inoculated in the same broth at 37˚C under aerobic or anaerobic conditions. Bacterial and fungal cells were harvested in the late logarithmic phase by centrifugation at 5,000 × g at 4˚C for 10 min and washed twice in phosphate-buffered saline (PBS; pH 7.2).

Bactericidal effects. Each bacterial strain or *C.albicans* [1×10^7 colony-forming units (CFU)/ml] was mixed with 1 ml of PBS, FW, ConCool F [containing 0.05% CHG, Weltec, Osaka, Japan], or LISTERINE (containing methyl salicylate, cineole, thymol, and l-menthol, Johnson & Johnson, New Brunswick, NJ, USA) for 30 s. I used two concentrations of ConCool F: undiluted, and the optimal concentration suggested by the manufacturer (0.0006% CHG). After mixing for 30 s, the mixture was diluted and 50 μl was plated on a BHI agar plate. The plates were inverted and cultured for 24 h in a 37˚C incubator, after which the bacterial colony numbers were counted.

Measurement of the activity of superoxide dismutase. *S. aureus* (1×10^7 CFU/ml) were treated with FW diluted with PBS (concentration: 10, 30, 50, or 70%) for 30 s. After treatment, I crushed the bacteria with zirconia beads (NIPPON Genetics, Tokyo, Japan) and a Beads Crusher μT-12 (TAITEC, Saitama, Japan), and the superoxide dismutase (SOD) activity was measured using Superoxide Dismutase Assay Kits (Cayman Chemical, Ann Arbor, MI, USA). The absorbance was measured on a microplate reader model 3550 at a wavelength of 450 nm (BioRad, Hercules, CA, USA). I counted the CFUs of *S. aureus* treated with each concentration of FW.

Human cell culture. Two types of cell lines (HSC-3 and HeLa) were obtained from the Health Science Research Resources Bank (Osaka, Japan). The HeLa cells were maintained in minimum essential medium supplemented with 10% fetal calf serum (FCS), 50 mg/ml streptomycin, and 50 U/ml penicillin in a 5% CO_2 incubator. The HSC-3 cells were maintained in RPMI1640 medium supplemented with 10% FCS, 50 μg/ml streptomycin, and 50 U/ml penicillin in a 5% CO_2 incubator.

Cell stimulation with FW and measurement of cytotoxicity. Both cell types were plated in 96-well plates at two densities (1×10^4 and 1×10^5 cells/well) on the day before the experiment, and treated with FW, ConCool F (undiluted only), or LISTERINE for 30 s. After treatment, the cells were washed with the appropriate cell culture medium and cultured for 1 h. The culture supernatant was harvested, and cell viability was measured with XTT Cell Proliferation Assay Kits (Cayman Chemical, Ann Arbor, MI, USA). I measured cytotoxicity as the cell viability after treatment. Cell viability was measured from the OD value based on PBS.

Statistical analysis. The collected data were imported to SPSS ver. 26.0 (SPSS, Chicago, IL, USA) for statistical analysis. All experimental data are presented as the mean ± SD,

and $n = 5$. Statistical analysis was performed using one-way analysis of variance with Tukey's multiple comparisons test; $p < 0.05$ was considered to indicate statistical significance. To evaluate the cytotoxicity of FW, I performed statistical analysis on the cell viability data only.

Results

Bactericidal effects. After treatment with FW, almost no colony formation was detected in any of the species investigated (Figure 1A-E). The same results were found following treatment with LISTERINE and undiluted ConCool F. Diluted ConCool F produced bactericidal effects only on *C. albicans* (Figure 1D).

SOD activity. I investigated the effect of FW treatment on SOD activity in *S. aureus*, a major causative agent of aspiration pneumonia. SOD activity significantly increased more than twice when the bacteria were treated with 50% FW ($p < 0.05$), peaked at this point, and then decreased (Figure 2A). Stimulation with 10% FW also showed a significant increase in SOD activity ($p < 0.05$, fold-change: 1.27).

A significant decrease in colony formation was observed after treatment with 30% FW, whereas an extreme decrease in the number of bacteria was observed after treatment with 50% FW. Almost no colony formation was observed with 70% FW (Figure 2B).

Cell viability. The three reagents produced significantly lower cell viability ($p < 0.05$ for both cell density) in HeLa cells than in the control PBS-treated cells. There was no significant

difference in the viability of HeLa cells among the three reagents when I treated low-density cells. When stimulated high-density cells were treated, FW produced higher cell viability than that of the other mouth washes (both $p < 0.05$, Figure 3A and B).

In HSC3 cells, the three mouth washes produced significantly lower cell viability than PBS ($p < 0.05$ for both cell density). There was a significant difference between FW treatment and LISTERINE treatment in low-density cells. Upon treatment of high-density cells, the viability of cells after treatment with FW was significantly higher than that of the other gargling agents (both $p < 0.05$, Figure 3C and D).

Discussion

There have been many studies on the use of FW in the field of dentistry. FW has been reported to be effective as an in root canal treatment (18, 19). There are, however, no reports of its use as a gargling agent for oral care of the elderly. The results showed that FW may be valuable in the maintenance of oral hygiene. Aspiration pneumonia is caused by a wide range of bacteria. I analyzed the effect of FW on five major causative organisms of aspiration pneumonia and found that it had excellent bactericidal effects against all five organisms. Its bactericidal effect was equal to or greater than that of conventional mouth washes.

Several mechanisms of action have been reported for the bactericidal effect of FW. In the guidelines published by the Society for Oral Functional Water, it is suggested that its bactericidal mechanism may be based on oxidation by hypochlorous acid (20). In this study, changes in the activity of SOD, which is an indicator of oxidative reactions (21), was used to determine whether an oxidative response occurred when the bacteria were treated with FW. I confirmed a change in SOD activity in *S. aureus*, one of the primary causative bacteria of aspiration pneumonia. Since treatment of *S. aureus* with 100% FW killed all bacteria, five different concentrations of FW were used, and SOD was measured

when the bacteria survived treatment. Treatment with 50% FW significantly increased the SOD activity, and the concentration was consistent with the concentration at which a significant bactericidal effect on *S. aureus* was observed. Following treatment with 70% FW, the SOD activity tended to be higher than that of the control, despite the small number of viable bacteria. These data supported the hypothesis that the bactericidal effect of FW arises from oxidative stress. These results suggested that pH possibly influenced the SOD activity; however, I believe this is unlikely, as reported by Nagamatsu *et al.* (22).

FW produced similar or lower rates of cell death as other mouth washes. Especially, FW showed significantly higher cell viability than the other two mouth washes in both cell lines with high-density cultures. This observation suggests that FW is less cytotoxic than the other gargling agents. Previous reports have shown that the use of FW has no adverse effects on the human body. Morita *et al*. reported that mice administered FW orally experienced no systemic effects (23). I envision that FW will be used for oral care of the elderly, and the fact that it is less cytotoxic than other gargling agents indicates that the elderly can use it safely.

The present study was preliminary, aiming to lay the groundwork for further clinical research, and is essentially a baseline report. However, the results suggest that FW may be more beneficial than other mouth washes.

CHG which is the main component of ConCool F is one of the most widely used mouth washes globally (24, 25). Most reports indicate that it is used in concentrations of 0.10 - 0.20% (26). However, the concentration used varies from country to country. In Japan, the maximum concentration used is 0.05%, due to the risk of anaphylactic shock and gingival necrosis (27). Further dilution is recommended; for ConCool F, the concentration after dilution is about 0.006%. CHG in Japan is diluted about 200 times more than the concentration used in other countries. It is not easy to directly quote the results of reports describing the effects of conventional CHG rinsing. The results also show that, except for *C. albicans,* treatment with diluted CHG did not have an adequate bactericidal effect. FW is an aqueous solution consisting mainly of hypochlorous acid, which is purified by the electrolysis of salt water, and the possibility of gingival necrosis caused by FW is low. Although the stimulation time was short, Morita *et al*. also reported no gingival necrosis (23). In my opinion, investigations into the safety of FW should be focused on aspects other than cytotoxicity. It has been reported that common mouth washes, such as LISTERINE and ConCool F used in this experiment, have a pungent taste, which can cause taste disorders (28-30). Taste disorders can cause a wide range of problems and reduce an individual's quality of life (31). Many elderly individuals are hesitant to use gargle products under these circumstances. It is believed that taste

disorders in gargling materials are mainly due to the effects of drugs (29). FW is produced by the electrolysis of salt water; thus the risk of taste disturbance is low. FW is also used for washing food, but no taste abnormalities have been reported due to washing. However, since it can be used for cleaning food, it appears that the risk of consuming FW is insignificant. In light of the above, FW may be a gargle material that can solve the problems faced by current gargle materials.

Although FW is a beneficial mouth wash, some clinical issues need to be addressed. First, there needs to be concern about dental demineralization by FW. Tooth demineralization develops due to a lack of balance with tooth remineralization (32, 33). I believe that the demineralization of teeth can be avoided by not allowing the oral pH to become acidic for long periods of time after rinsing with FW, by restoring it to neutral at an early stage. Therefore, after gargling with FW, it is essential to restore the pH of the oral cavity to neutral by rinsing with tap water. Morita *et al*. reported changes in tooth morphology due to FW impregnation for three weeks in an *in vitro* experiment (23). Still, no significant crown demineralization due to FW was observed in the short-term analysis. I may need to pay attention to gargling with FW in the long term.

FW has a short storage period, because it reverts to normal water after a period of time after purification (34), and is thus generally regarded as safe. However, this reversion

indicates that the sterilizing power will be lost. FW maintains its function for one month in cold, dark storage after purification following the guidelines of the Japan Society for Oral Functional Water (20). Ideally, FW should be used immediately after purification, thus it is not easy to distribute effective FW in the market. Therefore, it is necessary to purchase a generating device for use. I aim to use FW in the oral care of the elderly, but since it is difficult to purchase a single device for each household, it is realistic to use it only in nursing homes and dental clinics. For FW use to become widespread in general households, it is necessary to downsize the generating device, but I believe that this problem can be solved in the future.

A variety of mouth washes are available in the market and are used for oral care. my results confirmed that FW had a sufficient bactericidal effect and higher safety than other gargling agents. Safety is an essential factor in the use of gargles by the elderly. I believe that FW can become a new standard for gargling agents in the oral care of the elderly.

Conflicts of Interest

I report no conflicts of interest related to this work.

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Figures

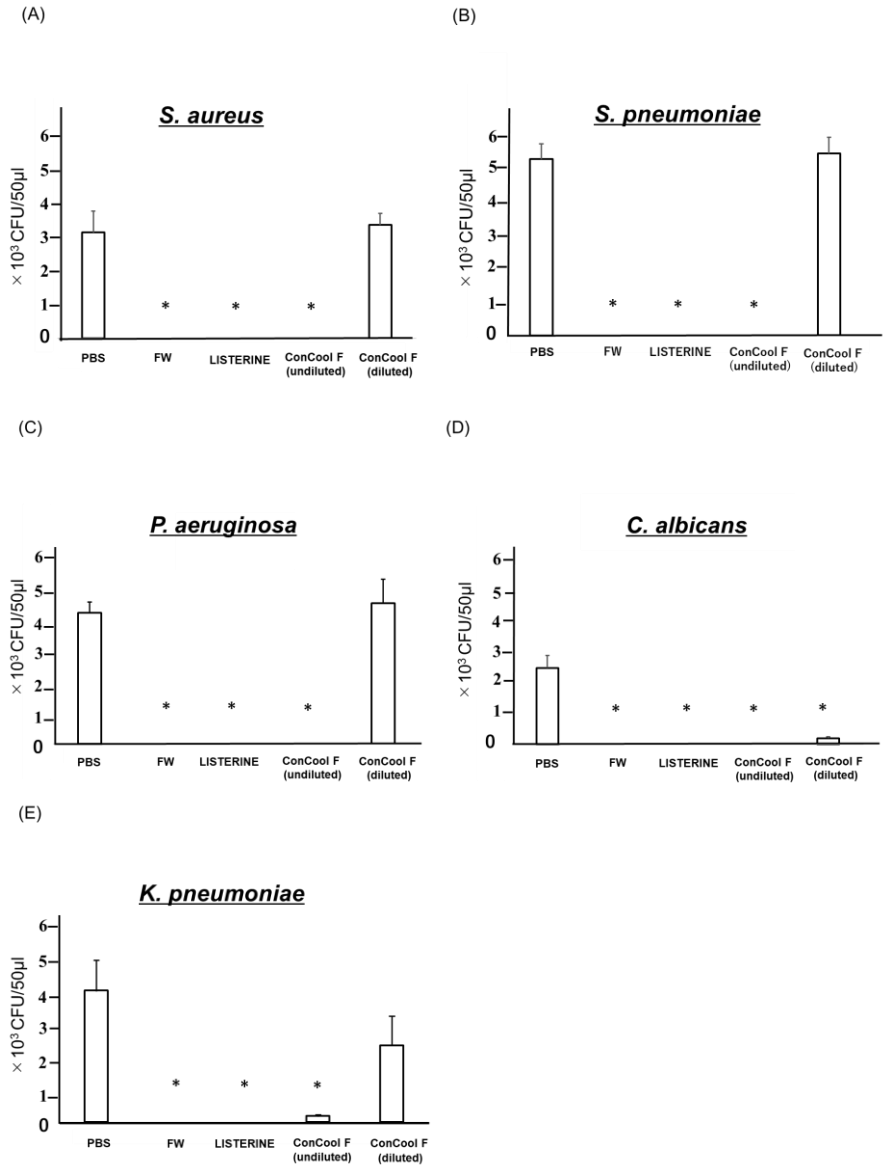

Figure 1. Bactericidal effects of each solution. The colony number was counted 24 h after plating, and the number of colony-forming units (CFUs) was also counted. (A) *S. aureus,* (B) *S. pneumoniae,* (C) *P. aeruginosa,* (D) *C. albicans,* and (E) *K. pneumoniae.*
The data are expressed as mean ± SD. **p* < 0.05 vs. PBS.

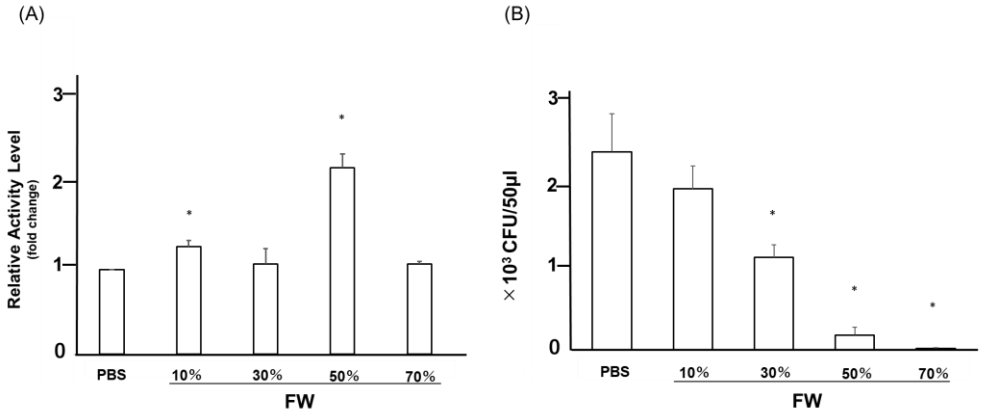

Figure 2. Effect of superoxide dismutase activity after treatment. (A) Comparison of superoxide dismutase activity of *S. aureus* after treatment with various concentrations of functional water. (B) The colony numbers were counted 24 h after treatment, and the colony-forming units were also calculated. The data are expressed as mean ± SD. **p* < 0.05 vs. PBS.

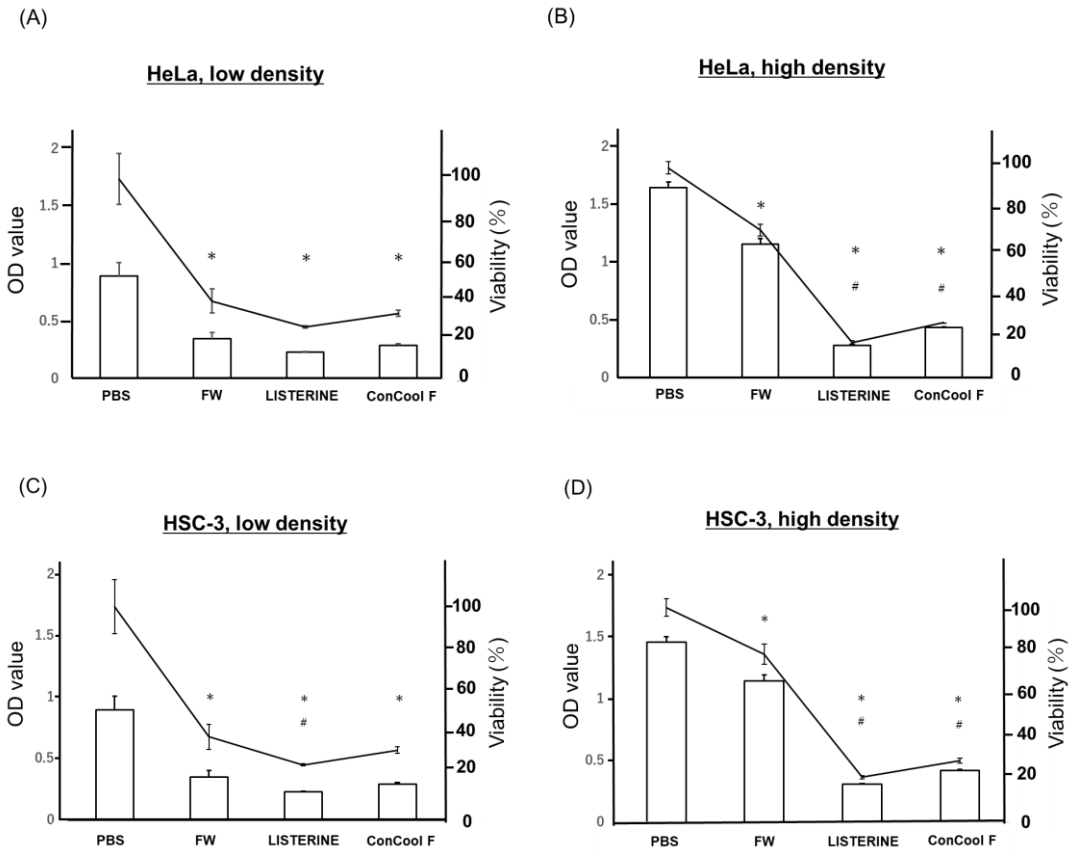

Figure 3. Viability of cells after treatment. (A) HeLa, low density (1×10^4 cells/well), (B) HeLa, high density (1×10^5 cells/well). I show the OD value and cell viability (line). (C) HSC-3, low density (1×10^4 cells/well), (D) HSC-3, high density (1×10^5 cells/well). I show the OD score and cell viability (line). The data are expressed as mean ± SD. *p < 0.05 vs. PBS, #p < 0.05 vs. FW.