Postantifungal-Like Effect of Sublethal Treatment of *Candida albicans* with Acid-Electrolyzed Water

Running title: Postantifungal-Like Effect of AEW

Takayuki Mokudai, Taro Kanno, Yoshimi Niwano*

Tohoku University Graduate School of Dentistry, 4-1 Seiryō-Machi, Aoba-ku, Sendai
980-8575, Japan

***Corresponding author**

Phone: +81-22-717-8298, Fax: +81-22-717-9299

E-mail: niwano@m.tohoku.ac.jp

Abbreviations: acid-electrolyzed water, AEW; postantifungal-like effect, PAFE;
postantibiotic effect, PAE; reactive oxygen species, ROS; hydroxyphenyl fluorescein,
HPF

ABSTRACT

Objective: Acid-electrolyzed water (AEW) has been applied to the treatment of oral candidiasis. We evaluated the postantifungal effect (PAFE)-like activity of AEW against *Candida albicans* under sublethal conditions by exposing *C. albicans* to dilute AEW.

Design: The growth of *C. albicans* after a short-term exposure to dilute AEW was evaluated in broth and on agar culture. The involvement of reactive oxygen species (ROS) in the PAFE was examined by flow cytometric analysis with hydroxyphenyl fluorescein (HPF) as a fluorescence probe.

Results: The dilute AEW exerted PAFE-like activity against *C. albicans*. ROS were produced in the cells treated with AEW diluted 16 times or fewer. The increase in HPF fluorescence after treatment with dilute AEW was cancelled by dimethyl sulfoxide, a hydroxyl radical ($\cdot\text{OH}$) scavenger.

Conclusion: It would be expected that the ROS, especially $\cdot\text{OH}$, produced in the *C. albicans* cells treated with sublethal dilutions of AEW could exert PAFE-like activity against the fungal cells.

Keywords: acid-electrolyzed water, *Candida albicans*, growth inhibition, reactive oxygen species

1. Introduction

Acid-electrolyzed water (AEW) is produced by the electrolysis of dilute sodium chloride (NaCl) or potassium chloride solution in the anode side of an instrument where the anode and cathode are separated by an ion-permeable diaphragm. AEW has been used mainly in the agricultural and medical fields as a disinfectant in farm and food hygiene (1-8) and for the disinfection of medical instruments such as dialyzers (9), patient-used endoscopes (10), and dentures (11) because of the potent antimicrobial potential of AEW (12-16). Recent studies showed the possible applications of AEW to various fields. It was reported that AEW could be used to improve hygiene of drinking water for dairy ewes (17), to sterilize biologic scaffolds with retaining their bioactivity for regenerative medicine in addition to its high efficiency for cell remnant removal (18), and to manage chronic rhinosinusitis by nasal irrigation (19).

The antimicrobial activity of AEW might be the result of the combined effects of the oxidation–reduction potential (ORP) and pH (20). The characteristic values of AEW, such as low pH (2.7 or lower) and high ORP (+1100 mV or higher), deviate from the tolerable range for microbial growth (pH 3–10, ORP +900–400 mV) proposed by Becking et al. (21). Regarding the potent oxidative power of AEW in relation to its antimicrobial potential, although the hydroxyl radical ($\cdot\text{OH}$), an oxygen radical species, is often suggested as a putative active ingredient for AEW antimicrobial activity (22), our previous study demonstrated that hydrogen peroxide (H_2O_2) is present in AEW as a source of $\cdot\text{OH}$, but the antimicrobial activity of AEW does not depend on the radical (23). To date, the main contributors to the antimicrobial activity of AEW have been thought to be hypochlorous acid (HClO), which has a low pH and a high ORP (24). Some studies have also suggested that HClO , as an undissociated form of chlorine,

penetrates microbial cell membranes and subsequently achieves its antimicrobial action through the oxidation of key metabolic enzymes (25-27). In addition to direct microbicidal activity of AEW, a recent study revealed that AEW may affect host immune defense system. That is, AEW could induce human β -defensins-2, a major family of cationic antimicrobial peptides, in oral epithelial cells (28).

Besides the bactericidal effect, diluted AEW is expected to inhibit the growth of remaining microorganisms that escaped the lethal effect. The lag of bacterial and fungal regrowth after a short-term exposure to antimicrobial agents is known as the postantibiotic effect (PAE) and postantifungal effect (PAFE), respectively (29-34). Although the terms PAE and PAFE were originally used for antibiotics and antifungals, the similar effect of disinfectants and antiseptics is also designated as PAE and PAFE (34-37). Regarding the PAE of disinfectants, it was reported that chloramine T, mandelic acid, chlorhexidine, and povidone-iodine induced PAE against nine bacterial pathogens, such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Enterococcus faecalis*, following a 2-min treatment with sublethal conditions (36). We have recently reported that a disinfection treatment by photolysis of H_2O_2 induces PAE in *S. aureus* and *Streptococcus salivarius* even though they were treated for only 10–30 s (38). Because the active ingredient of the disinfection treatment is thought to be $\cdot OH$, the PAE is likely induced by a chemical injury, and the resynthesis of macromolecules for repair, which is a slow process, becomes required. As is the case with $\cdot OH$, which causes lipid peroxidation and oxidative damage to DNA, RNA, and proteins (39-41), diluted AEW is expected to delay the microbial regrowth because $HClO$, an active ingredient of AEW, nonspecifically oxidizes several cell structures consisting of macromolecules.

Oral candidiasis is a clinical fungal infection that is the most common opportunistic infection of the human oral cavity (42). Several studies have applied AEW to oral hygiene (11, 43-45), and AEW was proven effective for the treatment of oral candidiasis (43). However, because salivary proteins attenuate the microbicidal efficacy of AEW in the oral cavity (44), it is critical to examine the sublethal effect of AEW on *Candida albicans*, a major pathogen of oral candidiasis. The purpose of this study was to evaluate the PAFE-like activity of AEW under sublethal conditions by exposing *C. albicans* to dilute AEW.

2. Materials and methods

2.1. Fungus

Candida albicans JCM 1537 was purchased from the Japan Collection of Microorganisms, RIKEN BioResource Center (Wako, Japan). A fungal suspension was prepared in sterile physiological saline from a culture grown on Sabouraud dextrose agar (SDA) consisting of 40 g/L dextrose, 10 g/L peptone, and 20 g/L agar at 37°C for 24 h. The fungal suspension was prepared at the time of use to be approximately 1×10^7 cells.

2.2. Preparation of AEW

NaCl solution (0.1% (w/v)) was electrolyzed for 15 min using a batch-type electrolyzed water generator (ALTRON MINI AL-700A, Altec Corporation, Nagano, Japan) at a regular AC voltage of 100 V and a rated current of 0.6 A. The characteristic values of the resultant AEW were determined using a pH/ORP meter (SG2, Mettler-Toledo, LLC, Columbus, OH, USA) for pH and ORP, and a residual chloride meter (HI196771C,

Hanna Instruments Japan, Tokyo, Japan) for residual chloride concentrations. Twofold serial dilutions of AEW were then prepared with pure water. The pH, ORP, and residual chlorine concentration of the undiluted AEW were 2.2–2.7, ≥ 1100 mV, and 50–60 mg/L, respectively. As for the concentrations of free available chlorine, including Cl_2 , HClO , and ClO^- , in undiluted AEW determined by the *N,N'*-diethyl-p-phenylenediamine standard method (46) were almost the same range of total residual chlorine concentration (50–60 mg/L). We also checked the concentration of H_2O_2 in the undiluted AEW determined by the colorimetric method based on the peroxide-mediated oxidation of Fe^{2+} followed by the reaction of Fe^{3+} with xylene orange (47). The concentration was around 100–200 μM , which is non-toxic level against *C. albicans*. When AEW was diluted, the residual chlorine concentration decreased in proportion to the dilution rate. The pH increased gradually with the dilution rate and the pH of AEW diluted 64 times became more than 4.0. The ORP decreased gradually with the dilution rate and the ORP of AEW diluted 64 times became less than 900 mV.

2.3. Growth curve evaluation after a short-term exposure to dilute AEW

The growth curve analysis of *C. albicans* after a 10-s exposure to the 2-fold serial dilutions of AEW was performed by measuring the optical density of the fungal suspension at 595 nm (OD595) with time. It was confirmed that the linear relationship was obtained between the number of *C. albicans* cells and OD595 at least within a range of 0–0.3 of OD595. An aliquot (0.5 mL) of *C. albicans* suspension in sterile physiological saline was mixed with 2 mL pure water or 2-fold serial dilutions of AEW for 10 s. Then the reaction was terminated by the addition of 0.5 mL fetal bovine serum (FBS, Life Technologies Corp., Carlsbad, CA, USA) because the bactericidal activity of

AEW could be abolished by organic materials such as proteins and amino acids through the rapid transformation of free available chlorine into *N*-chloro compounds (46). The suspension was centrifuged (3000 rpm for 10 min), and the resultant pellet was washed with sterile physiological saline. The washing procedure was repeated twice. The pellet was then resuspended in 3 mL RPMI 1640 medium buffered with 0.165 M morpholinopropanesulfonic acid (MOPS, pH 7.0). An aliquot (300 μ L) of the sample was transferred to a well of a 96-well culture plate. Immediately after the addition of the sample, the plate was placed in a microplate reader (FilterMax F5, Molecular Devices, Sunnyvale, CA, USA) with the temperature control at 37°C, and the OD595 was recorded. The OD595 was measured every 60 min until the control culture reached stationary phase. It was confirmed that the morphology of *C. albicans* observed by an inverted microscope was almost yeast-like. Since pseudomycelium was observed for the rare occasion, we judged that effect of pseudomycelium on the OD595 was negligible. All tests were performed in triplicate (three independent assays). In an experiment where the effect of dimethyl sulfoxide (DMSO; Wako Pure Chemicals, Osaka, Japan), a potent scavenger of $\cdot$ OH (48-51), on the growth curve of *C. albicans* was examined, the fungal cells were treated in the same way as described. The resultant pellet was resuspended in 3 mL RPMI 1640 medium buffered with 0.165 M MOPS containing 100 mM DMSO. An aliquot (300 μ L) of the sample was transferred to a well of a 96-well culture plate to monitor the fungal growth as described above.

To confirm the fungicidal effect of dilute AEW, a culture experiment was also conducted to determine the number of viable fungal cells. Immediately after the treatment of *C. albicans* with dilute AEW, a *C. albicans* suspension was prepared as described above. Then, 10-fold serial dilutions of the *C. albicans* suspension were

prepared using sterile physiological saline, and 10 μ L of each dilution was seeded onto SDA. After culture at 37°C for 48 h, the number of viable cells/mL was determined. All of the tests were performed in duplicate (two independent assays).

2.4 Evaluation of colony growth on agar plates after the short-term exposure to dilute AEW

The fungal growth after exposure to the dilute AEW was also examined using agar medium. The fungal suspension of *C. albicans* was treated with pure water or the 2-fold serial dilutions of AEW for 10 s as described above. After the addition of FBS, followed by washing twice as described above, the resultant pellet was resuspended in 3 mL sterile physiological saline. To avoid the conditions in which colonies grown on the agar plate become too numerous to count, some of the samples were further diluted with physiological saline. Then, 50 μ L each of undiluted and diluted samples was plated on SDA plates. After 16, 20, 24, and 36 h of incubation at 37°C, photographs of the agar plates were taken. Binary images of the photographs were processed, and the total area (mm^2) of the colonies was calculated using ImageJ (an image processing program) provided by the Research Services Branch of the NIH. Only the colonies with a diameter of 0.5 mm or more were analyzed. The total area of the colonies was divided by the number of colonies to calculate the average area (mm^2) per colony. All tests were performed in three independent assays.

2.5. Flow cytometric measurement of forward and side scatter (FS and SS) of light

The flow cytometric measurement of FS and SS of light was performed essentially as described in an earlier report (52). A fungal suspension of *C. albicans* was treated with

pure water or the two-fold serial dilutions of AEW for 10 s as described above. After the addition of FBS followed by washing twice as described above, the resultant pellet was resuspended in 3 mL sterile physiological saline followed by the flow cytometric measurement with a flow cytometer (Cytomics FC500, Beckman Coulter, Brea, CA, USA). The sample flow rate was set at medium. The instrument settings were as follows: FS, 637 V; SS 1000 V; and threshold value, 100 for FS. Each sample was analyzed for 10,000 events or yeast cells. Electronic gates were set up based on cells in control experiments (treated with pure water). Cell debris and clusters below the gates were not included in sample analyses. The samples were analyzed for FS and SS to obtain the percentile of the cells within the gated area.

2.6. Flow cytometric measurement of reactive oxygen species (ROS)

Single cell analysis of hydroxyphenyl fluorescein (HPF; Sekisui Medical Co. Ltd., Tokyo, Japan) fluorescence was performed to determine the level of ROS in the cell. As a fluorescent probe, aminophenyl fluorescein (APF) could be used instead of HPF because APF can detect not only ROS but OCI^- . However, it is proposed that OCI^- cannot penetrate into microbial cells because lipid bilayer of plasma membrane prevents ion from penetrating (53). Thus, to avoid the interference effect of extracellular OCI^- , HPF was used in the experiment. A fungal suspension of *C. albicans* was treated with pure water or the 2-fold serial dilutions of AEW for 10 s as described above. After the addition of FBS followed by washing twice as described above, the resultant pellet was resuspended in 3 mL 5 μM HPF dissolved in phosphate-buffered saline (PBS; pH 7.4). Immediately after and 24 h after the resuspension, samples were analyzed by the flow cytometer (Cytomics FC500), with excitation at 488 nm and emission at 525 nm. Ten

tho not only used cells were measured for each sample. The shift in fluorescence was calculated as the difference between the average fluorescence of the population in the AEW treatment groups and the average in the control group (pure water treatment). In an experiment where the quenching effect of DMSO on HPF fluorescence was examined, the fungal cells were treated in the same way as described above except that the pellet was resuspended in 3 mL of the mixture of 5 μ M HPF and 100 mM DMSO in PBS, followed by the flow cytometric analysis.

2.7. Statistical analyses

The statistical significance of the colony size of *C. albicans* between the control and 16-times diluted AEW group was assessed by the Student's *t* test. The statistical significance of the percentile of the cells within the gated area in the flow cytometric measurement of FS and SS and of the calculated shift in fluorescence in the flow cytometric measurement of ROS from the corresponding control groups was assessed by Dunnett's multiple comparison test. The statistical significance of the calculated shift in fluorescence in which the quenching effect of DMSO was examined among the groups was assessed by the Tukey–Kramer multiple comparison test. P values of <0.05 were considered to be significant.

3. Results

3.1. Growth curve in the broth and colony growth on the agar after the short-term exposure to dilute AEW

Figure 1 shows the growth curve of *C. albicans* treated with different dilutions of AEW for 10 s. AEW diluted 32 and 64 times did not affect the growth of *C. albicans*

compared with that of the control (pure water treatment). In contrast, undiluted AEW and AEW diluted two and four times completely inhibited the fungal growth. Delayed growth of the fungal cells was observed only when they were treated with AEW diluted 16 times. This delayed growth was reproducibly observed in additional experiments. In the culture experiment in which the fungicidal effect of dilute AEW was examined, only AEW diluted 16 times showed a sublethal or partially lethal effect on *C. albicans*. The recovered number of living *C. albicans* cells was 1.7×10^5 cells/mL (final volume was 3 mL) of control and 2.2×10^3 cells/mL when treated with AEW diluted 16 times. The recovered cells/mL of *C. albicans* treated with AEW diluted eight times or fewer was below the detection limit ($<1 \times 10^2$ cells/mL), and AEW diluted 32 and 64 times showed no lethal effect on *C. albicans*. To examine the effect of inoculum size on the growth of *C. albicans*, the growth of *C. albicans* at an inoculum size of 2×10^3 cells/mL was monitored. We observed delayed fungal growth compared with that of the control, but the delay was apparently milder than that of *C. albicans* treated with AEW diluted 16 times (data not shown). Figure 2 shows the quantitative analysis of colony size grown on the agar plates. The colony size of *C. albicans* treated with AEW diluted 16 times was significantly smaller than that of the control (pure water treatment) 24 h after incubation (Fig. 2). After incubation for 36 and 48 h, no differences in the colony size between the two groups were detected. The reproducibility was confirmed by an additional experiment.

3.2. Flow cytometric analyses of *C. albicans* after the short-term exposure to dilute AEW

Figure 3a shows the FS versus SS contour plot profiles of *C. albicans* treated with dilute

AEW for 10 s. When *C. albicans* cells were treated with undiluted AEW, the SS value of each cell tended to move toward a lower level than that of control cells, while the cytogram of *C. albicans* cells treated with AEW diluted 16 times was very similar to that of control cells. Figure 3b summarizes the percentage of the cells in the gated area in which more than 90% of control cells was involved. The percentage of gated *C. albicans* cells treated with undiluted AEW and AEW diluted four times was significantly lower than that of control cells, while that treated with AEW diluted 16 and 64 times was almost the same as that of control cells.

Figure 4a shows representative histograms of HPF fluorescence after treatment with dilute AEW for 10 s. The histogram of the cells was shifted when the cells were treated with AEW diluted 16 times or fewer; this shift was observed not only immediately after the treatment but also 24 h after the treatment, indicating that ROS was continuously present in the cells. Figure 4b summarizes the calculated shift in fluorescence as the difference between the average fluorescence of the population in the AEW treatment groups and the average in the untreated group without HPF load. Single-cell analysis by the flow cytometer showed a significant shift in HPF fluorescence in response to treatment with AEW diluted 16 times or fewer both immediately and 24 h after the treatment; in the both cases, the greatest shift was observed with undiluted AEW.

3.3. Effect of DMSO, an $\cdot\text{OH}$ scavenger, on the growth curve and HPF fluorescence of *C. albicans*

Figure 5 shows the growth curve of *C. albicans* exposed to AEW diluted 16 times in the presence or absence of 100 mM DMSO. When *C. albicans* exposed to pure water was

cultured in the presence of 100 mM DMSO, no growth delay was observed, indicating that 100 mM DMSO was not cytotoxic to *C. albicans* cells. When *C. albicans* was exposed to AEW diluted 16 times, the growth of *C. albicans* was delayed as in Figure 1, and the delayed growth of *C. albicans* was partially cancelled by the presence of 100 mM DMSO.

Figure 6a shows representative histograms of HPF fluorescence 24 h after treatment with AEW diluted 16 times for 10 s. The histogram of the cells was shifted when the cells were treated with AEW diluted 16 times as in Figure 4a, and this shift was completely prevented in the presence of 100 mM DMSO. Figure 5b summarizes the calculated shift in fluorescence as the difference between the average fluorescence of the population in the treated groups and the average in the untreated group without HPF load. A significant shift in HPF fluorescence in response to treatment with AEW diluted 16 times was observed as in Figure 4b, and this shift was completely abolished in the presence of 100 mM DMSO.

4. Discussion

This study demonstrated that the dilute AEW could exert PAFE-like activity against *C. albicans*. PAFE has been considered to be a valuable parameter for the evaluation of antifungals. In this study, treatment with AEW diluted 16 times clearly delayed the fungal growth in the broth culture. However, AEW diluted 16 times showed a slight fungicidal effect on *C. albicans* as demonstrated in the culture experiment, reducing the initial number of living cells in the broth culture study. Nonetheless, because the growth delay of *C. albicans* at an initial inoculum of 2×10^3 cells/mL was clearly milder than that of *C. albicans* treated with AEW diluted 16 times when the initial number of cells

was also approximately 2×10^3 cells/mL, the growth delay *C. albicans* cells treated with AEW diluted 16 times was at least partially attributable to impaired cell growth. In addition, to eliminate the effect of the initial number of living cells, the colony growth was then evaluated on agar medium. Similar to the broth culture, treatment with AEW diluted 16 times delayed the colony growth of *C. albicans*.

As is the case with PAE, PAFE is commonly defined as the time required for the fungal cell to recover from the transient injury sustained by the organism as a result of a brief exposure to an antifungal drug. Regarding antifungals, amphotericin B physically creates pores in the cytoplasmic membrane of fungal cells, rendering it biologically nonfunctional; the effect is rapid and requires only a short exposure to inflict a prolonged crippling effect on the cell (30). In the case of fungistatic triazoles, PAFE could be induced by the prolonged time required for the depletion of lanosterol in the fungal cell by inhibiting cytochrome P450-dependent lanosterol demethylase (30). Fuursted et al. suggested that two types of injury were possibly involved in the mechanism of PAE caused by disinfectants (6). One type of injury is a chemical injury by which the cell membrane is destroyed, and the resynthesis of macromolecules for repair, a slow process, is required. The other type of injury is a physical injury by which the configurations of membrane components are altered, and re-organization for repair, a rapid process, is required. Thus, the PAE is extended by the chemical injury more than the physical injury. Although our previous study showed that the active ingredient of AEW is HClO and not $\cdot\text{OH}$ (23), antibacterial mechanisms of HClO are thought to be partially attributable to ROS, including superoxide anion ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and highly reactive $\cdot\text{OH}$, which might be formed in the bacterial cells from HClO (54, 55). The present study supports the idea that ROS in the *C. albicans* cells are

involved in the antifungal activity of AEW because the intensity of HPF fluorescence increased in the fungal cells when they were pretreated with a range of AEW dilutions, which partially or completely inhibited the following fungal growth in broth culture. The increased HPF fluorescence was abolished in the presence of DMSO, a potent $\cdot\text{OH}$ scavenger (48-51), suggesting that the ROS that increased in the *C. albicans* cells was likely $\cdot\text{OH}$. This was also supported by the observation that the delayed growth of *C. albicans* exposed to AEW diluted 16 times was partially prevented in the presence of noncytotoxic concentrations of DMSO. In the flow cytometric measurement of FS and SS of light, treatment with AEW diluted 16 times did not affect the cytogram of the *C. albicans* cells. Because FS and SS reflect cell size and intracellular complexity, respectively (56, 57), AEW diluted 16 times did not cause notable morphological changes in the fungal cells. Nonetheless, ROS were produced in the cells treated with AEW diluted 16 times. Therefore, it would be expected that the ROS produced in the cell could induce PAFE to the extent that the ROS would not cause morphological changes in the fungal cell.

Regarding the underlying mechanism by which ROS are produced, three mechanisms might be involved. As an alternative unified mechanism of antibiotic killing, it was proposed that bactericidal compounds, irrespective of their mode of action, induce the formation of ROS, especially highly reactive $\cdot\text{OH}$, by activating the electron transport chain (58, 59). Similar to the case of bacteria, electron leakage from the electron transport chain of mitochondria in the AEW-treated *C. albicans* cells would reduce oxygen molecules (O_2) to $\text{O}_2^{\cdot-}$. Then, the reaction between HClO and $\text{O}_2^{\cdot-}$ generates $\cdot\text{OH}$ in the following way (60-63): $\text{HClO} + \text{O}_2^{\cdot-} \rightarrow \cdot\text{OH} + \text{O}_2 + \text{Cl}^-$. Another mechanism is a Fenton-like reaction. That is, H_2O_2 , which arises from the dismutation

of $O_2^{\cdot-}$ and trace metals such as ferrous ions, generating $\cdot OH$ in the following way:

$H_2O_2 + Fe^{2+} \rightarrow \cdot OH + OH^- + Fe^{3+}$. A third mechanism is the one-electron reduction of HClO in the presence of ferrous ion to produce $\cdot OH$ in the following way: $HClO + Fe^{2+} \rightarrow \cdot OH + Cl^- + Fe^{3+}$ (64, 65).

In this study, increased HPF fluorescence in response to a range of diluted AEW was observed 24 h after the treatment. Because it has been demonstrated that the free available chlorine in AEW is quickly transformed into *N*-chloro compounds in the presence of amino acids or proteins (46), it would not be expected that the increased HPF fluorescence after 24 h was derived from HClO. In addition, it was reported that HPF functions as a fluorescence probe to detect selectively highly reactive ROS such as $\cdot OH$ (66). Therefore, it is likely that a Fenton-like reaction, as proposed above, is at least partially involved in the production of $\cdot OH$. Because it is known that $\cdot OH$ causes lipid peroxidation and oxidative damage to DNA, RNA, and proteins (39-41), sublethal amounts of $\cdot OH$ would probably induce PAFE on the fungal cells. Of recent advances in the methods of intracellular ROS detection, electron spin resonance approaches have been tried by developing suitable spin probes (67). Thus, we will try to determine oxygen radical species in *C. albicans* cells exposed to AEW by applying such approaches in the future study, because determination of oxygen radical species would be a critical point to elucidate the mechanism by which dilute AEW exerts PAFE.

Concerning the application of AEW to the treatment of oral candidiasis, even if the fungi survive after the disinfection treatment with AEW, the regrowth of the surviving fungi could be delayed by the PAFE as demonstrated in this study. The PAFE would probably contribute to both an extended interval of application and the time for the host immune defense system to attack the fungi.

Funding

This work was supported by a grant from the Japan Science and Technology Agency (JST) A-STEP (No. 241FT0011).

References

1. Fabrizio KA, Cutter CN. Comparison of electrolyzed oxidizing water with other antimicrobial interventions to reduce pathogens on fresh pork. *Meat Sci* 2004;**68**(3):463-468.
2. Northcutt J, Smith D, Ingram KD, Hinton A, Jr., Musgrove M. Recovery of bacteria from broiler carcasses after spray washing with acidified electrolyzed water or sodium hypochlorite solutions. *Poult Sci* 2007;**86**(10):2239-2244.
3. Guentzel JL, Liang Lam K, Callan MA, Emmons SA, Dunham VL. Reduction of bacteria on spinach, lettuce, and surfaces in food service areas using neutral electrolyzed oxidizing water. *Food Microbiol* 2008;**25**(1):36-41.
4. Cao W, Zhu ZW, Shi ZX, Wang CY, Li BM. Efficiency of slightly acidic electrolyzed water for inactivation of *Salmonella enteritidis* and its contaminated shell eggs. *Int J Food Microbiol* 2009;**130**(2):88-93.
5. Park YB, Guo JY, Rahman SM, Ahn J, Oh DH. Synergistic effect of electrolyzed water and citric Acid against *Bacillus cereus* cells and spores on cereal grains. *J Food Sci* 2009;**74**(4):M185-189.
6. Lu ZH, Zhang Y, Li LT, Curtis RB, Kong XL, Fulcher RG, et al. Inhibition of microbial growth and enrichment of gamma-aminobutyric acid during germination of

- brown rice by electrolyzed oxidizing water. *J Food Prot* 2010;**73**(3):483-487.
7. Rahman SM, Ding T, Oh DH. Effectiveness of low concentration electrolyzed water to inactivate foodborne pathogens under different environmental conditions. *Int J Food Microbiol* 2010;**139**(3):147-153.
8. Rahman SM, Jin YG, Oh DH. Combination treatment of alkaline electrolyzed water and citric acid with mild heat to ensure microbial safety, shelf-life and sensory quality of shredded carrots. *Food Microbiol* 2011;**28**(3):484-491.
9. Tanaka N, Fujisawa T, Daimon T, Fujiwara K, Yamamoto M, Abe T. The use of electrolyzed solutions for the cleaning and disinfecting of dialyzers. *Artif Organs* 2000;**24**(12):921-928.
10. Lee JH, Rhee PL, Kim JH, Kim JJ, Paik SW, Rhee JC, et al. Efficacy of electrolyzed acid water in reprocessing patient-used flexible upper endoscopes: Comparison with 2% alkaline glutaraldehyde. *J Gastroenterol Hepatol* 2004;**19**(8):897-903.
11. Nagamatsu Y, Tajima K, Kakigawa H, Kozono Y. Application of electrolyzed acid water to sterilization of denture base part 1. Examination of sterilization effects on resin plate. *Dent Mater J* 2001;**20**(2):148-155.
12. Kiura H, Sano K, Morimatsu S, Nakano T, Morita C, Yamaguchi M, et al. Bactericidal activity of electrolyzed acid water from solution containing sodium chloride at low concentration, in comparison with that at high concentration. *J Microbiol Methods* 2002;**49**(3):285-293.
13. Sharma RR, Demirci A. Treatment of Escherichia coli O157:H7 inoculated alfalfa seeds and sprouts with electrolyzed oxidizing water. *Int J Food Microbiol* 2003;**86**(3):231-237.

14. Nisola GM, Yang X, Cho E, Han M, Lee C, Chung WJ. Disinfection performances of stored acidic and neutral electrolyzed waters generated from brine solution. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 2011;**46**(3):263-270.
15. Rodriguez-Garcia O, Gonzalez-Romero VM, Fernandez-Escartin E. Reduction of *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* with electrolyzed oxidizing water on inoculated hass avocados (*Persea americana* var. Hass). *J Food Prot* 2011;**74**(9):1552-1557.
16. Feliciano L, Lee J, Pascall MA. Transmission electron microscopic analysis showing structural changes to bacterial cells treated with electrolyzed water and an acidic sanitizer. *J Food Sci* 2012;**77**(4):M182-187.
17. Bodas R, Bartolome DJ, Tabernero De Paz MJ, Posado R, Garcia JJ, Rodriguez L, et al. Electrolyzed water as novel technology to improve hygiene of drinking water for dairy ewes. *Res Vet Sci* 2013;**95**(3):1169-1170.
18. Hussein KH, Park KM, Teotia PK, Hong SH, Yang SR, Park SM, et al. Sterilization using electrolyzed water highly retains the biological properties in tissue-engineered porcine liver scaffold. *Int J Artif Organs* 2013;**36**(11):781-792.
19. Jiang RS, Liang KL, Wu SH, Su MC, Chen WK, Lu FJ. Electrolyzed acid water nasal irrigation after functional endoscopic sinus surgery. *Amer J Rhinol Allergy* 2014;**28**(2):176-181.
20. Handojo A, Lee J, Hipp J, Pascall MA. Efficacy of electrolyzed water and an acidic formulation compared with regularly used chemical sanitizers for tableware sanitization during mechanical and manual ware-washing protocols. *J Food Prot* 2009;**72**(6):1315-1320.
21. Becking LGMB, Kaplan IR, Moore D. Limits of the natural environment in terms of

- pH and oxidation-reduction potentials. *J Geolog* 1960;**68**:243-284.
22. Hotta K. Acid electrolyzed saline solution: Its function and medical application. *J Jpn Soc Intensive Care Med* 2000;**7**:97-105.
 23. Mokudai T, Nakamura K, Kanno T, Niwano Y. Presence of hydrogen peroxide, a source of hydroxyl radicals, in acid electrolyzed water. *PLoS One* 2012;**7**(9):e46392.
 24. Al-Haq MI, Sugiyama J, Isobe S. Applications of electrolyzed water in qgriculture & food Industries. *Food Sci Technol Res* 2005;**11**(2):135-150.
 25. Albrich JM, Gilbaugh JH, 3rd, Callahan KB, Hurst JK. Effects of the putative neutrophil-generated toxin, hypochlorous acid, on membrane permeability and transport systems of Escherichia coli. *J Clin Invest* 1986;**78**(1):177-184.
 26. Barrette WC, Jr., Hannum DM, Wheeler WD, Hurst JK. General mechanism for the bacterial toxicity of hypochlorous acid: abolition of ATP production. *Biochemistry* 1989;**28**(23):9172-9178.
 27. Hurst JK, Barrette WC, Jr., Michel BR, Rosen H. Hypochlorous acid and myeloperoxidase-catalyzed oxidation of iron-sulfur clusters in bacterial respiratory dehydrogenases. *Eur J Biochem* 1991;**202**(3):1275-1282.
 28. Gojoubori T, Nishio Y, Asano M, Nishida T, Komiyama K, Ito K. Distinct signaling pathways leading to the induction of human beta-defensin 2 by stimulating an electrolytically-generated acid functional water and double strand RNA in oral epithelial cells. *J Recept Signal Transduct Res* 2014;**34**(2):97-103.
 29. Craig WA. The postantibiotic effect. *Clin Microbiol Newslett* 1991;**13**:121-128.
 30. MacKenzie FM, Gould IM. The post-antibiotic effect. *J Antimicrob Chemother*

1993;**32**(4):519-537.

31. Manavathu EK, Ramesh MS, Baskaran I, Ganesan LT, Chandrasekar PH. A comparative study of the post-antifungal effect (PAFE) of amphotericin B, triazoles and echinocandins on *Aspergillus fumigatus* and *Candida albicans*. *J Antimicrob Chemother* 2004;**53**(2):386-389.
32. Chryssanthou E, Loebig A, Sjolín J. Post-antifungal effect of amphotericin B and voriconazole against germinated *Aspergillus fumigatus* conidia. *J Antimicrob Chemother* 2008;**61**(6):1309-1311.
33. Samaranayake YH, Yau JY, Thein ZM, Jayatilake JA, Yeung KW, Samaranayake LP. The post-antifungal effect (PAFE) of amphotericin B, nystatin, ketoconazole and 5-fluorocytosine and its impact on the colonization traits of *Candida glabrata*. *Med Mycol* 2010;**48**(5):725-734.
34. Calamari SE, Bojanich MA, Barembaum SR, Berdicevski N, Azcurra AI. Antifungal and post-antifungal effects of chlorhexidine, fluconazole, chitosan and its combinations on *Candida albicans*. *Med Oral Patol Oral Cir Bucal* 2011;**16**(1):e23-28.
35. Juliano C, Piu L, Gavini E, Zanetti S, Fadda G. In vitro antibacterial activity of antiseptics against vaginal lactobacilli. *Eur J Clin Microbiol Infect Dis* 1992;**11**(12):1166-1169.
36. regrowth (postantibiotic effect) of five antiseptics on nine bacterial pathogens. *J Antimicrob Chemother* 1997;**40**(2):221-226.
37. Nagl M, Hengster P, Semenitz E, Gottardi W. The postantibiotic effect of N-chlorotaurine on *Staphylococcus aureus*. Application in the mouse peritonitis model. *J Antimicrob Chemother* 1999;**43**(6):805-809.

38. Odashima Y, Nakamura K, Ikai H, Kanno T, Meirelles L, Sasaki K, et al.
Postantibiotic effect of disinfection treatment by photolysis of hydrogen peroxide. *J Chemother* 2014;**26**(2):92-100.
39. Cabiscol E, Tamarit J, Ros J. Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int Microbiol* 2000;**3**(1):3-8.
40. Nakamura K, Yamada Y, Ikai H, Kanno T, Sasaki K, Niwano Y. Bactericidal Action of Photoirradiated Gallic Acid via Reactive Oxygen Species Formation. *J Agric Food Chem* 2012.
41. Hong R, Kang TY, Michels CA, Gadura N. Membrane lipid peroxidation in copper alloy-mediated contact killing of Escherichia coli. *Appl Environ Microbiol* 2012;**78**(6):1776-1784.
42. Lalla RV, Patton LL, Dongari-Bagtzoglou A. Oral candidiasis: pathogenesis, clinical presentation, diagnosis and treatment strategies. *J Calif Dent Assoc* 2013;**41**(4):263-268.
43. Tanaka M, Nishida K, Akiyama S. Basic Research on Strong Acid Electrolytic Water. Microbiological Examination. *Hosp Dent Oral —Maxillofac Surg* 1999;**11**(2):119-122.
44. Shiba A, Tsukasaki A, Kanaishi A, Shiba K, Chida T, Okamura N. Electrolyzed Strong Acidic Water as a New Harmless Disinfectant Agent and its Application to Dentistry. *J Showa Univ Dent Soc* 2000;**20**(2):173-179.
45. Qing Y, Akita Y, Kawano S, Kawazu S, Yoshida T, Sekine I. Cleaning efficacy and dentin micro-hardness after root canal irrigation with a strong acid electrolytic water. *J Endod* 2006;**32**(11):1102-1106.

46. Oomori T, Oka T, Inuta T, Arata Y. The efficiency of disinfection of acidic electrolyzed water in the presence of organic materials. *Anal. Sci* 2000;**16**(1), 365-369.
47. Jiang ZY, Woollard AC, Wolff SP. Hydrogen peroxide production during experimental protein glycation. *FEBS Lett* 1990;**268**, 69-71.
48. Panganamala RV, Sharma HM, Heikkila RE, Geer JC, Cornwell DG. Role of hydroxyl radical scavengers dimethyl sulfoxide, alcohols and methional in the inhibition of prostaglandin biosynthesis. *Prostaglandins* 1976;**11**(4):599-607.
49. Suthanthiran M, Solomon SD, Williams PS, Rubin AL, Novogrodsky A, Stenzel KH. Hydroxyl radical scavengers inhibit human natural killer cell activity. *Nature* 1984;**307**(5948):276-278.
50. Winterbourn CC. The ability of scavengers to distinguish OH. production in the iron-catalyzed Haber-Weiss reaction: comparison of four assays for OH. *Free Radic Biol Med* 1987;**3**(1):33-39.
51. Herskind C, Westergaard O. Variable protection by OH scavengers against radiation-induced inactivation of isolated transcriptionally active chromatin: the influence of secondary radicals. *Radiat Res* 1988;**114**(1):28-41.
52. Taguchi H, Tanaka R, Miyahi M, Nishimura K, Kanno H, Ishiyama H. Studies on the effect of combination of amphotericin B and flucytosine on *Candida albicans* by flow cytometry. *Kansensho Zasshi* 1992;**66**(4):516-521.
53. Fukuzaki S. Mechanisms of actions of hypochlorous acid as cleaning and disinfecting agents in relation to injury to bacteria. *Jpn J Food Microbiol*

- 2009;**26**(2):76-80.
54. Dukan S, Touati D. Hypochlorous acid stress in Escherichia coli: resistance, DNA damage, and comparison with hydrogen peroxide stress. *J Bacteriol* 1996;**178**(21):6145-6150.
 55. Dukan S, Belkin S, Touati D. Reactive oxygen species are partially involved in the bacteriocidal action of hypochlorous acid. *Arch Biochem Biophys* 1999;**367**(2):311-316.
 56. Meyer RA, Brunsting A. Light scattering from nucleated biological cells. *Biophys J* 1975;**15**(3):191-203.
 57. Visser JW, van den Engh GJ, van Bekkum DW. Light scattering properties of murine hemopoietic cells. *Blood Cells* 1980;**6**(3):391-407.
 58. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 2007;**130**(5):797-810.
 59. Dwyer DJ, Kohanski MA, Collins JJ. Role of reactive oxygen species in antibiotic action and resistance. *Curr Opin Microbiol* 2009;**12**(5):482-489.
 60. Ramos CL, Pou S, Britigan BE, Cohen MS, Rosen GM. Spin trapping evidence for myeloperoxidase-dependent hydroxyl radical formation by human neutrophils and monocytes. *J Biol Chem* 1992;**267**(12):8307-8312.
 61. Fujimoto S, Kawakami N, Ohara A. Formation of a hydroxyl radical by the myeloperoxidase-NADH-oxygen system. *Biol Pharm Bull* 1993;**16**(6):525-528.
 62. Winterbourn CC, Hampton MB, Livesey JH, Kettle AJ. Modeling the reactions of superoxide and myeloperoxidase in the neutrophil phagosome: implications for microbial killing. *J Biol Chem* 2006;**281**(52):39860-39869.

63. Yaekashiwa N, Sato E, Nakamura K, Iwasawa A, Kudo A, Kanno T, et al.
Determination of reactive oxygen species generated by phorbol 12-myristate 13-acetate-stimulated oral polymorphonuclear cells from healthy human volunteers without any dental problems. *Arch Oral Biol* 2012;**57**(6):636-641.
64. Candeias LP, Stratford MR, Wardman P. Formation of hydroxyl radicals on reaction of hypochlorous acid with ferrocyanide, a model iron(II) complex. *Free Radic Res* 1994;**20**(4):241-249.
65. Folkes LK, Candeias LP, Wardman P. Kinetics and mechanisms of hypochlorous acid reactions. *Arch Biochem Biophys* 1995;**323**(1):120-126.
66. Setsukinai K, Urano Y, Kakinuma K, Majima HJ, Nagano T. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. *J Biol Chem* 2003;**278**(5):3170-3175.
67. Dikalov SI, Harrison DG. Methods for detection of mitochondrial and cellular reactive oxygen species. *Antioxid Redox Signal* 2014;**20**(2):281-294.

Figure legends

Figure 1

Growth curve of *Candida albicans* in RPMI 1640 medium buffered with 0.165 M MOPS. *C. albicans* was pretreated with two-fold serial dilutions of acid-electrolyzed water (AEW) for 10 s. Each value represents the mean of three independent assays.

Figure 2

Time course of changes in the colony growth of *C. albicans* on Sabouraud dextrose agar. *C. albicans* was pretreated with AEW diluted 16 times for 10 s. Each value represents the mean of three independent assays with standard deviation. A significant difference between the two groups is shown as $P < 0.01$ (**).

Figure 3

Representative cytograms of *C. albicans* treated with designated dilutions of AEW (a), and the percentage of the cells within the gated area in which more than 90% of control cells was involved (b). Each value in (b) represents the mean of three independent assays with standard deviation. Significant differences from the control group are shown as $P < 0.01$ (**).

Figure 4

Representative histograms of hydroxyphenyl fluorescein (HPF) fluorescence immediately and 24 h after treatment with designated dilutions of AEW (a), and the calculated shift in fluorescence as the difference between the average fluorescence of the population in the AEW treatment groups and the average in the untreated group without HPF load (b). Each value in (b) represents the mean of three independent assays with standard deviation. Significant differences from the corresponding control groups are shown as $P < 0.01$ (**).

Figure 5

Growth curve of *C. albicans* in RPMI 1640 medium buffered with 0.165 M MOPS in the presence of DMSO. *C. albicans* was pretreated with AEW diluted 16 times for 10 s.

Each value represents the mean of three independent assays.

Figure 6

Representative histograms of HPF fluorescence in the presence or absence of DMSO 24 h after treatment with AEW diluted 16 times (a), and the calculated shift in fluorescence as the difference between the average fluorescence of the population in the treatment groups and the average in the untreated group without HPF load (b). Each value in (b) represents the mean of three independent assays with standard deviation. Significant differences ($P < 0.01$) between two groups are indicated by the same letter.

Fig. 1

Fig. 2

a

Control (pure water)

b

Fig. 3

Fig. 4

Fig. 5

a

Relative fluorescence intensity (RFI)

b

Fig. 6