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A comparison of the bactericidal effects and cytotoxic activity of three types of oxidizing water, prepared by electrolysis, as chemical dental plaque control agents

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Abstract

Acid oxidizing water (AOW), neutral oxidizing water (NtOW) and acid oxidizing water with a low available chlorine concentration (AOW-LC) may be obtained by electrolyzing a solution of tap water containing various quantities of NaCl and HCl. This study compared the bactericidal effects of these waters on cariogenic and periodontopathogenic bacteria and their cytotoxicities against epithelial cells. AOW, NtOW and AOW-LC showed considerable bactericidal effects. The cytotoxicity of AOW-LC was significantly lower than the other solutions tested ($P < 0.0001$). The results indicated that the three types of oxidizing water had similar activity in inhibiting bacterial plaque formation as conventional chemical plaque-control agents.
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Keywords: Oxidizing water; Bactericidal effect; Cytotoxicity; Chemical plaque control; Dental caries; Periodontal disease

1. Introduction

Bacterial dental plaque is involved in the initiation of gingivitis, and the progression of periodontitis and plaque control is of great importance in the prevention of these diseases [1–3]. Adequate plaque control using mechanical methods is difficult to achieve for most individuals. Therefore, a number of chemical dental plaque-control agents have been used to supplement routine oral procedures such as mouth washing or oral irrigation [4]. Antimicrobial agents such as antibiotics, enzymes, chlorhexidine and phenol have shown antiplaque effects both in vitro and in vivo. However, these agents have side effects such as unpleasant tastes, tooth discolouration, irritation and induction of painful lesions of the oral mucosa. Bacteria can also develop resistance to such agents [5–7].

Recently, it has been reported that three types of oxidizing water produced by electrolysis apparatus, have bactericidal effects [8]. These are: acid oxidizing water (AOW), with a pH of 2.8, an oxidation–reduction potential (ORP) of more than 1100 mV and an available chlorine concentration (ACC) of approximately 35 ppm; neutral oxidizing water (NtOW), with a pH of 5.0–5.5, an ORP of approximately 830 mV and an ACC of approximately 80 ppm; and acid oxidizing water with a low ACC (AOW-LC), with a pH of 2.8, an ORP of more than 1100 mV and an ACC of approximately 10 ppm. In the electrolysis apparatus for producing AOW, a solution with a small quantity of NaCl added to tap water is electrolysed. AOW is obtained from the anode and is separated via a diaphragm in the electrolysis apparatus. NtOW is obtained by electrolyzing a solution of tap water containing small quantities of NaCl and HCl using an electrolysis apparatus without a diaphragm. AOW-LC is obtained from the anode, separated via a diaphragm, of an electrolysis apparatus that electrolyses only tap water. However, advantages and disadvantages of oxi-

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dizing water compared with conventional chemical disinfectants and the differences between the bactericidal effects and cytotoxicities of these three types of oxidizing water have not been reported.

The objectives of this study, therefore, were to compare their bactericidal effects on cariogenic and periodontopathogenic bacteria, and to compare their cytotoxicities against epithelial cells with those of conventional chemical disinfectants.

2. Materials and methods

2.1. Test solutions

AOW was prepared using an Aquachid NDX-60KMW electrolysis apparatus, (Omco O.M.C. Co., Saitama, Japan), NtOW using an Aquachid NDX-60KH (Omco O.M.C. Co.), and AOW-LC using a Minestar 201 (Minestar Co., Tokyo, Japan). Povidone-iodine (PI) (0.35%; Meiji Seika Co., Tokyo, Japan), 0.2% chlorhexidine (CHX) (Sigma Chemical Co., St. Louis, MO, USA), Listerine (LST) (Warner Lambert Co., Selangor, Malaysia) and 70% ethyl alcohol (Et) (Wako Pure Chemical Industries, Tokyo, Japan) were used as controls. Phosphate-buffered saline (PBS(−)) (Nissui Pharmaceutical Co., Tokyo, Japan) served as a negative control.

2.2. Bacteria and culture

The test bacteria, *Streptococcus mutans* PS 14, *Streptococcus sobrinus* NIDR 6715, *Streptococcus mitis* ATCC 9811, *Streptococcus salivarius* ATCC 7073, *Streptococcus sanguis* ATCC 10556, *Actinomyces viscosus* B 236, *Actinomyces naeslundii* H 272, *Fusobacterium nucleatum* ATCC 28726, *Porphyromonas gingivalis* 381, *Prevotella nigrescens* ATCC 25261, *Prevotella loeschii* ATCC 15930, *Prevotella melaninogenica* GAI 5596 and *Actinobacillus actinomycetemcomitans* ATCC 33384, were a gift from the Department of Bacteriology, Nihon University School of Dentistry.

Brain heart infusion broth (Difco Laboratories, Detroit, MI, USA) was the culture media used for streptococci and actinomyces, while GAM broth (Nissui Pharmaceutical Co.) was used for the other bacteria. Incubation was conducted at 37°C aerobically for streptococci and actinomyces, and anaerobically (N_2, 80%; H_2, 10%; CO_2, 10%) for the other bacteria. All bacteria were cultured to the logarithmic phase before being used in the tests.

2.3. Cells and cell cultures

The epithelial cells, Ca 9-22 JCRB 0625-derived human gingiva and HO-1-u-1 JCRB 0828-derived human mouth floor mucosa, were purchased from Health Science Research Resources Bank (Osaka, Japan). Ca 9-22 cells were grown on Eagle's minimum essential medium (Iwaki Glass Co., Chiba, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Cancera, Ont., Canada) and 1% antibiotics (Sigma Co.) in 25 cm^2 tissue culture flasks (Becton Dickinson & Co., Lincoln Park, NJ, USA). HO-1-u-1 cells were grown on Dulbecco's MEM-F12 medium (Iwaki Glass Co.) under the same conditions. The culture flasks were incubated at 37°C in an atmosphere of 5% CO_2–95% air. When cultures reached confluency, the cells were detached by trypsin-ethylenediamine tetraacetic acid (EDTA) (Life Technologies, Grand Island, NY, USA) treatment and transferred to 75 cm^2 flasks (Becton Dickinson & Co.). The 75 cm^2 flasks were incubated until confluent growth was obtained.

2.4. Bactericidal effects

The logarithmic phase test organisms were centrifuged at $3000 \times g$ for 15 min and the supernatant removed. The residue was washed two or three times with sterilized sodium phosphate buffer (pH 7.0) and the cells harvested by centrifugation. Fresh sterilized buffer was added, and cell suspensions (approximately 10^8 cells/ml) prepared. The samples were recentrifuged and the supernatant removed. After culturing streptococci and actinomyces with 1.0 ml of a test solution for 30 s, one platinum loop of sample was spread onto a BHI agar plate and the bacteria incubated aerobically for 48 h at 37°C. The other types of bacteria were cultured anaerobically on GAM agar plates for 96 h at 37°C. The bactericidal effects were evaluated by counting the colonies formed. Tests were done in triplicate.

2.5. Cytotoxicity

The confluent cells in 75 cm^2 flasks were detached by trypsin-EDTA, centrifuged at $800 \times g$ for 5 min and then the supernatant removed. Fresh medium was added to the cells to prepare a cell suspension of approximately 2.0×10^5 cells/ml. One hundred microlitres of the cell suspension were inoculated into 96-well plates (Becton Dickinson & Co.), and incubated at 37°C for 24 h in an atmosphere of 5% CO_2–95% air. The medium was removed and the cells washed twice with PBS(−). The cells were incubated with 100 μl test solutions for 30 s, the solution removed, and the cells washed twice with PBS(−). One hundred microlitres of medium and 10 μl colouring reagent from a cell counting kit (Dojindo Laboratories, Kumamoto, Japan) [9,10] were pipetted into the wells and the plates incubated at 37°C for 2 h in an atmosphere of 5% CO_2–95% air. The absorbance of each well at 450 nm was measured with a reference wavelength at 620 nm in a

spectrometer (Multiskan MS, Labsystems, Finland). The absorbance was adjusted according to the number of cells. All tests were repeated five times.

2.6. Statistical analysis

Differences between the mean cell numbers were analyzed statistically using one-way factorial analysis of variance and Scheffe's method with STATVIEW software (Abacus Concepts, Berkeley, CA, USA). Statistical significance was accepted at $P \leq 0.05$.

3. Results

3.1. Bactericidal effects

Bacteria incubated with NtOW, AOW, AOW-LC, LST, PI or Et did not form any colonies. *S. salivarius*, *A. viscosus* and *F. nucleatum* incubated with CHX did not form any colonies. The other bacteria incubated with CHX did form some colonies (+). Many colonies (+ +) for each strain of bacteria grew on the control plates.

3.2. Cytotoxicity

The cytotoxicities of the test solutions are presented in Fig. 1. The cell counts for Ca 9-22 incubated with AOW-LC and controls were $(3.2 \pm 0.3) \times 10^4$ and $(5.4 \pm 0.1) \times 10^4$, respectively. The counts for the other solutions were less than 0.2×10^4. The cell counts for HO-1-u-1 incubated with AOW-LC and the controls were $(1.4 \pm 0.5) \times 10^4$ and $(3.6 \pm 0.5) \times 10^4$, respectively. The counts for the other solutions were all less than 0.1×10^4. Statistically significant differences were observed between the control and test solutions, and between AOW-LC and the other test solutions ($P < 0.0001$)

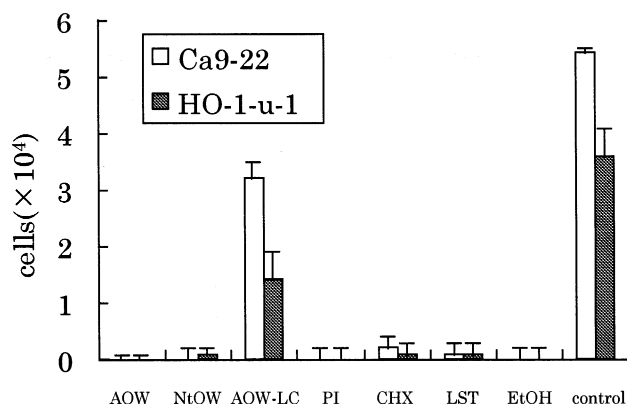


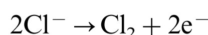
Fig. 1. Cytotoxic activity of three types of oxidizing water and chemical disinfectants. Statistically significant differences between the control and test solutions, and between AOW-LC and the other test solutions ($P < 0.0001$) were observed.

4. Discussion

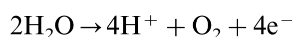
The bactericidal effects of AOW, NtOW and AOW-LC were stronger than CHX but similar to the other disinfectants tested. The cytotoxicities of AOW and NtOW were similar to the conventional disinfectants tested. The cytotoxicity of AOW-LC was lower than that of all solutions tested.

A difference between ACC of AOW and AOW-LC was observed, due to the differences in the Cl^- concentrations of the solutions before electrolysis. Chlorine is added to tap water in Japan as a disinfectant and Cl^- as NaCl was added to the electrolysis solution when producing AOW. The same chemical reactions occur in the AOW and AOW-LC electrolysis apparatus. The following chemical reactions are believed to take place.

At the anode:



$$2Cl^- \rightarrow Cl_2 + 2e^-$$



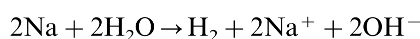
$$2H_2O \rightarrow 4H^+ + O_2 + 4e^-$$

$$Cl_2 + H_2O \rightarrow HClO + H^+ + Cl^-$$

$$HClO \rightarrow H^+ + ClO^-$$

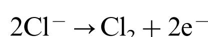
At the cathode:

$$Na^+ + e^- \rightarrow Na$$

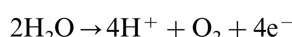


$$2Na + 2H_2O \rightarrow H_2 + 2Na^+ + 2OH^-$$

On the other hand, the following chemical reactions are thought to occur in the NtOW electrolysis apparatus, which does not have a diaphragm.

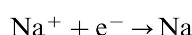


$$2Cl^- \rightarrow Cl_2 + 2e^-$$

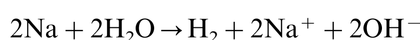


$$2H_2O \rightarrow 4H^+ + O_2 + 4e^-$$

$$Cl_2 + H_2O \rightarrow HClO + H^+ + Cl^-$$

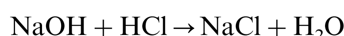


$$Na^+ + e^- \rightarrow Na$$



$$2Na + 2H_2O \rightarrow H_2 + 2Na^+ + 2OH^-$$

$$Na^+ + OH^- \rightarrow NaOH$$



$$NaOH + HCl \rightarrow NaCl + H_2O$$

HCl added before electrolysis neutralizes the NaOH produced at the cathode so the pH of NtOW becomes 5.0–5.5 [11,12]. The chlorine is converted to HClO from Cl_2 in the NtOW due to the pH value of 5.0–5.5. HClO does not readily vaporize and has a stronger bactericidal effect than Cl_2 [13].

The bactericidal effects of oxidizing waters are thought to be caused by their high chlorine concentrations and not the low pH or high ORP. We previously reported [8] that the bactericidal effect of NtOW (high pH and low ORP) was stronger than that of AOW (low pH and high ORP). However, the results of the present study indicate that the bactericidal effects of AOW, NtOW and AOW-LC are not different, and are similar

to those of LST, PI and Et but stronger than CHX. It has been reported that CHX is active against Gram-positive and Gram-negative bacteria and yeast cells [14], and that the effects of CHX linger in the mouth because it attaches to the teeth or oral mucosa [15,16]. We believe the weak CHX effect observed was due to the short period of time the bacteria were exposed to CHX in the method used in this study.

Ito et al. [17] reported on the inhibitory effects of AOW on early dental plaque formation in vitro. NtOW and AOW-LC were thought to possess similar bactericidal effects, and AOW also had an inhibitory effect on early dental plaque formation. Thus, the bactericidal effects of oxidizing waters on cariogenic and periodontopathogenic bacteria are similar to those of chemical dental plaque control agents and are sufficient to justify their use in clinical therapy. However, the bactericidal effects of oxidizing waters are known to decrease when they come into contact with proteins because chlorine reacts with protein [18]. There are many types of proteins in saliva, dental plaque and gingival crevicular fluid. From the point of view of killing bacteria using a mouthwash or oral irrigation, the ACC of oxidizing waters must be high to be effective.

Oxidizing waters used as a mouthwash come into direct contact with the oral mucosa and gingiva. Therefore, it is necessary to determine their cytotoxicities against epithelial cells derived from the oral mucosa or gingiva. The results showed that the counts of both cell types incubated with test solutions other than AOW-LC were less than one-tenth that of control, while the counts of cells incubated with AOW-LC were 40–60% of the control. It was shown that the test solutions other than AOW-LC had strong cytotoxic activities. Katsuragi et al. [19] reported that AOW is cytotoxic to fibroblasts, but no-one has investigated the cytotoxicity of NtOW and AOW-LC

It has been reported [5–7] that conventional chemical dental plaque control agents are cytotoxic in vitro. The results are similar to those of this study but even though they are cytotoxic, they can still be used in clinical therapy. It is thought that the cytotoxic effects are due to the presence of chlorine in the oxidizing waters. Cytotoxic activity of the oxidizing waters are thought to decrease when binding occurs with the many proteins in the mouth, such as in the saliva, dental plaque and gingival crevicular fluid, which decreases the ACC. Thus, it has been suggested that NtOW and AOW, whose in vitro cytotoxic actions are similar to those of conventional chemical agents, may control plaque formation without producing any side effects. AOW-LC is thought to be useful and without side effects because its in vitro cytotoxicity is weaker than that of conventional chemical agents.

In conclusion, the results of the present study indicate that the three types of oxidizing water examined are approximately as potent at inhibiting bacterial plaque formation as conventional chemical plaque control agents, with the added benefit of no side effects. Therefore, oxidizing waters may be applicable as antiplaque agents However, future clinical research should focus on evaluating the efficacy and safety of mouthwashes containing oxidizing waters and their effects on the oral mucosa and gingiva.

Acknowledgements

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