Abstract: Sodium hypochlorite (NaOCl) is widely used as an antimicrobial agent; however, it has cytotoxic and neurotoxic effects. For these reasons, development of new, safe irrigants other than NaOCl is long overdue. In the present study, the antimicrobial and noxious effects of NaOCl were evaluated and compared with those of NaOCl. Enterococcus faecalis, Streptococcus mutans, Porphyromonas gingivalis, or Candida albicans were mixed with each tested solution for 30 s. The mixtures were then plated on brain-heart infusion agar plates, after which colony numbers were counted. Serially diluted solutions of NaOCl were used to determine the actual chloride concentration (ACC) required for a bactericidal effect. Noxious effects were evaluated by measuring lactate dehydrogenase released from HeLa cells. Acid FW and NaOCl had similar bactericidal effects against all bacterial species but not against C. albicans. An ACC of at least 10 ppm was required in order to ensure effective bacteriocidal activity and induce significant lactate dehydrogenase release. Acid FW-treated HeLa cells exhibited healthy growth, with slight retardation as compared with non-treated cells. Because of its efficient bactericidal, and less noxious, effects on human cells, acid FW may be a useful irrigant for effective root canal treatment.

Keywords: sodium hypochlorite, Enterococcus faecalis, apical periodontitis, lactate dehydrogenase

Introduction

Apical periodontitis is an inflammatory disease with multiple causes [1]. Most lesions heal after appropriate root canal treatment; however, treatment failure may lead to chronic apical periodontitis [2]. Apical periodontitis is usually asymptomatic and discovered by chance during routine dental examination. Radiographically it presents as a demarcated radiolucent lesion in contact with the tooth root. Surgical intervention is required for removal of persistent apical periodontitis. Although complete elimination of intraradicular infectious agents is essential, these agents may remain in the region because of anatomical complexities such as accessory canals, ramifications, and anastomoses [3]. All root canal surfaces, especially oval roots, are inaccessible to instruments [3]. In such cases, irrigation is essential. Sodium hypochlorite (NaOCl) is widely used as an antimicrobial irrigant. Although it has proteolytic properties and is effective in reducing bacterial numbers [4], NaOCl has cytotoxic and neurotoxic effects when extruded into periapical tissues [5,6]. Therefore, development of alternative irrigants is long overdue. Acidic and alkaline electrolyzed functional waters (FWs) are produced by electrolyzing low concentrations of saline waters (FWs) are produced by electrolyzing low concentrations of saline (prevalence, 22%–77% [14-20]) and is associated with persistent endodontic infections [21]. Elimination of E. faecalis is therefore of utmost importance for successful prevention of chronic apical periodontitis.

This study compared the bactericidal and noxious effects of NaOCl with those of acid and alkaline FWs. The bactericidal effect likely depends on the actual chloride concentration (ACC). Therefore, to determine the ACC required for an effective bactericidal effect, FWs were diluted with distilled water before the experiments. To assess noxious effects, the human cervical cancer-derived fibroblastic cell line HeLa was directly treated with FWs. The present findings may lead to clinical use of FWs.

Materials and Methods

Reagents

Acid (ACC 30 ppm; pH 2.7; oxidation-reduction potential [ORP] >1,100 mV) and alkaline (ACC 0 ppm; pH 11.5; ORP ≈800 mV) FW were kindly provided by Miura Denshi (Nikaho, Japan). The NaOCl solution (6% solution, pH 11.7) was purchased from Yoshida Pharmaceutical (Tokyo, Japan) and diluted with distilled water to 1%, (final ACC, ≈2,000 ppm). ACC was measured with an ACC measuring kit (Sibata, Soka, Japan). ORP and pH were measured with a handy pH/ORP measuring device (DKK-TOA, Tokyo, Japan). The Handy Sonic UR-20P (Tomy Seiko, Tokyo, Japan) with an active ultrasonic tip (diameter, 2.5 mm) was used as the ultrasonic unit and was operated at a fixed driving frequency of 28 kHz with an output power of 10 or 20 W.

Cell culture

The HeLa cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 50 mg/mL streptomycin, and 50 U/mL penicillin (10% FCS-MEM).

Microorganism culture and treatment

Four strains of oral microorganisms—S. mutans ATCC25175, P. gingivalis FDC381, E. faecalis JCM5803, and C. albicans ATCC18804—were used in this study. S. mutans, E. faecalis, and C. albicans were maintained on brain-heart infusion (BHI; BD Biosciences, Rockville, MD, USA) agar. Gifu anaerobic medium (Nissui, Tokyo, Japan) supplemented with hemin and menadione (5 and 0.5 ppm, respectively) broth was used for P. gingivalis. Subcultures were freshly prepared before use. Each strain was cultured for 24 to 48 h in nutrient and inoculated in the same broth at 37°C under aerobic or anaerobic conditions. Bacterial and fungal cells were har-
vested in the late logarithmic phase by centrifugation at 5,000 g at 4°C for 10 min and washed twice in phosphate-buffered saline (pH 7.2).

Bactericidal effect
Ten microliters of a bacterial strain or C. albicans (1 × 10^8 CFU/mL) was mixed with 1 mL of acid FW, alkaline FW, or NaOCl solution for 30 s. After treatment, the mixture was diluted and plated on a BHI agar plate. The plates were inverted and cultured for 48 to 72 h in a 37°C incubator, after which bacterial colony numbers were counted.

Cell stimulation and LDH measurement
The cells were plated in 24-well plates at a density of 1 × 10^5/well on the day before the experiment and treated with NaOCl solution (5%) or acid or alkaline FW for 30 s. After stimulation, the mixture was diluted and plated on a BHI agar plate. The plates were inverted and cultured for 48 to 72 h in a 37°C incubator, after which bacterial colony numbers were counted.

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Statistical analysis
Statistically significant differences were determined with one-way analysis of variance and the Tukey test (Figs. 1a, c, 3a, 5b) or with the Mann-Whitney U test with Bonferroni correction (Figs. 2a, 5a, 6). Statistical significance was defined as P < 0.05. All data are expressed as means ± standard deviation (SD).

Results
Comparison of bactericidal effects
The NaOCl solution had obvious effects on all three of the examined bacterial species (S. mutans (a), P. gingivalis (b) and E. faecalis (c)): almost no bacteria were observed after treatment with this agent (Fig. 1). The effects of acid FW on S. mutans, P. gingivalis, and E. faecalis were equivalent to those of the NaOCl solution: colony numbers were significantly reduced (20%, 0%, and 3.8% respectively) (Fig. 1). Alkaline FW showed strong bactericidal effects only for P. gingivalis, but the effects were nevertheless substantial, as compared with control, for S. mutans (72.7%) and E. faecalis (84.6%) (Fig. 1).

Effects against C. albicans
Because of the prominent bactericidal effects of NaOCl and acid FW, their effect on C. albicans was examined. The C. albicans colony number was reduced to 66.3% of the control after treatment with acid FW for 30 s (Fig. 2a). Further treatment for longer periods yielded a time-dependent decrease in viability; no colony was present after 20 min of treatment (Fig. 2a). Similar titration experiments with NaOCl showed that a treatment
duration of only 30 s was sufficient to kill all microorganisms (Fig. 2b).

Effect of diluted acid FW

Subsequently, the ACC required for a bactericidal effect was determined by diluting acid FW and NaOCl with distilled water and subjecting them to experiments. *E. faecalis* was mixed with the diluted solutions, and viability was measured as shown in Fig. 1. Bactericidal effects were significant at acid FW concentrations of 30% and higher, i.e., viability was less than 5% (Fig. 3a). In contrast, 51.5% of *E. faecalis* remained unaffected when 10% acid FW was used (Fig. 3a). The pH values of acid FW dilutions were measured, and 30% and 10% dilutions had pH values of 3.0 and 3.4, respectively (Fig. 3a, indicated by the line). Similar experiments with NaOCl solution showed significant bactericidal effects, and the effects of a 10% diluted solution were equivalent to those of a 100% solution (Fig. 3b). The line in Fig. 3b shows the pH of the diluted solutions.

ACC

Next, acid FWs with various ACCs were generated. Each acid FW was diluted, as shown in Fig. 3, and the bactericidal effect was examined. Marked changes were observed for 30% acid FW solutions with 100, 80, 60, and 40 ppm of acid FW. For the 20-ppm solution, however, the change was obvious at a dilution of 50% (Fig. 4).

Noxious effects

HeLa cells were treated with each solution to clarify noxious effects and released LDH in each solution. The cells immediately peeled off the culture dish after NaOCl treatment, and LDH could therefore not be measured (data not shown). HeLa cells release LDH even in a resting state (49 m units/mL). In the acid FW-treated sample, the LDH value was 580 m units/mL (Fig. 5a). However, alkaline FW treatment did not affect LDH release (48.5 m units/mL). The LDH-releasing effect paralleled the bactericidal effect of each solution; thus, HeLa cells were treated with serially diluted NaOCl and acid FW. NaOCl treatment resulted in cells rapidly peeling off the plate, even after treatment with the 10% diluted solution (data not shown). Acid FW treatment resulted in concentration-dependent LDH release (Fig. 5b). At 30%, 50%, and 70% dilutions, LDH levels reached 157.9, 266.2, and 375.2 m units/mL, respectively (Fig. 5b).

The effect of acid FW on cell growth was further examined. Cells were seeded at 1 × 10^5/10-cm dish. After 30 s of treatment with acid FW, cells were cultured for 4 days and cell numbers were counted. Non-treated HeLa cells exhibited culture period-dependent growth, reaching 1 × 10^6 cells at day 4. Although the growth rate was lower in the acid FW-treated cells, it was nevertheless healthy, and no morphological changes were observed at day 4 (Fig. 6).
Discussion

The present study found that the bactericidal effects of acid FW were equivalent to those of NaOCl—all bacterial species examined were dead within 30 s of treatment. However, the effects of these treatments on C. albicans significantly differed. Treatment with acid FW resulted in only a 30% reduction in viability. Although 20 min of treatment completely killed C. albicans, the effect was much lower than that for NaOCl. A previous study reported that acid FW was effective in killing C. albicans [22]. Differences in experimental setting might explain this discrepancy between past and present findings.

Alkaline FW had very weak bactericidal effects, which accords with the findings of a previous study that reported relatively weak bactericidal and biofilm-removing effects with alkaline FW [23]. In the present experiments, the three different bacterial species were treated for only 30 s. Although longer treatment might have had a greater effect, prolonged treatment is not practical during root treatment in clinical settings. In addition, brief contamination by organic agents remaining in the infected root canal drastically reduces the bactericidal effects of FWs [24]. To maintain acid FW activity during root canal treatment, solutions should be frequently replaced with fresh solutions, for effective irrigation in clinical applications.

Chlorine-related substances such as chlorine (Cl2), hypochlorous acid (HOCl), and hypochlorous acidic ion (ClO−) are thought to be responsible for the bactericidal effect of FWs. To determine the ACC required for these effects, acid FW was serially diluted. The effect was preserved up to a dilution of 30% (chloride concentration 9 ppm), when the original chloride concentration was 30 ppm. Although pH affects the bactericidal effects of acid FW, the pH of the 30% diluted solution (3.0) was similar to that of the original acid FW solution (2.7). To confirm these results, acid FW (pH 2.7) with various ACCs was produced. For a 100-ppm acid FW, a 10% dilution (ACC 10 ppm) resulted in 30% recovery of viability. In contrast, 20-ppm acid FW (50% dilution, ACC 10 ppm) showed only 5% bacterial viability. Taken together, these findings indicate that the ACC must be greater than about 10 ppm to be effective.

The degree of cell damage was compared by measuring the amount of LDH released by HeLa cells. NaOCl resulted in immediate peeling off of cells from the culture dishes, indicating enormous damage. LDH release was observed with acid FW, the cells did not peel off the dish. The treated cells did not die; instead, they grew at a reduced proliferation rate, as compared with non-treated cells. Because 10 ppm was the lowest ACC for effective bactericidal activity, serially diluted acid FW solutions were examined for LDH release, which increased in a concentration-dependent manner. However, LDH release in the 10% diluted solution (ACC 10 ppm) was not higher than that of the control solution.

In summary, the present results suggest that acid FW is safe and has a bactericidal effect equivalent to that of NaOCl. For continuous, effective bactericidal effects, acid FW should be changed frequently, although the best method of supplying fresh acid FW during root canal treatment remains to be determined. These issues must be addressed to ensure effective clinical use of acid FW and ultrasonic irrigation.

Acknowledgments

This study was supported in part by research grants from the Sato Fund of the Nihon University School of Dentistry, a grant from the Dental Research Center of Nihon University School of Dentistry for 2018, a Nihon University multidisciplinary research grant and Individual Research Grant, and by the MEXT-Supported Program for the Strategic Research Foundation at Private Universities 2013–2017 (Grant-in-Aid for Scientific Research (C), MEXT KAKENHI, 15K11086).

Conflict of interest

The authors declare no conflict of interest regarding the authorship or publication of this article.

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Fig. 6 Effect of acid FW on cell growth. HeLa cells were treated with or without acid FW for 30 s. The cells were washed and further cultured with 10% FCS-DMEM. The cell numbers were counted on days 1, 2, 3, and 4 after acid FW treatment. Data are expressed as mean ± SD. Three independent experiments were performed.