



Bactericidal Effect of a Novel Alkaline EDTA Root Canal Cleaning Solution

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Abstract

Objectives In this study, we aimed to evaluate the bactericidal effect and cytotoxicity of an ethylenediaminetetra-acetic acid (EDTA)-based root canal irrigant solution capable of efficiently removing both the organic matter and the smear layer. We prepared a strong alkaline EDTA (AE) solution with an acid buffer capacity similar to that of sodium hypochlorite.

Materials and Methods AE was used at concentrations of 1%, 2%, and 3%. The bactericidal effect of AE on *Enterococcus faecalis* was evaluated by determining the colony number and biofilm removal rate. Biofilms were visualized using a Live/Dead BacLight bacterial viability kit. Viability of AE-treated cells were determined using a CCK-8 cell counting assay.

Statistical Analysis One-way analysis of variance followed by a Dunnett's multiple comparison test were used for comparisons among groups.

Results Significant reduction in cell viability and biofilm formation were observed in case of 3% and 2% AE. AE exerted bactericidal effects in a concentration-dependent manner. Damage of normal human fibroblasts was not observed at any of the AE concentrations.

Conclusions Our results suggest that the AE solution could be used as an effective canal irrigant for the removal of bacterial biofilm.

Keywords

- ▶ ethylenediaminetetraacetic acid
- ▶ sodium hypochlorite
- ▶ biofilm
- ▶ root canal irrigant

Introduction

Endodontic treatment involves the removal of pulp infected with bacteria in case of dental caries and trauma to prevent apical periodontal disease. Additionally, it prevents reinfection of the root canal to avoid redundancy. The root canal is anatomically curved and narrow, exhibiting a complex

structure with intricate branches at the apex, making it difficult for X-rays and imaging tools to diagnose root canal infections.¹ The complexity of the root canal system makes it difficult to eliminate the source of infection. Recently, the development of cone beam computed tomography (CBCT) and optical coherence tomography (OCT) techniques have

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made it possible to image the complex morphology of the root canal. Moreover, progress in the development of innovative therapeutic devices for endodontic treatment, such as rotary nickel titanium files, contributed to an effective and time-efficient means of shaping the canal.² However, mechanical instrumentation alone does not prepare the entire root canal surface and is insufficient to remove bacteria, owing to the complexity of the root canal system.^{1,3-5} Therefore, in addition to the mechanical cleaning, supplemental chemical cleaning with root canal irrigants is essential to achieve the desired prognosis in case of endodontics.⁶

Sodium hypochlorite (NaOCl) is the most commonly used root canal irrigant, owing to its disinfecting capacity and its ability to disrupt biofilms and dissolve organic tissues. The lysing action of hypochlorous acid dissolves the pulp tissue and eradicates the residual bacteria in the root canal. It is necessary to effectively remove smear layers, smear plugs, and cutting debris attached to the root canal wall, which cannot be performed by mechanical cleaning alone. Therefore, ethylenediaminetetra-acetic acid (EDTA), which is capable of dissolving inorganic substances, is used in combination with another reagent in endodontic procedures. Therefore, the combination of chemical cleaning and mechanical cleaning leads to a successful root canal treatment.⁷

NaOCl exhibits an excellent organic-dissolving effect in root canal treatment; however, it could cause severe damage to the soft tissue if the solution scatters or leaks out of the apex.⁸ Cleaning agents are delivered to the apex of the root canal through the extra-articular area. This causes severe pain and acute inflammation, and in worse cases, it requires hospitalization owing to the high toxicity of NaOCl to vital tissues.⁹ Moreover, long-term exposure to EDTA could lead to root fractures or overdemeralization. Therefore, it is necessary to develop a root canal cleaning agent that is safe, easy to handle, and capable of exerting potent bactericidal effects.

In this study, we aimed to develop a method for safe and effective removal of both the organic matter and the smear layer by using a strong alkaline EDTA (AE) solution adjusted to the same acid buffer capacity as that of NaOCl. The aim of this study was to evaluate the bactericidal effect and cytotoxicity of the root canal irrigation solution using EDTA solutions with different pH values to establish a next-generation root canal irrigation method.

Materials and Methods

Bactericidal Effect

Enterococcus faecalis (ATCC 19433) was cultured in brain heart infusion (BHI; Becton Dickinson and Company, Sparks, MD, USA) broth, supplemented with yeast extract (5 mg/mL) for 18 hours under anaerobic conditions (CO₂: 10%, H₂: 10%, N₂: 80%) at 37°C. A total volume of 10 µl (4.1 × 10⁸ colony-forming unit [CFU]/mL) of bacterial suspension was treated with 1 mL of endodontic irrigants to determine their bactericidal effect. The effects of the following solutions of endodontic irrigants were investigated: Alkaline EDTA solution (AE: 0.09 mol/liter EDTA) was adjusted to 1%

(1% AE, pH 11.9), 2% (2% AE, pH 12.1) and 3% (3% AE, pH 12.3), respectively; 3% Smear clean (SC: pH 9.5, EDTA-2Na solution; Nippon Shika Yakuhin Co., Ltd, Yamaguchi, Japan), and 3% Antiformin (AF: pH 12.3, NaOCl, Nippon Shika Yakuhin Co., Ltd). Phosphate-buffered saline (PBS: pH 7.5) was used for the control treatment. Following the treatment of bacterial cells with endodontic irrigants, 10-fold serial dilution was performed using fresh BHI broth, and 100 µl was then spread onto a BHI agar plate. Bactericidal effect was determined by evaluation of the number of CFU after 24 hours of incubation in anaerobic conditions. The experiment was performed in triplicate.

Biofilm Removal Assay

Bacterial biofilms were grown on sterilized coverslips for 18 hours. Following incubation, nonadherent cells were removed by washing with PBS, and *E. faecalis* biofilm was treated with various types of endodontic irrigants for 0 to 5 minutes to evaluate their biofilm removal effect. Biofilms were stained using the Live/Dead BacLight bacterial viability kit (Life Technologies; Eugene, OR, USA) and observed under a fluorescence microscope (BZ-X700; Keyence Corp., Osaka, Japan). The coverslips were stained with 1% crystal violet (CV). After CV staining, each coverslip was distilled with 1 mL of 95% ethanol. Further, the optical density (OD) of CV-stained solution containing the biofilm was measured at 550 nm using a spectrophotometer (DU800; Beckman coulter, Brea, CA, USA). The experiment was performed in triplicate.

Cell Survival Assay

Human gingival fibroblasts (HGFs) were isolated from healthy gingival tissues of patients. The experimental procedures were approved by the Ethical Committee of Kanagawa Dental University (approval no. 455). HGFs were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co.; LTD, Tokyo, Japan) and supplemented with 0.35% glucose (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 0.28 mM ascorbic acid (FUJIFILM Wako Pure Chemical Corporation), 4 mM L-glutamine (Sigma-Aldrich; St. Louis, MO, USA), 0.18% NaHCO₃ (FUJIFILM Wako Pure Chemical Corporation), 50 U/mL penicillin (Sigma-Aldrich), 50 µg/mL streptomycin (Sigma-Aldrich), and 10% fetal bovine serum (FBS; S1820, Biowest, France), at 37°C under 5% CO₂. The medium was replaced every 2 days, and cells from passages 3 to 7 were cultured in 100-mm diameter cell culture dishes (Corning, NY, USA). Further, 1 × 10⁴ cells were seeded onto a 96-well plate. Cell survival assay was measured using Cell Counting kit-8 (CCK-8; CK04-11, Dojindo, Kumamoto, Japan) at 30 second, 1 minute, 4 minutes, 5 minutes, and 10 minutes following the manufacturer's instruction. Cells were stained with 10 µl of CCK-8 solution in each well and incubated at 5% CO₂ and 37°C for 4 hours. The OD values of each samples were then measured at 450 nm using a microplate reader (iMark microplate reader; Bio-Rad Laboratories, Inc., Hercules, CA, USA). This assay was performed in triplicate.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test were performed to analyze the results. Test results were considered significant when probability (p) values were < 0.05 .

Results

The bacterial cells were treated with various endodontic irrigants. We observed that SC and 1% AE did not exhibit any bactericidal effect, similar to PBS. However, hypochlorous acid exerted a significant bactericidal effect and reduced the number of viable *E. faecalis* cells after 1 minute of exposure. Additionally, 3% AE reduced the viable *E. faecalis* count after 5 minutes of treatment, while 2% AE significantly reduced the count of viable bacteria by half (►Fig. 1). Our results demonstrated that AE inhibited the growth of floating bacteria in a concentration-dependent manner.

Further, we examined the bactericidal effect of various solutions on biofilm formation. We observed that 73.2% of the biofilm was removed following treatment with 3%

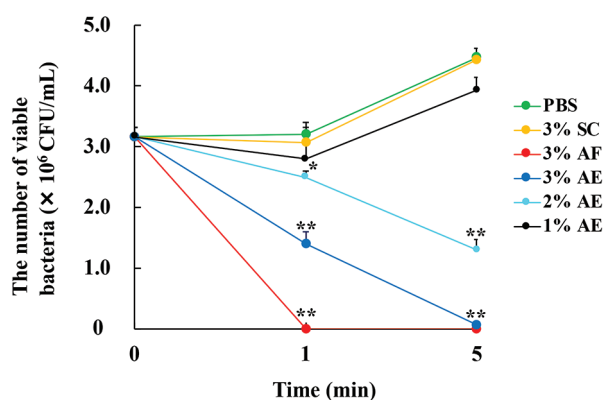


Fig. 1 Bactericidal effect of alkaline ethylenediaminetetra-acetic acid (EDTA) (AE). The number of viable bacteria after treatment with different concentrations of AE. *: $p < 0.05$, **: $p < 0.01$.

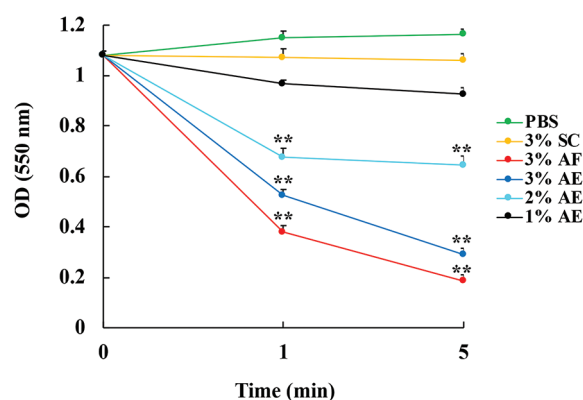


Fig. 2 Removal of *E. faecalis* biofilm. The percentage of biofilm removal after treatment with different concentrations of alkaline ethylenediaminetetra-acetic acid (EDTA) (AE). ** $p < 0.01$

AE for 5 minutes (►Fig. 2). We also observed reduction in the number of bacterial cells after 1 minute of treatment (►Fig. 3C), and lysis of bacteria was observed after 5 minutes (►Fig. 3I). On the contrary, SC and PBS only removed 1.9% of the biofilm after 5 minutes of treatment (►Fig. 2). Besides, we did not observe any bactericidal effect in case of SC or PBS even after 5 minutes (►Fig. 3G, 3H). On the other

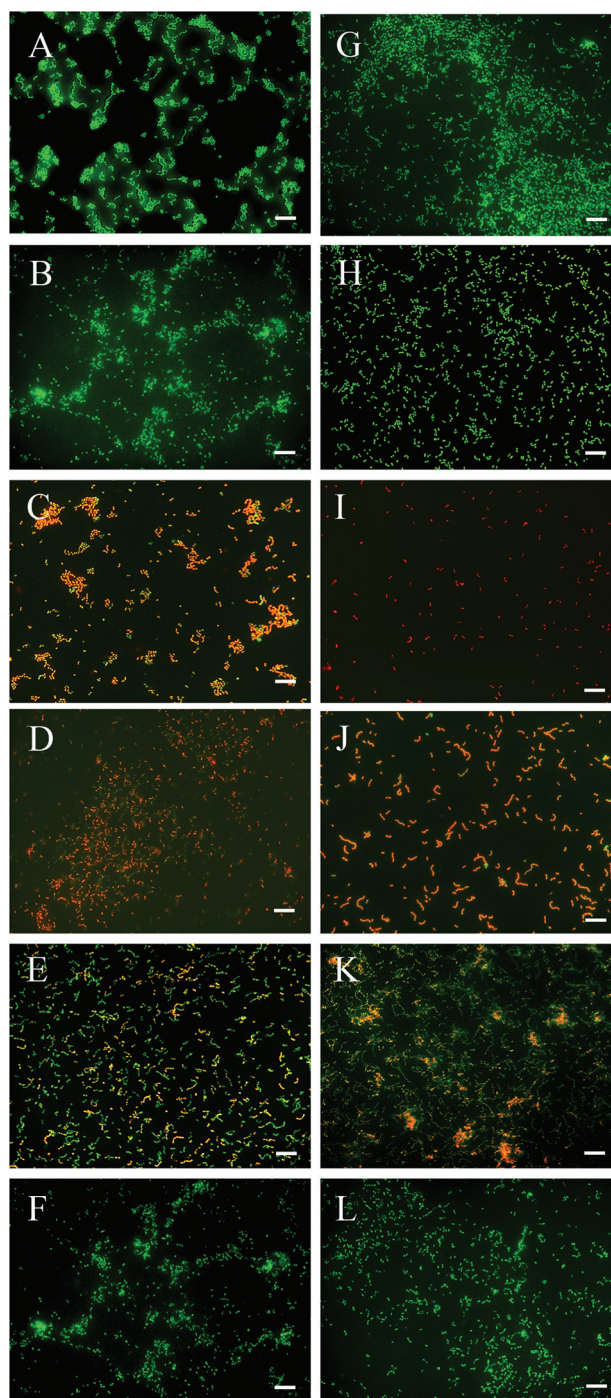


Fig. 3 Fluorescence microscopic images of *E. faecalis* biofilm stained using crystal violet (CV). The viable (green) and dead (red) bacteria are clearly visible. A,G; phosphate-buffered saline (PBS), B,H; 3% Smear clean (SC), C,I; 3% Antiformin (AF), D,J; 3% alkaline ethylenediaminetetra-acetic acid (EDTA) (AE), E,K; 2% AE, F,L; 1% AE. Bars: 10 μ m. (bar; x100).

hand, reduction in bacterial counts was observed in AE in a concentration-dependent manner (►Fig. 2), and most bacteria were killed upon treatment with 3% AE for 5 minutes (►Fig. 3 J). Therefore, AE is expected to be as effective as AF in the removal of biofilm.

Accidental escape of the cleaning fluid outside the apical foramen can occur during root canal cleaning. Therefore, it is necessary to consider the effect of the washing solution on the periodontal tissue of the root apex. Therefore, in this study, we aimed to investigate the effect of effective cleaning agents on human fibroblasts. We observed that most fibroblastic cells treated with AF were affected 4 minutes after the addition of NaOCl (►Fig. 4). However, we did not observe any cell damage in case of AE, similar to that in the control.

Discussion

The success of endodontic treatment depends on the eradication of microbes from the root-canal system and prevention of the infection. The complex root canal morphology makes it difficult to eliminate *E. faecalis*, which penetrates dentinal tubules and forms biofilms.^{10,11} Surface adhesins and gelatinases of *E. faecalis* are involved in bacterial attachment, colonization, and biofilm formation.^{12,13} Therefore, it is essential to prevent secondary endodontic infections by eliminating this species from the root canal system. Refractory periapical periodontitis is complicated due to the presence of a biofilm formed by the bacterial flora near the apex. *E. faecalis* lipoteichoic acid (LTA) detected in refractory periapical periodontitis is thought to be one of the causes of the irreversible suppression of osteoblast proliferation and apoptosis via JAK2-STAT3 signaling.^{14,15} *E. faecalis* has been reported to be detected commonly by persistent endodontic infections.¹⁶ Therefore, we designed the bactericidal effects of alkalized EDTA on biofilms using *E. faecalis*. Our results suggest that alkalized EDTA could be used as an effective cleaning agent for the treatment of intractable root canals, as it suppresses the growth of *E. faecalis*.

Root canal lavage is one of the important requirements for determining the success or failure of the treatment that involves various cleaning agents. An ideal root canal cleaner

should efficiently remove debris, act as a lubricant to reduce friction due to the instrument during endodontic procedures and facilitate dentin removal, dissolve inorganic tissue and organic matter, kill bacteria and yeasts, inhibit biofilm production, avoid damage to vital periapical tissue, and prevent caustic or cytotoxic effects.⁶ There are no root canal cleaning agents that meet all of these requirements. EDTA, which is a cleaning solution, is mixed with hypochlorous acid or chlorhexidine, resulting in its precipitation and rendering it ineffective.⁶ On the other hand, EDTA Na₄/NaOCl solution supplemented with the quaternary ammonium compound cetrimide does not interfere with the antibacterial activity of NaOCl.¹⁷ Several studies have reported the development of hypochlorite solution that lacks chlorinated odor.¹⁸ Therefore, in this study, we aimed to develop an effective cleaning agent that reduced the unique scent and taste by using AE.^{19,20} Irrigation activation techniques have been proposed to improve their distribution through the canal system and enhance the antibacterial and antibiofilm capacity of root canal irrigants.^{21,22} However, since NaOCl has a high surface tension, the effect of penetrating dentinal tubules cannot be expected.²³ To exert an effective cleaning effect, the concentration and amount of agent used, and the effects of temperature on the interactions between agents, must be considered in combination to develop effective cleaning of the root canal. These findings suggest that further research is required to develop an appropriate cleaning method.

Root canal cleaning is effectively performed by appropriately supplying and stirring the cleaning agent into the root canal.²⁴ Sonics ultrasonic, sonics photoactivation systems, and laser are widely used methods for irrigation activation that applies external mechanical force to the irrigants.²⁴⁻²⁶ Passive ultrasonic irrigation utilizes small noncutting files oscillating freely in shaped canals at ultrasonic frequencies, activating irrigates by acoustic streaming, whereas sonic irrigation produces a hydrodynamic phenomenon through oscillating movement by frequencies of 1 to 10 kHz. Laser-activated irrigation using Er:YAG laser has been introduced as an alternative method to activate root canal irrigation unique ability, in order to utilize micro-explosion to produce cavitation in the root canal agent by strong absorption of the laser energy. The combination of these cleaning methods could enhance their efficiency. Further studies should be performed using animal models of infected root canal to study the effect of the irrigant.

Conclusions

The AE solution promotes the inhibition of *E. faecalis* and effectively removes bacterial biofilms. These results indicate that the AE solution might have the potential to be applied as an endodontic irrigant in root canal treatment.

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Conflict of Interest

None declared.

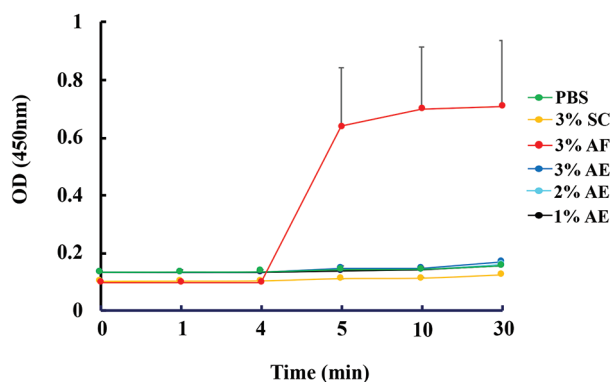


Fig. 4 Proliferation of cells treated with alkaline ethylenediaminetetraacetic acid (EDTA) (AE). The survival rate of human gingival fibroblast cells treated with AE.

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