Original Article

A Final Flush with EDTA Effectively Enhances the Disinfection of NaOCl in Non-Instrumented Root Canals

Sumana Chetthavatcharapan[1], Ruchanee Salingcarnboriboon Ampornaramveth[2], Pavena Chivatxaranukul[3]

[1]Department of Operative Dentistry, Chulalongkorn University, Bangkok, Thailand

[2]Department of Microbiology Research Unit on Oral Microbiology and Immunology, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand

[3]Department of Operative Dentistry, Chulalongkorn University, Bangkok, Thailand

Abstract

The objective of this study was to compare the effectiveness of regenerative endodontic irrigation procedures using ethylenediaminetetraacetic acid (EDTA) to those using normal saline as final flush agents for disinfecting non-instrumented large root canals. Sterilized root samples (0.8-mm-wide apical foramen) (n=53) were prepared from extracted human mandibular premolars and 2 samples were used as sterile controls. Fifty-one samples were infected with *Enterococcus faecalis* for 21 days and randomly assigned into 3 groups (n=17 per group) with the following irrigation procedures, respectively: no irrigation (initial), irrigation with 1.5% NaOCl and a final flush with normal saline (N-NS), and irrigation with 1.5% NaOCl and a final flush with 17% EDTA (N-EDTA). Subsequently, the root canal walls were shaved and processed for microbial analysis, while 2 samples from each group were split and processed for observation using scanning electron microscopy. The number of remaining bacteria (CFUs) were determined and analyzed using one-way ANOVA. All irrigation procedures significantly reduced bacterial numbers when compared with the initial group ($P<0.001$). The N-EDTA group (5.43 CFUs/mL) had significantly fewer bacteria than the other groups ($P<0.001$), with approximately 373-fold lower than the N-NS (2.02×10^3 CFUs/mL) group and 1.3×10^5-fold lower than the initial group (7.13×10^5 CFUs/mL). Using a non-instrumented large root canal model, a final flush using EDTA after 1.5% NaOCl irrigation was more effective for root canal disinfection than using normal saline. Therefore, to improve the effectiveness of root canal disinfection in regenerative endodontic procedures, EDTA should be chosen for a final flush agent.

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Correspondence to:

Pavena Chivatxaranukul, Department of Operative Dentistry, Faculty of Dentistry, Chulalongkorn University, Henri-Dunant Road, Bangkok 10330, Thailand. Tel: 081-819-2321 E-mail: pavena.c@chula.ac.th; pavenachi@gmail.com

Introduction

Regenerative endodontic procedures (REPs) are a biologically based approach for immature permanent teeth with pulpal necrosis. REPs aim to resolve clinical signs and symptoms, regenerate pulp-like tissue in the root canal, and allow further root development.[1,2] Successful treatment requires effective bacterial reduction to provide a suitable environment for revascularization and periapical healing.

Root canal infections are typically characterized by bacterial biofilms that attach to the root canal surface.[3] Biofilms are communities of bacteria embedded in an extracellular polysaccharide matrix, providing a physical barrier against disinfecting agents.[4] Other properties of biofilm bacteria, e.g., higher resistance to antimicrobial agents, also make root canal disinfection challenging.[5] Generally, biofilm elimination is achieved by chemo-mechanical root canal preparation or exposure to high concentration of NaOCl.[6,7] However, the thin root canal walls and wide apical foramen of immature teeth pose limitations to the routine debridement protocol.[1] Mechanical preparation could weaken the root and render the root more prone to fracture. Therefore, root canal disinfection during REPs primarily depends on root canal irrigation and root canal medication, with no or minimal root canal preparation.[1,8]

Although exposure to a higher concentration of NaOCl is more effective in biofilm eradication[6], it should be avoided to preserve the stem cells that are involved in the regeneration process.[9] In the first visit, the REP irrigation procedure uses 1.5% NaOCl for 5 min to disinfect the root canal, followed by a final flush with either normal saline or ethylenediaminetetraacetic acid (EDTA) to dilute the residual NaOCl or to reverse the deleterious effect of NaOCl on stem cells.[8,9]

Normal saline is an isotonic agent without antibacterial properties; however, EDTA is a chelating agent with some antibacterial/antibiofilm effect against some Gram-negative bacteria, including Haemophilus influenzae and Pseudomonas aeruginosa.[10,11] Although there is a discrepancy among studies on its antibacterial and antibiofilm effect [12,13], a final flush using EDTA may have adjunctive roles in bacterial reduction.[10,14] The objective of this study was to compare the effectiveness of irrigation protocols using EDTA to those using normal saline as a final flush agent to reduce bacteria from non-instrumented large root canals.

Materials and Methods

Sample Preparation and Specimen Inoculation

This study was approved by the Human Research Ethics Committee (#073/2018). Fifty-three intact mandibular premolars with single straight root canals, extracted for orthodontic reasons, were collected from patients <25 years old. The sample size was determined by power analysis.[7] The root samples, demonstrating wide root canal and unprepared root canal surface of immature teeth, were prepared according to the study by Sasanakul *et al,* with modifications.[7] The apical and coronal tooth portions were removed using a precision saw (ISOMET 1000; Buehler, Lake Bluff, IL) to generate 10-mm-long samples with a 0.8-mm apical foramen diameter. The pulp tissue was removed with a barbed broach and H-type file (Dentsply Maillefer, Ballaigues, Switzerland) without removing the root canal dentin. The root samples were apically sealed with composite resin (Filtek Z350; 3M EPSE, St Paul, MN) and externally coated with nail polish (OPI Products, Calabasas, CA). The root canals were irrigated with irrigants in the following order: 5 mL 2.5% NaOCl (Chulalongkorn University, Bangkok, Thailand), 5 mL 17% EDTA (Endo Clean; Mahidol university, Bangkok, Thailand), 5 mL 2.5% NaOCl, 5 mL 10% sodium thiosulfate (Emsure, Darmstadt, Germany) (to inactivate the bactericidal effect of NaOCl), and 5 mL sterile distilled water. Custom silicone blocks were constructed to secure the roots in an upright position. The samples and silicone blocks were sterilized with ethylene oxide gas.

Root canal infection with *E. Faecalis*

An isolated *Enterococcus faecalis* (ATCC 29212) colony grown on BHI agar was inoculated in BHI broth and incubated overnight at 37°C with 5% CO_2. The root samples (n=51) were individually immersed in 5 mL *E. faecalis* culture (optical density=0.5, ~1.5 x 10^8 colony-forming units (CFUs)/mL) in brain-heart infusion (BHI) broth

(Himedia, Mumbai, India), and 2 samples were immersed in sterile BHI media as sterile controls. The samples were incubated at 37°C in a 5% CO_2 atmosphere. During the 21-day incubation period, the bacterial suspension was 90% replaced with sterile BHI every other day. The purity of *E. faecalis* culture was periodically checked using Gram staining and observing the colony-forming morphology on BHI agar plates.

Experimental Procedures

After 21 days, the infected samples (n=51) were rinsed with 15 mL of 1% phosphate-buffered saline (PBS) and fixed in their custom silicone blocks. The samples were randomly assigned into 3 groups (n=17) as follows:

Group 1: No irrigation (Initial). This group represented the initial number of bacteria in the root canal. In groups 2 and 3, the experimental protocols were assigned following the AAE clinical considerations for regenerative procedures.[8]

Group 2: Final flush with normal saline (N-NS). Root canals were irrigated with 20 mL 1.5% NaOCl for 5 min, followed by a final flush with 20 mL normal saline for 5 min.

Group 3: Final flush with EDTA (N-EDTA). Root canals were irrigated with 20 mL 1.5% NaOCl for 5 min, followed by a final flush with 20 mL 17% EDTA for 5 min.

The irrigants were delivered into each root canal using a 25-gauge side-vented needle (ProRinse; Dentsply Tulsa Dental Specialties, Tulsa, OK) at 4 mL/min. The needle tip was placed 1 mm from the samples' apical end. After the assigned irrigation procedures, the root canals in the experimental groups and the sterile controls were gently flushed with 10 mL PBS.

Microbiological Evaluation

The remaining bacteria in the root canal and inner root dentin were collected from 2 sterile and 45 experimental samples (n=15 per group). The root canal wall was shaved to depth of 250 µm using a #4 Peeso-reamer (Dentsply Maillefer, Switzerland), and 3 sterile paper points were sequentially placed into the root canal to absorb the remaining fluid. The dentine shavings and the paper points were transferred into an Eppendorf tube (Eppendorf North America, Hauppauge, NY) containing 1 mL PBS. The contents were sonicated for 30 sec, serially diluted, plated on BHI agar, and incubated for 24 hours as described above. The CFUs/mL counts representing the number of remaining bacteria were recorded. All procedures were performed by one operator.

SEM observation of the remaining bacteria

Two root canal samples from each experimental group were split longitudinally into two halves and processed for scanning electron microscopy (Quanta 250 FEG; FEI, Hillsboro, OR) examination. Images of the residual bacteria on the root canal wall at the upper part of the apical third level were randomly captured at 10000x magnification.

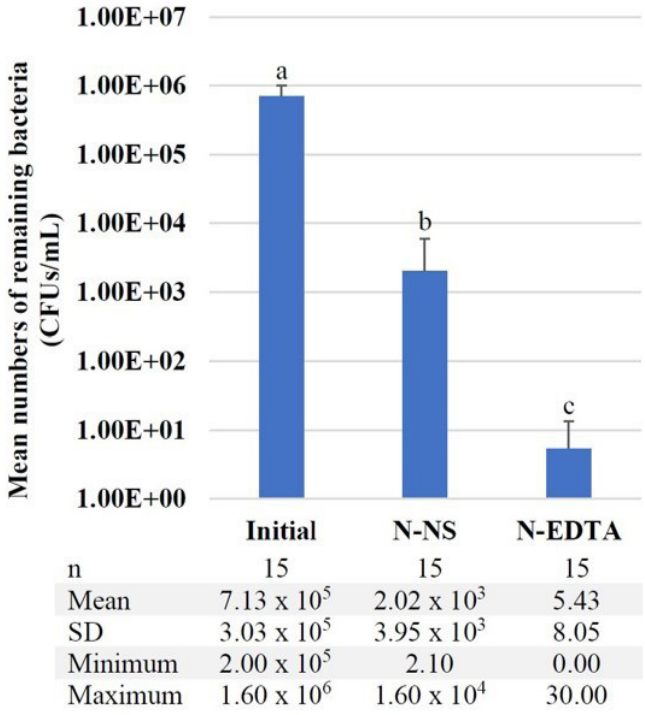
Statistical analysis

The data was analyzed using IBM SPSS Statistics for Windows, Version 22.0 (IBM, Armonk, NY). $P<.05$ was considered statistically significant. The numbers of remaining bacteria were $\log_{10}$ transformed for statistical analysis. The homogeneity of variances was verified using Levene's test. The differences in the remaining bacteria among the 3 groups were analyzed using one-way ANOVA. The Games-Howell post-hoc analysis was performed to identify significant differences between groups. The magnitude of bacterial reduction was calculated from the ratios of the average numbers of remaining bacteria of the two groups.

Results

Microbiological evaluation

The flushing procedure, recommended by the AAE clinical considerations for regenerative procedures, was confirmed to be effective in reducing bacteria from non-instrumented infected teeth with large root canal. All irrigation procedures demonstrated significantly > 99% lower numbers of remaining bacteria than the initial group ($P < 0.001$). The mean numbers of remaining bacteria of each group (Fig. 1) and the mean differences in numbers of remaining bacteria between groups (Table 1) were determined. Furthermore, the magnitude of bacterial reduction was different between groups.



	Initial	N-NS	N-EDTA
n	15	15	15
Mean	7.13×10^5	2.02×10^3	5.43
SD	3.03×10^5	3.95×10^3	8.05
Minimum	2.00×10^5	2.10	0.00
Maximum	1.60×10^6	1.60×10^4	30.00

Figure 1 *Mean numbers of remaining bacteria (CFUs/mL) in each group, shown in log scale. Different lowercase letters indicate significant differences between the groups (P < .05). The error bars represent the standard deviation (SD) of the mean numbers of remaining bacteria from 15 samples. All irrigation procedures significantly reduced bacteria from the initial group. The N-EDTA group had the lowest mean number of remaining bacteria*

Table 1 *Mean Differences in the Number of Remaining Bacteria (Log_{10} Values) between Each Group, P-Value, and 95% Confidence Interval (Games-Howell Post-hoc Analysis)*

Group (A)	Group (B)	Mean difference (A-B)	*P* value	95% Confidence interval	
				Lower bound	Upper bound
Initial	N-NS	3.22*	< .001	2.41	4.03
	N-EDTA	5.33*	< .001	4.98	5.7
N-NS	N-EDTA	2.11*	< .001	1.26	2.96

**, significant difference; Initial, no irrigation; N-NS, NaOCl & normal saline; N-EDTA, NaOCl & EDTA*

The N-EDTA group demonstrated the lowest mean number of remaining bacteria (5.43 CFUs/mL), which was significantly lower than other groups (P<0.001). In the N-EDTA group, 6 from 15 samples were bacteria-free. The mean number of remaining bacteria in the N-NS group was 352-fold less than the initial group, while the mean number of remaining bacteria in the N-EDTA group was 1.3×10^5-fold lower than the initial group and 373-fold lower than the N-NS group. When bacterial growth was present, Gram staining and colony-forming morphology indicated a pure *E. faecalis* culture. No bacteria were detected in the sterile samples.

Biofilm Verification and Evaluation

The SEM images of the initial group illustrated clusters and chains of bacterial biofilm on the infected root canal walls (Fig. 2a). Few bacterial cells were observed on the root canal walls in the N-NS group (Fig. 2b), whereas the N-EDTA group had root canal walls with hardly any bacteria (Fig. 2c).

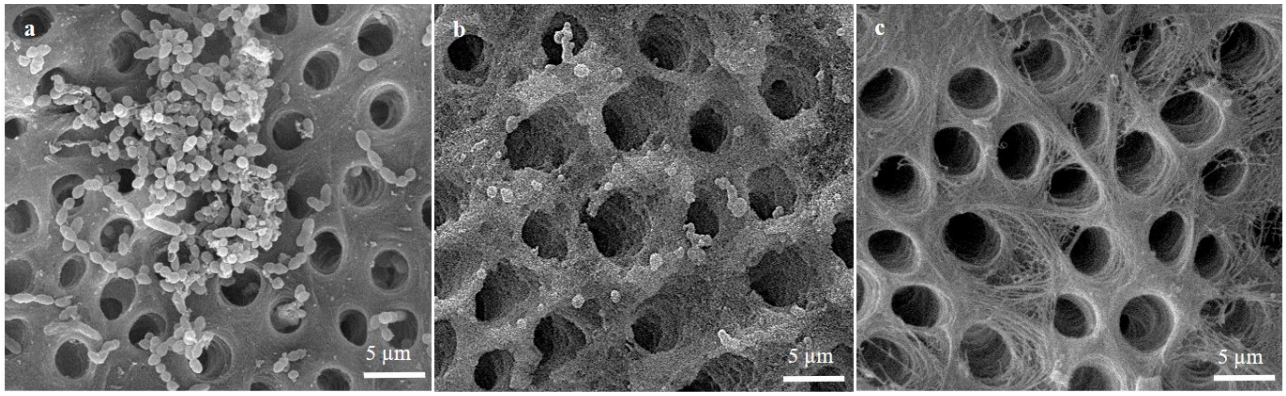


Figure 2 *Representations of SEM images at 10000x magnification. (a) Chains and bacterial biofilm clusters on the infected root canal walls of the initial group. (b) Scattered bacterial cells remained on the root canal wall of the N-NS group, after the root canal irrigation procedures. (c) Bacterial cells were scarcely present in the N-EDTA group, after the root canal irrigation procedures*

Discussion

Our study compared the effectiveness of different regenerative endodontic irrigation procedures to reduce bacteria in the large, non-instrumented root canals.[8] The results revealed the superiority of a final flush using 17% EDTA for bacterial reduction compared to normal saline.

NaOCl is an effective broad-spectrum antibacterial agent. However, its activities depend on usage parameters such as concentration and exposure time.[15] Our findings were in agreement with the previous study showing the limited antibacterial effect of 1.5% NaOCl irrigation in non-instrumented root canals.[7] Although high concentrations of NaOCl effectively removed the biofilm[6], exposure to these concentrations should be avoided during REPs because of its deleterious effect on stem cells.[9]

Our results revealed that an EDTA final flush significantly reduced root canal bacteria. The lowest numbers of remaining bacteria and scarce bacteria found in SEM images suggest the potential efficacy of the N-EDTA irrigation procedure to reduce bacteria in large, non-instrumented root canals. EDTA is a chelating agent[16] that is widely used in endodontic treatments for smear layer removal.[17,18] Although bacteria embedded in the smear layer could be eradicated concurrently with the smear layer removal[19], it is not the case for bacterial reduction in REPs, especially where mechanical instrumentation is not performed, and smear layer is not generated. It was proposed that EDTA exerted its antibacterial activity by attacking the Mg^{2+} on bacterial cell walls and disrupting Gram-negative bacterial cell integrity.[16] Although some studies differed[12,13], EDTA's effect on bacterial cell integrity and viability was reported for both Gram-negative and positive bacteria.[16,20]

Bacteria in biofilms are shielded in the extracellular matrix and harder to kill than their planktonic counterparts.[4] Therefore, the ideal properties of effective endodontic irrigants should not only include antibacterial effects, but also anti-biofilm effects. The ability of EDTA to reduce biofilm biomass was illustrated in several studies.[10,14] EDTA could loosen or release calcium or metal ions that are essential for cell adhesion and stabilization of biofilm extracellular polysaccharide matrix, leading to biofilm breakdown and biofilm detachment.[16,21] However, the susceptibility of biofilm to the irrigating agents was affected by the biofilm structure, agent type, concentration[14,20], and exposure time.[22]

The discrepancies in the biofilm response to EDTA among studies were also due to bacterial species[23], binding substratum, and biofilm growth condition.[10] Despite the disagreement over the anti-biofilm effect of EDTA, it is interesting that biofilm dispersal was typically found when root canals or hydroxyapatite were used as a substratum.[14,24] These results may be because a) *E. faecalis* adheres to collagen and hydroxyapatite[25], b) *E. faecalis* forms a distinct calcified biofilm in a calcium

carbonate and calcium phosphate-rich microenvironment[26], and c) the strong EDTA chelating leads to the loss of mineralization content and can cause dentine erosion.[27] Therefore, EDTA's strong chelating effect may alter the binding substratum or biofilm structure, resulting in biofilm dislodgement.

In this study, the root samples were prepared to simulate the large root canals of immature teeth. The length of root samples was standardized to 10-mm representing 2/3 of mature premolar's root length.[28] Although the infection of necrotic immature permanent teeth is polymicrobial[29], the single-species biofilm model was applied to minimize variations possibly caused by bacterial interaction. *E. faecalis* was selected as a test organism because of its ability to form a biofilm on root canal walls, invade dentinal tubules, and tolerate calcium hydroxide's antimicrobial effects.[30] *E. faecalis* was also detected both in primary root canal infection as well as in failed endodontic cases.[31,32] Therefore, *E. faecalis* was chosen as a representative for bacteria that may escape from the endodontic procedure and survive in the root canal system.

Although irrigating solutions could penetrate and exert an antibacterial effect in the dentinal tubules[33], some bacteria are still able to survive and further migrate to re-infect the root canal space.[34] To evaluate the extent of remaining bacteria that pose the possibility of interfering with the healing process, the number of remaining bacteria was assessed from the shaved dentine to include bacteria residing both at the root canal wall and within the dentinal tubules.

Clinically, residual bacteria can have a critical negative effect on the regenerative endodontic outcomes.[35] Therefore, it is essential to reduce as many bacteria from the root canal as possible. Interestingly, although 0.5-3% NaOCl exposure did not completely render bacteria-free root canals[6], favorable REP outcomes were observed when 1.5-2.5% NaOCl was used for irrigation in non-instrumented root canals.[36] The possible contributing factors are the synergistic effect of flush agents, medicaments, and host defense.[37]

Our study illustrated that using EDTA as a final flush agent effectively reduced the bacterial biofilm in non-instrumented root canals. EDTA also promotes growth factor release[38], which may induce stem cell differentiation.[39] Although the *in vitro* results from single-species biofilm cannot be directly extrapolated to the clinical situation and should be interpreted cautiously, EDTA should be a reasonable choice for a final flush and could be considered as an adjunct for bacterial/biofilm reduction during REPs. However, the long-standing presence of multispecies bacterial biofilm in clinical situation can make the root canal disinfection more challenging.[40,41] Further studies using multispecies biofilm as well as confocal laser scanning microscopy will provide more insight into the effect of clinical procedures for bacterial management in non-instrumented root canals.

Conclusions

The results confirmed that the current REP disinfection procedures significantly reduced root canal bacteria. However, a final flush using 17% EDTA was more effective than NS to reduce bacteria from non-instrumented large root canals.

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