Effectiveness of EDTA and Modified Salt Solution to Detach and Kill Cells from *Enterococcus faecalis* Biofilm

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Abstract

Introduction: Disruption of the matrix of endodontic biofilms will aid in their removal from a root canal. Therefore, the aim of this study was to investigate the efficacy of EDTA and a modified salt solution (MSS) to detach bacteria from biofilms. **Methods:** Forty-eight-hour-old *Enterococcus faecalis* biofilms were grown on glass coverslips and then treated for 1 hour by immersion in 17% EDTA or MSS. Phosphate-buffered saline served as a negative control. Then, residual biofilm cells on the substrate and the detached cells in the supernatant were collected. Viability was verified by the colony-forming unit (CFU) counting method. Propidium monoazide (PMA) treatment in conjunction with quantitative polymerase chain reaction (qPCR) was also performed to detect the presence of *E. faecalis* 16S ribonucleic RNA genes. Data were analyzed using 1-way analysis of variance and Tukey or Kruskal-Wallis and Dunn tests. The Pearson R test evaluated the correlation between results from CFU and PMA ($\alpha = 5\%$). **Results:** qPCR showed that EDTA detached 99% of biofilm cells, and MSS detached 94% of biofilm cells (both $P < .001$). In contrast to EDTA, MSS was highly antimicrobial. The treatment promoted an ample log 7 reduction of the attached cells ($P < .001$), and almost no live cells were detected in the supernatant ($P < .001$). Positive correlations between CFU and qPCR with PMA were observed ($r = 0.959$ and $r = 0.729$). **Conclusions:** EDTA detached cells in biofilms with a minor antimicrobial effect. Besides a great antimicrobial effect, MSS also detached biofilm cells. These dispersals of biofilms give insights into new endodontic biofilm removal strategies. *(J Endod 2016;42:320–323)*

Key Words

Biofilm detachment, biofilm disruption, disinfection, EDTA, *Enterococcus faecalis*, modified salt solution, propidium monoazide, quantitative polymerase chain reaction

Irrigants have been used in combination with endodontic instruments during root canal preparation aiming to resolve apical periodontitis, a biofilm-induced inflammatory response (1). Although during root canal treatment it is possible to reduce the root canal bacterial load, most irrigants fail to completely eradicate microorganisms (2). This is because of the reduced susceptibility of these microorganisms when they are organized as a biofilm (3). Another reason for inadequate disinfection is that irrigants such as sodium hypochlorite and chlorhexidine are excellent surface disinfectants that fail to penetrate fully into biofilm plaques (4, 5).

In general, biofilms consist of communities of bacteria attached to surfaces and encased in an extracellular polymeric substance (EPS). EPS consists essentially of water but also proteins, polysaccharides, extracellular DNA, and other components (6). Polycationic metals, such as calcium (Ca^{2+}), are also present and play an important role in maintaining the stability, architecture, viscosity, and strength of the biofilm (7).

EDTA, a chelator, is already used during root canal therapy to remove the inorganic component of the smear layer produced by instrumentation (8). Its biofilm-dispersing property (9, 10) means that EDTA can also be used to "loosen" or clean endodontic biofilms. Its antimicrobial action seems limited although viability culture assays have not been often performed and data acquired by viability staining may not be accurate (11). To date, in the field of endodontics, little work has been performed to investigate and quantify the biofilm cleaning/killing effect of EDTA.

Hypertonic salt solutions induce cell death (12) and also reduce the cohesion of biofilm matrices (13, 14). The modified salt solution (MSS) is hypertonic, and its great efficacy in inactivating biofilms is caused by the simultaneous application of hyperosmotic, weak acid, and sorbic acid stress (5, 15). Its efficacy has been tested in models in which bacteria formed biofilms attached to the surface of a substrate. In these models, after MSS treatment, the metabolic activity and viability of the residual biofilms on the substrate were assessed (5, 15). Therefore, it remains unclear whether the bacterial reduction was caused by the killing of bacteria, the detachment of biofilm cells from the substrate, or both. After investigation, when MSS appears to be able to disperse biofilms, then it may also aid in cleaning the root canal system.

In brief, EDTA is mainly known for its biofilm-dispersing properties, whereas MSS is an antimicrobial. The aim of this study was to evaluate the effectiveness of EDTA or MSS to detach bacteria from *Enterococcus faecalis* biofilms. Also, the antimicrobial properties of MSS and EDTA were re-evaluated.

Materials and Methods

After the treatments, with the colony-forming unit (CFU) counting method, the viability of the bacterial cells that were still attached to the substrate as well as the cells

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that had detached from biofilm or substrate into the supernatants was evaluated. In addition, in a quantitative polymerase chain reaction (qPCR) assay, the presence of *E. faecalis* 16S ribosomal RNA genes was detected in the remaining biofilms and their supernatants. To distinguish between live and dead cells in the detected 16S ribosomal DNA, propidium monoazide (PMA) was used to block the DNA from the dead cells in the qPCR assay (16).

Bacterial Strain and Growth Conditions

A clinical isolate of *E. faecalis E2* (17) was retrieved from the −80°C stock, and a fresh culture was obtained by incubating 500 μL of this stock in 10 mL semidefined broth (pH = 7.1) containing 0.2% glucose at 37°C in an anaerobic jar with an atmosphere of 80°C N_2, 10% H_2, and 10% CO_2. At OD600 ∼ 0.9, the suspension contained ∼5 × 10^8/mL cells. Biofilms were grown in semidefined broth (pH = 7.1) containing 0.2% sucrose. The composition of semidefined broth has been previously published (18).

Biofilm Growth

For culturing the biofilms, the Amsterdam active attachment model was used (5, 19) following a previously published protocol (5). Twenty-four-well plates (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) were inoculated with 1.5 mL/well growth medium containing approximately 10^8 cells/mL *E. faecalis* and closed with a custom-made stainless steel lid with 24 clamps. The clamps hold glass coverslips (12 mm in diameter; Thermo Scientific, Braunschweig, Germany), which served as substrate for biofilm growth. The model was incubated for 48 hours under anaerobic conditions at 37°C with medium refreshments after 8, 24, 32, and 42 hours.

Treatments and Bacterial Suspensions

To remove nonadherent cells, the lid was transferred to a new 24-well plate with 1.5 mL/well phosphate-buffered saline (PBS). Then, the biofilms were treated for 1 hour by immersion in 1.7 mL/well 17% EDTA (Sigma-Aldrich, St Louis, MO), MSS, or PBS under anaerobic conditions at 37°C. PBS served as a negative control. MSS had been prepared by dissolution of sodium chloride (Sigma-Aldrich) and potassium sorbate (Sigma-Aldrich) in demineralized water. Immediately after the 1-hour period, the treatments were neutralized for 5 minutes in 2% buffered peptone water (Oxoid, Basingstoke, UK) with 1% sodium thiosulfate (Merck, Darmstadt, Germany). Biofilms were subsequently washed 3 times in PBS. Each coverslip was collected in vials containing 2 mL PBS. The biofilms were removed from the substrate by sonication on ice for 45 seconds at an amplitude of 40 W (VibraCell; Sonics and Materials, Newtown, CT).

The detached cells were retrieved by collecting the supernatants of the treatment, buffered peptone water, and the PBS washes and centrifuging those at 4,500 rpm × 3,395*g* for 10 minutes. The formed pellet was then washed in 1.5 mL PBS, and the suspension was again centrifuged for 10 minutes. The pellet was resuspended once more in 2 mL PBS, and then each suspension was sonicated for 30 seconds because *E. faecalis* tends to form short chains of bacteria, which would appear on the culture plate as 1 CFU (unpublished data May 2013, Suzette V. van der Waal, acquired by microscopic examination [Axiolab; Carl Zeiss Microscopy GmbH, Munich, Germany; 1000× magnification with an oil-immersion lens] of a drop of the suspension).

Viability Assay

After vortexing for 5 seconds, both the attached and detached bacterial suspensions were diluted, and 50-μL aliquots were seeded in duplicate onto brain-heart infusion agar plates (Bacto, Le Pont-de-Claix, France). The plates were incubated at 37°C anaerobically for 48 hours, and the number of CFUs was counted and logarithmically transformed.

The experiment was performed 3 times. Each experimental run contained 6 biofilms per group.

PMA Treatment

A total of 1 mg PMA (Biotum Inc, Hayward, CA) was dissolved in 100 μL 20% dimethyl sulfoxide to make a 20-mmol/L stock solution (20). From the remaining bacterial suspensions as mentioned previously, 2 aliquots of 500 μL were dispensed in Eppendorf tubes. Of the PMA stock, 1.3 μL was added to one 500-μL sample to obtain a final concentration of 50 μmol/L PMA. The other sample received no PMA. The Eppendorf tubes were placed horizontally on ice and incubated in the dark for 5 minutes. Subsequently, they were placed at a distance of 25 cm from a 650-W halogen lamp and were then exposed to the light for 2 minutes. Afterward, the Eppendorf tubes with the suspensions were stored at −80°C until further analysis.

DNA Extraction and Real-time Polymerase Chain Reaction

The frozen non-PMA and PMA suspensions were thawed at room temperature. Then, 200 μL were collected for bacterial DNA extraction according to manufacturer's instructions of the AGOWA mag DNA-isolation kit (AGOWA, Berlin, Germany). The presence of the *E. faecalis* genes was determined using the previously described *E. faecalis*–specific 16S ribosomal RNA gene primer pairs (Eurogentec Nederland BV, Maastricht, The Netherlands) (21). The qPCR was run using the LightCycler 480-II Instrument (Roche Diagnostics, Hofmann La Roche Ltd, Basel, Switzerland) at 40 cycles for 10 minutes at 95°C and then per cycle for 20 seconds at 95°C, 15 seconds at 68°C, and 15 seconds at 72°C. For each reaction, 10 mL SYBR Green mix (Roche Diagnostics), 1 mL of each primer (Eurogentec Nederland BV), and 5 mL Mili-Q water (Merck) was used.

For quantification, with 100-μL serial 10-fold dilutions of an *E. faecalis* V583 culture in the midexponential phase, standard curves were generated that were quantified through the CFU method. For quantification of the total bacterial load, a standard curve of *Escherichia coli* was used. PCR water served as a negative control. After each qPCR assay, DNA melting curves for 16S ribonucleic DNA amplification products were assessed for qPCR artifacts or nonspecific PCR products. This experiment was performed twice. Each experimental run contained 6 biofilms per group.

Statistical Analysis

Parametric data obtained from the qPCR without PMA treatment were compared using 1-way analysis of variance and post hoc Tukey tests. When the assumption of normality was violated, which occurred in the viability assay and qPCR with PMA treatment data, Kruskal-Wallis and post hoc Dunn tests were used. The Pearson R correlation test was used to evaluate correlation between results from CFU and PMA treatment. Analyses were performed using SPSS software version 21.0 (SPSS Inc, Chicago, IL), and P values <.05 were considered statistically significant.

Results

Viability Assay (CFU)

Control biofilms attached to the glass surface contained 1.6 × 10^8 ± 1.6 × 10^7 CFUs per biofilm (Fig. 1). Compared with the control biofilms, after EDTA treatment, 1% of cells were still attached (P < .001). In the MSS group, on average 21.7 ± 68.8 CFUs could be recovered from the glass coverslips. This log 6 reduction was significant (P < .001).