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Research Article

Influence of Various Irrigating Regimens on the Force of Adhesion of Enterococcus Faecalis to Root Dentin using Atomic Force Microscopy - An *In Vitro* Study

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Abstract

Introduction: Adherence of bacteria to a substrate is the first stage in biofilm formation. This investigation aimed to study the effects of final Irrigants on the force of adhesion of *E. faecalis* to dentin by using atomic force microscopy. This information could aid in understanding the force on interaction between *E. faecalis* and dentin and subsequently facilitate in designing treatment strategies which would prevent further bacterial recolonization in the root canal.

Methods: The irrigants tested in our study were 1.3% NaOCl, 1.3% NaOCl + 17% EDTA, 1.3% NaOCl + MTAD, 1.3% NaOCl + 18% HEBP and untreated samples (control). The force of adhesion was measured by using atomic force microscopy. One way ANOVA followed by Post Hoc multiple comparision Tukey HSD tests were used to analyze the data.

Results: There was a significant increase in the adhesion force after irrigation of dentin when EDTA and HEBP were used as the final irrigants whereas the NaOCl-treated dentin was similar to the control group.

Conclusion: With the use of MTAD as a final irrigant, a repulsive force was seen between *E.faecalis* and dentin. This study highlighted that chemicals that alter the physicochemical properties of dentin will influence the nature of adhesion force and subsequent biofilm formation of *E. faecalis* to dentin.

Keywords: Adhesive Force; Repulsive Force; Atomic Force Microscopy; MTAD; HEBP; EDTA; NaOCl

Introduction

Successful root canal therapy relies on the combination of proper instrumentation, disinfection and obturation of the root canal [1]. The chemo mechanical preparation concept relates to the use of chemically active agents in combination with mechanical cleansing [2]. Sodium hypochlorite (NaOCl) solutions remain the most widely recommended irrigants in endodontics based on their unique capacity to dissolve necrotic tissue remnants [2] and their excellent antimicrobial property [2] Formation of smear layer on the dentin surface during instrumentation has deleterious effects and its removal becomes mandatory. Hence, a chelating agent that removes the smear layer is essential as a final irrigant [3]. Commonly used final irrigants in endodontics are ethylenediaminetetraacetic acid (EDTA) [4]. Mixture of tetracycline acid and detergent (MTAD) [5] and 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP) [6]. EDTA has been proved to effective in removing the inorganic component of the smear layer [7]. However, the use of EDTA as a final rinse caused a progressive dissolution of dentin at the expense of peritubular and intertubular areas [7-9]. MTAD contains 3% doxycycline, 4.25% citric acid and detergent (Tween 80) and is commercially available as Bio Pure MTAD (Dentsply Tulsa Dental, Tulsa, OK). It is biocompatible [10] less erosive [11] and is also efficient in removing the smear layer [5,12,13]. The manufacturer recommends that MTAD should neither be removed nor inactivated, instead it should be left inside the canal for a sustained antimicrobial activity [1415]. A yellow precipitate was formed on root treated dentin when root canals were irrigated with 1.3% NaOCl as an initial rinse followed by MTAD as a final rinse and this precipitate was found have a high affinity to hydroxyapatite [16].

HEBP, also known as etidronic acid or etidronate, has been proposed as a potential alternative to EDTA or citric acid because this agent shows no short-term reactivity with sodium hypochlorite [17]. Like EDTA, it is a chelator commonly used as an adjunct in household and personal care products such as soaps [18,19]. Measuring force of adhesion between E. faecalis and chemically treated dentin would contribute to a further understanding of how this bacterium can persist in a post-treatment endodontic environment. The atomic force microscope (AFM) has been used recently to study the forces of interaction between bacteria cells and between the bacterial cell and the substrate [20,21]. The bacterial cell or substrate particle can be attached onto an AFM tip and the forces of interactions between bacterial cells and between the bacterial cell and the substrate can be determined. As the AFM tip approaches the substrate and the gap between the 2 interacting bodies close to nanometer range, the interacting forces developed are registered by the AFM tip [22,23].

In our previous studies Gopikrishna., *et al*. [24], Kandaswamy., *et al*. [25] we employed various final irrigants and proved that EDTA showed higher bond strength, AH plus sealer to dentin when compared to MTAD and HEBP. Hence, we followed the same irrigating regimen for this study. So far no study has been done against the role of MTAD and HEBP on the force of adhesion of E. faecalis to root dentin. Hence, the aim of our study was to investigate the role of final irrigants (EDTA, HEBP and MTAD) on the force of adhesion of E. faecalis to root dentin using AFM.

Materials and Methods

Sample preparation

Fifty single-rooted human mandibular premolars extracted for orthodontic reasons were taken for this study. Residual soft t issues were removed with a scalpel, and the dental surfaces were wiped down with 70% ethanol. The teeth were sectioned vertically by using a water-cooled diamond- impregnated saw (Isomet, Buehler Ltd., Lake Bluff, IL) under copious water cooling and the root canal lumen was flattened by using progressively 1000-4000 grit silicon carbide paper. The samples were then ultrasonically cleaned in deionized water for 30 minutes to obtain root canal dentin specimens without the presence of smear layer. Silicon carbide paper was used on the external root surface to make those surfaces parallel to the root canal wall in the section [26].

Harvestation of *E. faecalis* And Functionalization of the AFM Tip

Enterococcus faecalis cells (ATCC 29212; Difco Laboratories, Detroit) were grown in Brain Heart Infusion Broth (BHI; Difco Laboratories, Detroit) and harvested in mid-exponential phase by centrifugation at 8,000 rpm for 10 min and washed in distilled deionized water (pH 6.5). A drop of 2.4% vol/vol electron microscope-grade glutaraldehyde (Merck, Darmstadt, Germany) was added to the cell suspension and incubated at 4°C overnight. To immobilize bacteria onto cantilevers, a pellet of cells was manually transferred onto gold coated silicon nitride tips and incubated at 4°C for 1-2 hr. Cantilevers were then rinsed in water and air dried. Scanning electron microscopy (Philips XL30E5, Philips, Eindhoven, Netherlands) was performed on all tips coated with bacteria after AFM measurements to verify the presence of bacteria on the silicon nitride tip [27].

Irrigation protocols

Dentin samples were divided into five groups (n = 50) where Group 1 consisted of the untreated dentin samples (control), Group 2 was irrigated with 1.3% NaOCl (Chlorox; The Chlorox Co, Oakland, CA) for 20 minutes, Group 3: 1.3% NaOCl for 5 minutes followed by 17% EDTA (Sigma,St. Louis, MA) for 1 minute, Group 4: 1.3% NaOCl for 5 minutes followed by MTAD (Dentsply Tulsa Dental, Tulsa, OK) for 5 minutes and Group 5: 1.3% NaOCl for 5 minutes followed by 18% HEBP for 5 minutes. HEBP solutions were freshly prepared by mixing HEBP powder (Zschimmer and Schwarz Mohsdorf GmbH and Co KG, Burgstadt, Germany) with bi-distilled water to w/v concentrations of 18%. After each chemical treatment, the specimen was carefully rinsed in deionized water without physical contact of the surfaces under study, mounted on a specimen mount with double-sided tape and the force curves were recorded.

AFM operation and force measurements

Agilent Pico LE 5100 atomic force microscope (Agilent Technologies Inc., Santa Clara, CA) was used to image cells and measure interaction. Gold coated silicon nitride tips (Digital Instruments, Veeco, Plainview, NY) with a curvature radius <20 nm and a spring constant of 0.06 N/m were used. Experiments were conducted in a fluid cell filled with distilled deionized water (pH 6.5). Force measurements were carried out by engaging the AFM without touching the surface to prevent any tip contamination from the sample. The tip was then approached to the surface in 100-nm increments with the specified Z scan size of 300 nm at a frequency of 1 Hz. After contact with dentin, retraction of the AFM tip was delayed by 10 seconds to allow interaction Surfaces were imaged after every force curve to confirm the presence of a continuous bacterial lawn. Gold silicon nitride tips were checked for cracks or breaks under an optical microscope before and after force measurements [27]. Force

curves recorded as the tip approached the surface were analyzed to determine the initial interactions between surfaces and bacteria. All force curves were normalized so that the tip deflection was 0 nm where there was no interaction, and the slope of the constant compliance region (portion of curve where cantilever moves with the surface) was equal to the rate of piezo displacement [21,23]. The slope of the retraction force curves in the region where probe and sample were in contact was used to convert the voltage into cantilever deflection. The conversion of deflection into force was carried out as described by Dufrene (Dufrene YF, 2000) where F = -kx where F is the force; k was the spring constant of the cantilever; x is the deflection. Approach curves were fitted to an exponential function. Retraction curves generally showed multiple adhesion peaks, and the magnitude of the peaks was recorded and averaged. Representative force curves for all samples were plotted together by aligning the zero deflection and constant compliance portions of the curves [21,23] (Figure 1).

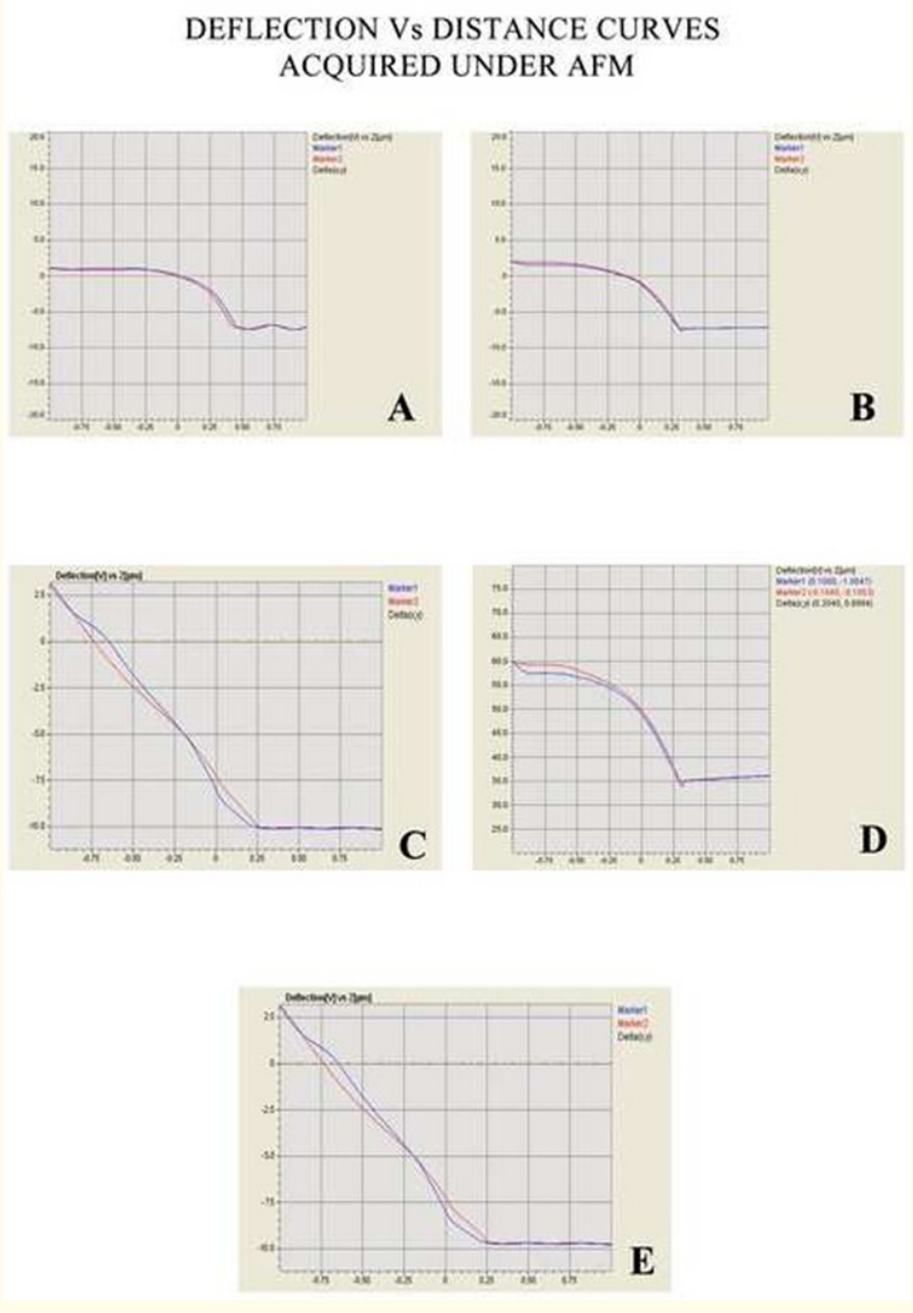

Figure 1

Statistical Analysis

One-way ANOVA followed by Post Hoc multiple comparison Tukey HSD Tests were used to analyze the data. Significance was established at $p < 0.01$ level.

Results and Discussion

- EDTA (Group II - 0.61 nN) showed the highest adhesive force followed by HEBP (Group IV - 0.59 nN), NaOCl (Group I - 0.45 nN) and Control (Group V - 0.42 nN). MTAD showed a repulsive force (Group III - 2.02 nN) ($p < .001$). (Table 1)
- Statistical Analysis with post hoc multiple comparison HSD tests showed that there was no statistical significance between force maxima observed in Control and NaOCl-treated dentin; EDTA- treated dentin and HEBP- treated dentin ($p < .001$).
- MTAD (Group III - 2.02 nN) showed statistically significant difference with all the groups ($p < .001$).

Irrigation regimens	Force of adhesion (n N) *(Mean ± Standard Deviation)*
Group I - NaOCl	0.45 ± 0.0137
Group II - NaOCl + EDTA	0.61 ± 0.0108
Group III - NaOCl + MTAD	2.02 ± 0.1032
Group IV - NaOCl + HEBP	0.59 ± 0.0108
Group V - Untreated Dentin (Control)	0.42 ± 0.0134

Table 1: Force of adhesion of *E. faecalis* to dentin for various final irrigant.

Discussion

Concepts of chemo mechanical preparation imply that in addition to mechanical cleansing, chemicals should be applied on instrumented root canal surfaces to remove the smear layer [3,28]. Complete removal of the smear layer requires the use of irrigants that can dissolve both the organic and inorganic components. Since, no single solution is capable of providing both effects alone [29], the use of acids and/or chelating agents followed by tissue solvents has been advocated [30]. NaOCl is one of the most commonly used endodontic irrigants because of its ability to destroy a broad spectrum of microbes and dissolve organic materials. NaOCl dissolves necrotic pulp tissue and denatures collagen [6]. The alternating use of NaOCl and calcium chelators has gained wide acceptance as an effective irrigation protocol [31]. NaOCl has been advocated as an initial rinse and various irrigants such as EDTA, MTAD and HEBP have been employed as final rinses [2,32]. The objective of our investigation was to assess the role of final rinse on the force of adhesion of E. faecalis to dentin. The accepted protocol for MTAD is 1.3% NaOCl for 20 minutes followed by MTAD for 5 minutes [11]. Hence, the irrigating regimens in all the test groups were standardized to 1.3% NaOCl for 20 minutes as an initial rinse.

E. faecalis is the most common and, occasionally, the only single isolated bacteria from root canals of teeth with persistent periapical periodontitis [33-36]. Its inherent antimicrobial resistance, ability to adapt to harsh environmental changes, and its growth in root canal walls as biofilm is considered as the major factor attributing to its survival in post endodontic environment [37-40]. Microbial adherence to a substrate has been suggested to occur in 2 distinct phases. In phase 1, the interaction between microbial cell and substrate is mediated by the attractive force and/or repulsive force as the function of separating distance between the surfaces involved. Hydrophobic interactions between microbial cell and substrate are thought to contribute largely to the attractive forces, whereas repulsive forces are due to the net negative charges (zeta potential) on the surfaces of both microbial cell and substrate. The phase 1 interaction is the initial interaction (in seconds to minutes) that occurs as the cell approaches the substrate surface, whereas the phase 2 interaction (in hours to days) occurs between the polymeric microbial cell surface structures on which adhesion molecules are expressed (eg, fimbriae and pili), and the substrate. The phase 2 interaction makes adherence between bacteria and the substrate firmer [26]. Our study investigated the initial stage of interaction between E. faecalis and the chemically treated dentin. From our results, we noticed that there was no statistical significant difference between Group V (Control) and Group I (NaOCl) in adhesive force of E. faecalis to dentin. The individual collagen fibrils within dentin are encapsulated by hydroxyapatite. NaOCl cannot destroy hydroxyapatite, but it can remove the proteinaceous part (collagen) of dentin. The unexposed collagen is not readily accessible to NaOCl. The action of NaOCl involves the slow dissolution of encapsulated collagen, leaving unbound hydroxyapatite crystals. This means that the dentin surface after deproteinization responded largely similar to that of untreated dentin [41]. This could have been a possible explanation for statistically no significant values in adhesive forces between Control and NaOCl group in our study.

When EDTA was used as a final rinse, it showed highest force of adhesion (0.6nN) which was statistically not significant

with HEBP but a statistically significant difference was seen in comparison with Group V (Control), Group I (NaOCl) and Group III (NaOCl + MTAD). This feature could be attributed to the dentin demineralizing property of EDTA, which exposes collagen and creates an ideal substrate for adherence by E. faecalis [42,43]. The collagen binding protein Ace, serine protease and gelatinase are potential virulence factors that give E. faecalis the ability to bind to dentin [44]. The force of adhesion in Group II (EDTA-0.6nN) in our study was lower when compared to a study done by Kishen., *et al.* (0.97nN) [26]. The possible explanation for this finding could be because of the differences in timing for which EDTA had been employed for 1 minute in our study whereas Kishen et al used EDTA for 5 minutes which might have caused extensive demineralization and further exposure of collagen, thus favoring increased E. faecalis adhesion to dentin.

Comparing EDTA and HEBP, the adhesive forces of HEBP is lower than EDTA but it was not statistically significant from EDTA. This difference in adhesive forces might be contributed to the weak chelating ability of HEBP in comparison to EDTA. But a statistically not significant result between the weak (HEBP) and strong chelator (EDTA) could be attributed to the dissimilarity in durations of irrigation of HEBP and EDTA. HEBP was employed for 5 minutes which was higher in comparison to 1 minute of EDTA. De-Deus et al showed that 17%.

EDTA specimens were completely smear-free after 60 seconds of etching followed by enlargement of the dentinal tubules over time whereas HEBP specimens at 18% concentration were completely smear-free only after 300 seconds of etching [32]. The etching times and concentrations are similar to that used in our study.

MTAD treated samples showed a repulsive force between E. faecalis and dentin (2.02nN) whereas other groups showed adhesive forces between E. faecalis and dentin. The possible explanation could be the formation of a yellow dentin - bound precipitate as a result of the interaction between NaOCl and doxycycline of MTAD which might have acted as a physical barrier between the exposed collagen matrices and E. faecalis, causing a repulsive force between the bacteria and the dentin surface. The antibacterial efficacy of MTAD could have influenced the interaction between E. faecalis and dentin, thus exhibiting a repulsive behavior.

Conclusion

Within the limitations of our study, we can conclude that EDTA and HEBP when employed as a final rinse increased the force of adhesion of E. faecalis to dentin. A repulsive force occurred between E. faecalis and dentin when MTAD was employed as a final rinse.

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Conflict of Interest

No financial interest or any conflict of interest exists.

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