Original Article

Bacterial reduction of mature Enterococcus faecalis biofilm by different irrigants and activation techniques using confocal laser scanning microscopy. An *in vitro* study

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Abstract

Introduction: The aim of this study was to evaluate the effect of ultrasonic and sonic activation using three irrigants on mature *Enterococcus faecalis* biofilm.

Materials and Methods: Seventy single-rooted premolars were prepared and sterilized. Mature *E. faecalis* biofilm was developed. Roots were randomly divided into three groups ($n = 21$) according to activation technique: ultrasonic, sonic, and positive control. Each group was further subdivided into three subgroups ($n = 7$) according to the irrigant used: 4% propolis, 2% chlorhexidine (CHX), and 2.5% sodium hypochlorite. Samples were cut and scanned using confocal laser scanning microscopy. The fluorescent images were analyzed using Zen imaging software. Data analysis was performed using one way analysis of variance and Tukey's honestly significant difference test for pairwise comparison. Statistical significance was set at 5%.

Results: Both activated groups showed a statistically significant bacterial reduction ($P \leq 0.001$). CHX showed the highest antibacterial effect.

Conclusions: Irrigant activation is an essential step in reduction of bacterial counts. CHX has a potent antibacterial effect against mature *E. faecalis* biofilm.

Keywords: Bacterial biofilm, confocal laser scanning microscopy, irrigating solution, sonic, ultrasonic

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INTRODUCTION

Microorganisms are the main cause for pulp and periapical pathosis. Failure to effectively eliminate microorganisms and their byproducts might result in persistent irritation and impaired healing.[1]

Enterococcus faecalis is one of the most persistent microbial species in endodontic infection, which can survive in extreme environmental conditions. Several studies have shown the prevalence of *E. faecalis* in endodontically treated teeth to reach up to 90% of the cases.[2] *E. faecalis* has been reported to be resistant to most of the intracanal medications used such as calcium hydroxide, clindamycin, tetracycline, and erythromycin.[3]

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One of the main virulence factors of bacteria is the biofilm-forming ability. Biofilm organization offers a number of advantages to bacteria such as exchange of genetic material, bacterial communication, diverse growth range, metabolic diversity, and protection from external environment. As much as thousand times higher concentration of antibiotics is needed to kill bacteria in a mature biofilm than that needed to kill it in a planktonic state.[4]

Disinfection of root canal is maintained through combination of mechanical and chemical procedures. The introduction of rotary nickel–titanium instruments has significantly affected endodontic cleaning and shaping procedures. Yet, after meticulous shaping of root canal systems using novel rotary nickel–titanium instruments, about 30% of the root canal walls remain untouched.[5] Application of irrigating solutions helps in providing more effective cleaning of complex root canal anatomy.

Sodium hypochlorite (NaOCl) is one of the most commonly used irrigants owing to its well-known tissue dissolving effect and antibacterial properties.[6] Chlorhexidine (CHX) is unique for its substantivity in addition to its superior antibacterial effect.[7,8] Natural herbal irrigating solutions have been introduced to overcome some drawbacks of other irrigants such as tissue toxicity and allergy. Propolis, one of the natural resin irrigating solutions, is extracted from honey bee and known for its antimicrobial effect, antioxidant, antitumor, and anti-inflammatory properties.[9]

The antibacterial effect of most irrigating solutions used is ineffective if it does not reach the complex root canal anatomy. Conventional passive irrigation allows penetration of solution only 1–2 mm beyond the syringe tip which is not sufficient for achieving the objectives of irrigation. Therefore, different activation techniques were introduced.

Ultrasonic-activated irrigation was induced by acoustic streaming and cavitation with frequency >20,000 Hz.[10] Sonically activated irrigation provides noncutting polymer tip ranging in frequency from 1500 Hz to 6000 Hz that quickly and vigorously agitates irrigant solution.[11] Activation techniques were shown to improve the antibacterial effect of some irrigants; therefore, the aim of this study was to evaluate the effect of ultrasonic and sonic activation techniques using NaOCl, CHX, and propolis against mature *E. faecalis* biofilm using confocal laser scanning microscopy. The null hypothesis tested was that mature *E. faecalis* biofilm would not be affected by ultrasonic and sonic activation techniques of NaOCl, CHX, and propolis.

MATERIALS AND METHODS

The current study was approved by the Research Ethics Committee of Ain Shams University (Cairo, Egypt) (approval number 01022017).

Seventy single-rooted permanent mandibular premolars extracted for periodontal reasons were used in this study. Teeth were inspected using × 8 magnifications by a dental operating microscope (Zumax, Suzhou New District, China) and radiographed. Teeth that showed more than one root canal, caries, cracks, fracture, resorption, or calcification were excluded.

Teeth were cleaned mechanically using an ultrasonic scaler (Satelec, Cedex, France) to remove calculus or soft tissues. Then, decoronation was done using a diamond wheel stone mounted on a high-speed handpiece at the cementodentinal junction with copious amount of water. Roots' length was adjusted to 16 mm. Roots were immersed in 5.25% NaOCl solution for 30 min and then stored in saline solution (El Nasr Pharmaceutical Chemicals Co., Cairo, Egypt) until used. Chemomechanical preparation of all root canals was done using WaveOne Gold (Dentsply Tulsa Dental, Tulsa, OK, USA) and copious irrigation with 2.5% NaOCl. Autoclaving of all roots was performed for 30 min at 121°C twice.

E. faecalis (29212, ATCC) (Nemro Co., Cairo, Egypt) was introduced in 7 mL of brain–heart infusion (BHI) and incubated at 37°C for 24 h. Suspensions were then prepared. Bacteria were resuspended in saline and adjusted to the #1 McFarland turbidity standard using sterile loops.

Five milliliters of the inoculum was mixed with 5 mm of sterilized BHI. All of the prepared root canals were inoculated with *E. faecalis* for 60 h using sterilized micropipettes. After 72 h, re-inoculation was done using pure culture prepared and adjusted to the #1 McFarland turbidity standards. Roots incubation was done at 37°C and humid environment for 2 weeks to ensure biofilm maturation.

Roots were classified into three groups (A, B, and C) according to the activation method: ultrasonic, sonic, and positive control, respectively. Each group was further subdivided into three subgroups (1, 2, and 3) according to the irrigant used: 4% propolis, 2% CHX (Dental Company, Cairo, Egypt), and 2.5% NaOCl (Egyptian Company for household detergents, Cairo, Egypt), respectively, as detailed in Table 1. Seven roots were used as a negative control without irrigation.

Table 1: Sample classification

Groups	Negative control (without irrigation)	Group A Ultrasonic activation	Group B Sonic activation	Group C Positive control without activation	Total
Subgroup 1 (4% propolis)		7	7	7	21
Subgroup 2 (2% CHX)		7	7	7	21
Subgroup 3 (2.5% NaOCl)		7	7	7	21
Total	7	21	21	21	70

NaOCl: Sodium hypochlorite, CHX: Chlorhexidine

Each root canal was irrigated using 5 ml of the corresponding irrigant using a 23-gauge plastic syringe as a final flush. Group A received no activation. In Group B, irrigant activation was done using IRRS ultrasonic tip (VDW GmbH, Bayerwaldstr, Munich, Germany) for 2 min. In Group C, irrigant activation was done using the Eddy sonic tip (VDW GmbH, Bayerwaldstr, Munich, Germany) for 2 min.

To obtain a 4% propolis solution, four grams of propolis powder (Imtenan, Cairo, Egypt) was dissolved in 100 ml of dimethyl sulfoxide (Tedia Company Inc., Fairfield, USA). The solution was incubated for 24 h to ensure mixture dissolution. Then, filtration of propolis solution was done several times using filter papers (at least 3 times) to remove any remaining undissolved particles.

All samples were then sectioned using a 0.3-mm IsoMet saw (IsoMet 4000 Precision Saw, Secunderabad, Telangana, India) under constant cooling using sterile distilled water. Impression compound was used to fix the roots on an IsoMet platform. Two 1-mm-thick sections were taken from each root corresponding to the apical and middle thirds.

Root sections were washed using 100 µl of sterile distilled water for 1 min and dried smoothly. The washed root sections were put in the bottom of Eppendorf tubes (Eppendorf, Hamburg, Germany). A 100 µl of 0.01% acridine orange (AO; Shanghai Yueteng Biotechnology Co., Ltd., Shanghai, China) (green fluorescence) and 10 µL propidium iodide (PI; Shanghai Yueteng Biotechnology Co., Ltd., Shanghai, China) (red fluorescence) were added to the specimen in a dark room to stain the specimen and remained for 15 min after centrifuging for 10 s. Specimens were removed from the tube and washed twice with sterile 100 µl of distilled water. Specimens were transferred to glass coverslips and then covered with immersion oil before imaging.

Argon laser microscope was used for confocal illumination: 500 nm laser for AO and 460 nm emissions for PI. Confocal laser scanning microscope (Carl Zeiss, ZEISS, Jena, Germany) was used to view fluorescence from the stained cells. This dye binds to RNA producing red fluorescence and binds to DNA producing green fluorescence. AO stains the live cells producing green fluorescence. PI penetrates dead cells' membranes producing red fluorescence. DNA excitation and emission was 500 and 526 nm and RNA excitation and emission was 460 and 650 nm, respectively, for the AO staining. Sequential frame scan mode was applied to avoid crosstalk.

Specimens were examined using ×40 magnification oil immersion objective with a numeric aperture of 1.4 and confocal pinhole of 88 Mm for channel one and 164 Mm for channel two.

The fluorescent images were analyzed with Zen imaging software (Zen 2012 blue edition). Deep scans were obtained 5–10 µm inside the dentin structure from each specimen (20–40 sections of 2 µm step size in a format of 1024 × 1024 pixels). Percentage of dead biofilm cells was calculated.

Mean and standard deviation were calculated. The Kolmogorov–Smirnov test was used to test for normality. One-way analysis of variance (ANOVA) was used to compare between subgroups within the same group, irrigating materials, followed by Tukey's honestly significant difference (HSD) test for pairwise comparison. One-way ANOVA was used to compare between groups, activation methods, followed by Tukey's HSD test for pairwise comparison (α =0.05). Statistical analysis was performed with Statistical Package for the Social Sciences (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY, USA).

RESULTS

Ultrasonic and sonic activation showed the highest percentage of dead cells within all subgroups which significantly higher than the positive control group. There was no statistically significant difference between both activation techniques, as seen in Table 2.

Within Group A with ultrasonic activation, 4% propolis and CHX showed the highest percentage of dead cells, 60.24% ±3.87 and 61.56% ±2.56, respectively, which were statistically significantly higher than NaOCl, 57.96 ± 3.21.

No statistically significant difference was shown between propolis and CHX, as seen in Table 2 and Figure 1.

Within Group B with sonic activation, CHX showed the highest percentage of dead cells, 61.09% ±3.68, which was significantly higher than propolis, 58.06 ± 5.70, and NaOCl, 56.81 ± 5.06. No statistically significant difference was shown between propolis and NaOCl, as seen in Table 2 and Figure 2.

Table 2: Mean and standard deviation for the percentage of dead cells for different tested activation methods and irrigating solutions

	Mean±SD			*P*
	Ultrasonic	Sonic	Positive control	
Propolis	60.24^{aA}±3.87	58.06^{aB}±5.70	21.61^{bB}±3.07	≤0.001*
CHX	61.56^{aA}±2.56	61.09^{aA}±3.68	31.62^{bA}±8.34	≤0.001*
NaOCl	57.96^{aB}±3.21	56.81^{aB}±5.06	28.97^{bA}±5.22	≤0.001*
P	≤0.001*	≤0.001*	0.002*	

Different lower case letters within each row indicate significant difference. Different upper case letters within each column indicate significant difference. *Significant. NS: Nonsignificant, NaOCl: Sodium hypochlorite, CHX: Chlorhexidine, SD: Standard deviation

Within Group C without activation, CHX and NaOCl showed the highest percentage of dead cells, 31.62% ±8.34 and 28.97% ±5.22, respectively, which were significantly higher than propolis, 21.61 ± 3.07. No statistically significant difference was shown between CHX and NaOCl, as seen in Table 2. The negative control group yielded a percentage of dead cells of 12.37 ± 1.40.

DISCUSSION

E. faecalis can survive extreme challenges. Inside root canals, these bacteria are partly shielded from the host defense. It possesses virulence factors such as lytic enzymes, pheromones, aggregation substance, cytolysin, and lipoteichoic acid in addition to biofilm-forming ability. It does have the ability to suppress lymphocytes leading to endodontic failure.[12] Mature *E. faecalis* biofilm was used in our study to mimic the actual clinical situation rather than planktonic bacteria which is very simple and easy to eradicate.[13]

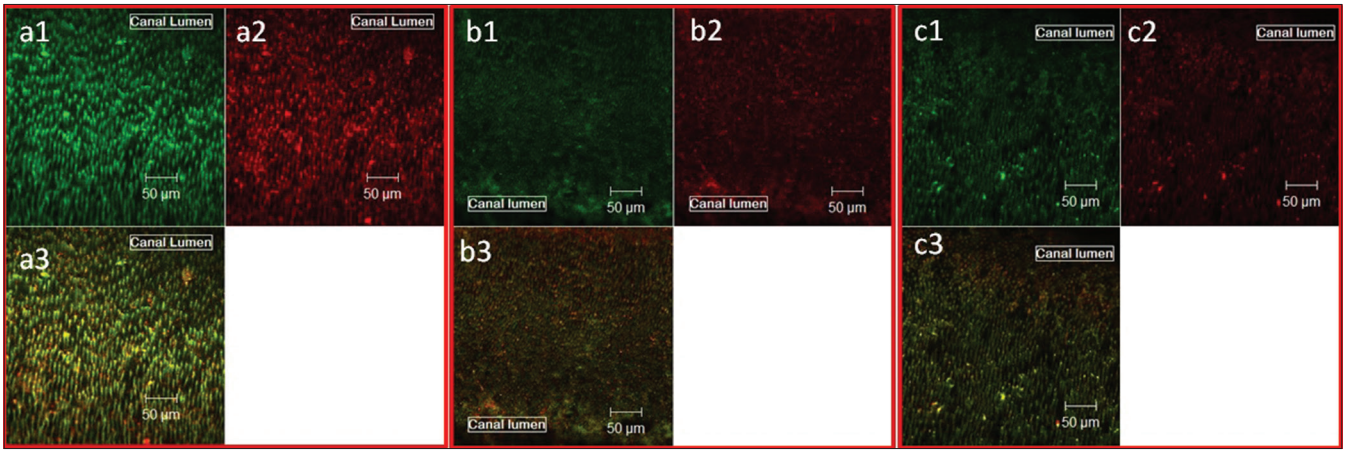

Figure 1: Live and dead bacteria in Group A, ultrasonic activation as green channel represents live bacteria and red channel shows dead bacteria, (a1, a2, and a3) subgroup 1, propolis; (b1, b2, and b3) subgroup 2, chlorhexidine; (c1, c2, and c3) subgroup 3, sodium hypochlorite

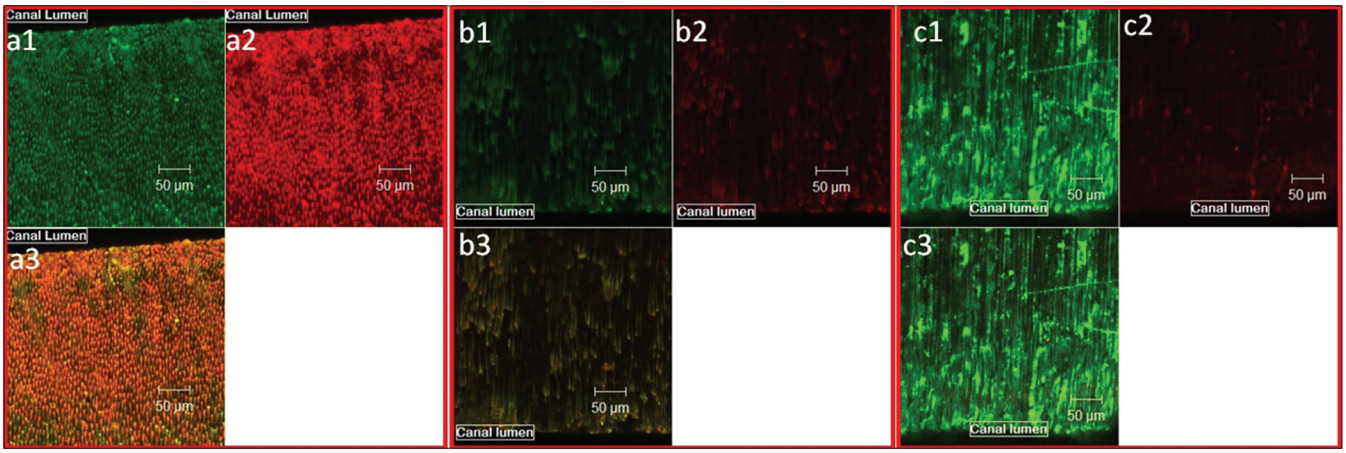

Figure 2: Live and dead bacteria in Group B, sonic activation as green channel represents live bacteria and red channel shows dead bacteria, (a1, a2, and a3) subgroup 1, propolis; (b1, b2, and b3) subgroup 2, chlorhexidine; (c1, c2, and c3) subgroup 3, sodium hypochlorite

In vivo studies are best used to study antimicrobial properties; yet, difficulty in standardization and ethical considerations made alternative models as extracted teeth, *in situ*, animal and *ex vivo* models more convenient.[14] Extracted human single-canalled teeth were used to best simulate the clinical situation excluding anatomical variations and complexity factors.[15]

Propolis is gaining interest in the endodontic literature owing to its antibacterial properties. The antibacterial action is attributed to its flavonoid content.[9] Flavonoids, phenolics, and other various aromatic compounds are the main components of propolis. Flavonoids possess antibacterial, antiviral, antifungal, antioxidant, and anti-inflammatory properties.[16] CHX 2% possesses effective broad-spectrum antibacterial properties.[17]

NaOCl demonstrates a potent antimicrobial activity.[18] Warming the solution, increasing the volume of the irrigant, use of agitation/activation methods, and lowering the pH of the irrigating solution improve the effectiveness of NaOCl. Ultrasonic activation increases the temperature of the irrigant and thus may improve its antibacterial effectiveness. NaOCl is able to totally eradicate intracanal biofilms.[19] However, Rosen *et al*.[20] showed NaOCl to yield viable bacteria in biofilms which may cause the persistence of disease and endodontic failure.

Confocal microscopy allows clear examination for biofilm without destruction of the ecosystems and the hydrated spatial organization. Live bacteria on root canal walls and in dentinal tubules can be easily observed in addition to identification of labeled bacteria and three-dimensional visualization of structural organization in biofilms.[21]

Specific stains differentiate between live and dead bacteria. Yet, specimen processing may cause bacterial death.[22] Nevertheless, three-dimensional reconstruction is not that accurate in studying biofilm disruption. Similar to light microscopy, cellular ultrastructure could not be examined.[22]

Both activation techniques resulted in significantly better bacterial eradication. This is in full agreement with Ghoddusi *et al*.[23] and van der Sluis *et al*.[10] who demonstrated significantly better bacterial eradication of sonically and ultrasonically activated NaOCl compared to no activation. This is simply related to the deeper penetration of the irrigant due to activation.[23] This could be explained on the basis of the shear stresses generated which leads to biofilm detachment from the dentinal walls and therefore facilitating their killing.[24] This could also be attributed to the disagglomeration of the biofilm, changing it to a planktonic form which is more susceptible to antibacterial agents.[25] Cavitations produced by ultrasonic activation causes weakening of the bacterial cell membrane increasing the permeability to antibacterial agents.[23,26] The use of ultrasonic activation improves intracanal bacterial eradication as the streaming field disrupts organic tissues and moves irrigating solution creating shear stress that damages bacterial cells.[27]

However, our results are inconsistent with Huffaker *et al*.[28] and Townsend and Maki[29] who showed no difference between activated and nonactivated irrigation. Yet, both studies used colony-forming unit for assessment of bacterial eradication. Colony-forming unit shows very low sensitivity in detecting viable cells in low concentrations and cannot detect bacteria in viable but noncultural state.[23]

Although the number of viable bacterial cells was less with ultrasonic activation, no statistically significant difference was demonstrated between both activation techniques. No technique was able to totally eradicate bacteria from inside the root canal creating a sterile root canal system.

All of the three tested irrigants were effective against the mature *E. faecalis* biofilm expressed by significant reduction in mean bacterial count. These results are in agreement with Baca *et al*.[30] The antibacterial activity of propolis is attributed to its flavonoid content.[9,16] The antibacterial activity of CHX is attributed to the ability to be adsorbed onto hydroxyapatite component of the dentine surface and its gradual release, protecting the canal against microbial colonization and also by inhibiting bacterial growth through leakage of the intercellular components.[7,8,18] The antibacterial activity of NaOCl is explained by the release of hypochlorous acid which is a powerful oxidizing agent that produces an antimicrobial effect by irreversible oxidation of hydrosulfuric groups of bacterial enzymes. As essential enzymes are inhibited, metabolic functions of the bacterial cell are impaired resulting in bacterial cell death. Chlorine can also adhere to bacterial cytoplasm components forming highly toxic N-chloro composites that destroy microorganisms.[8]

Using ultrasonic activation, propolis and CHX have shown significantly better bacterial eradication than NaOCl. Using sonic activation, CHX was superior to propolis and NaOCl. These findings could not be directly compared to earlier studies as none could be found in the literature comparing all three irrigants using ultrasonic or sonic activation.

Without activation, CHX and NaOCl showed significantly better bacterial eradication than propolis. Our results

are in agreement with Shveta *et al*.[31] who demonstrated the antibacterial efficacy of propolis against *E. faecalis* to be inferior to that of NaOCl and CHX. This could be attributed to the low pH value of the propolis, 4.9–5.2. *E. faecalis* growth was shown to be retarded at a pH value of 10.5–11 and totally destroyed at pH value of 11.5 or greater.[31] Our results are inconsistent with Baca *et al*.[30] who showed better bacterial reduction of NaOCl compared to CHX. This is attributed to the difference in methodology used as they determined the residual activity by exposing treated dentin blocks to *E. faecalis*. Our results were also not in full agreement with Bukhary and Balto[6] who demonstrated CHX antibacterial activity but inability to disrupt the biofilm structure.[6] This could be attributed to the difference in the study design, dentin discs rather than root canal. Bukhary and Balto[6] have also shown NaOCl to be superior to CHX; yet, they used NaOCl 5.25%, and the biofilm disruption ability of NaOCl was shown to be concentration dependent.[19]

The null hypothesis is rejected as bacterial counts in mature *E. faecalis* biofilm were significantly reduced by irrigant activation. Within the limitations of the current study, it can be concluded that irrigant activation is an essential step in reduction of bacterial counts in heavily infected canals. CHX has a potent antibacterial effect against mature *E. faecalis* biofilm. Propolis is a promising natural antimicrobial alternative.

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Conflicts of interest

There are no conflicts of interest.

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