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Antimicrobial activity of Er,Cr:YSGG and Ultrasonic on *E. faecalis* biofilm in the mesial root canal systems of lower molars

Atividade antimicrobiana de Er,Cr:YSGG e Ultrassônico no biofilme de *E. faecalis* nos sistemas de canais radiculares mesiais de molares inferiores

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ABSTRACT

The internal topography of the root canal is complex, especially for the permanent molar's mesial root. In response to such issues, improved irrigation techniques have been created, which use laser pulses to agitate fluids and improve microbial deposit removal. **Objective:** To assess the effectiveness of the Er,Cr:YSGG laser with a wavelength of 2,780 nm via photon-induced photoacoustic streaming (PIPS) protocol which agitated of 2% chlorohexidine (CHX) in removing mature Enterococcus faecalis (*E. faecalis*) biofilm in root canal systems of lower molars. **Material and Methods:** The mesial roots of lower first and second molars were separated and inoculated with *E. faecalis* bacterial suspension for 30 days. The roots were irrigated with CHX, some of them were agitated with a passive ultrasonic device (PUI), while the other roots were agitated by an Er,Cr:YSGG laser in PIPS at 60 μs/pulse, 5 Hz, (0.25, 0.5, 0.75, and 1) W. An atomic force microscope (AFM) was used as a new method to get the results in the isthmus area; the obtained results from each group were compared with each other. **Results:** Based on the AFM and SEM analyses, laser and ultrasonic activation groups showed higher antimicrobial efficacy than the conventional syringe irrigation group ($P<0.05$). **Conclusion:** Based on the investigation's findings, the activation of 2% CHX solution by Er,Cr:YSGG laser in PIPS and PUI offers better mature bacterial biofilm removal in the mesial root of lower human molars than the same irrigant with the SI technique.

KEYWORDS

2% Chlorhexidine gluconate; Atomic force microscope; Enterococcus faecalis biofilm; Er,Cr:YSGG laser; Passive ultrasonic activation.

RESUMO

A topografia interna do canal radicular é complexa, especialmente para a raiz mesial do molar permanente. Em resposta a esses problemas, foram criadas técnicas aprimoradas de irrigação, que utilizam pulsos de laser para agitar fluidos e melhorar a remoção de depósitos microbianos. **Objetivo:** Avaliar a eficácia do laser Er,Cr:YSGG com comprimento de onda de 2.780 nm via protocolo de streaming fotoacústico induzido por fótons (PIPS) que agitou clorohexidina a 2% (CHX) na remoção de Enterococcus faecalis maduro (*E. faecalis*) biofilme em sistemas de canais radiculares de molares inferiores. **Material e Métodos:** As raízes mesiais de 28 primeiros e segundos molares inferiores foram separadas e inoculadas com suspensão bacteriana de *E. faecalis* por 30 dias. As raízes foram irrigadas com CHX, sendo algumas delas agitadas com aparelho ultrassônico passivo (PUI), enquanto as demais raízes foram agitadas com laser Er,Cr:YSGG em PIPS a 60 μs/pulso, 5 Hz (0,25, 0,5, 0,75 e 1) W. Um microscópio de força atômica (AFM) foi utilizado como um novo método para obter os resultados na área do istmo; os resultados obtidos de cada grupo foram comparados entre si. **Resultados**: Com base nas análises de AFM e SEM, os grupos de ativação por laser e ultrassom apresentaram maior eficácia antimicrobiana do que o grupo de irrigação com seringa convencional ($P<0.05$). **Conclusão:** Com base nos achados da investigação, a ativação da solução de CHX a 2% pelo laser Er,Cr:YSGG em PIPS a (60 μs/pulso, 5 Hz, 0,75 W) oferece melhor remoção de biofilme bacteriano maduro na raiz mesial da raiz humana inferior molares do que o mesmo irrigante com as técnicas SI e PUI.

PALAVRAS-CHAVE

2% Gluconato de clorexidina; Ativação ultrassônica passiva; Biofilme de Enterococcus faecalis; Er,Cr: laser YSGG; Microscópio de força atômica.

INTRODUCTION

The primary pathogenic agents of the dental pulp and periapical infections are bacterial and other microbial pathogens in the root canal system. Enterococcus faecalis (*E. faecalis*) is a gram-positive, facultative and anaerobic bacteria which is the most commonly identified after failed root canal therapy and considered to be extremely difficult to clear with conventional methods [1]. The most commonly used antibacterial irrigation solution is chlorhexidine gluconate (CHX), which is a broad-spectrum antibacterial fluid that works to combat both gram-positive and gram-negative bacteria as well as yeasts [2]. CHX has been utilized as a final irrigant or intracanal medicament in endodontics. Also, it is recommended as an alternative to NaOCl, particularly in cases of open apex, root resorption, and root perforation.

CHX's antibacterial action is pH-dependent, with an optimal range of 5.5 to 7, as well as concentration-dependent, so irrigating with 2 percent chlorhexidine is better than 0.12 percent [3]. It is critical to work on novel strategies to ensure irrigation fluid reaches inaccessible places, thereby improving endodontic outcomes. Recently, the laser photon-induced photoacoustic streaming (PIPS) technology has demonstrated encouraging results in the elimination of biofilm from the root canal surface [4,5]. This method involves activating the irrigation fluid within the root canal via complex photoacoustic and photomechanical phenomena [6]. Each impulse generated by the PIPS tip gets absorbed by the water particles, resulting in the production of a powerful "shock wave" that results in the creation of effective fluid streaming within the root canal system [7]. PIPS technique is used by an Erbium laser family at sub-ablative settings. An erbium, chromium: yttrium scandium gallium garnet (Er,Cr:YSGG) is a type of water-absorbing laser with a wavelength of 2,780 nm [8]. It has been suggested that the hydrokinetic energy employing promotes dental canal disinfection with no thermal harm to the underlying tissues [9,10].

Atomic force microscopy (AFM) is a useful technique for studying the shape and texture of various surfaces. Surface texture includes roughness, waviness, and flaws [11]. This method has been widely utilized to investigate the mechanisms of antibacterial substance activity on bacteria [12]. Many kinds of research using AFM have been used in a variety of dental fields of study [13,14].

The most previous work on irrigation activation methods has been done about the improvement of biofilm removal by the laser agitation of chlorhexidine in an infected single root canal with Enterococcus faecalis biofilm obtained after a short incubation period [15,16]. The goal of this study was to examine whether mechanical agitation by Er,Cr:YSGG laser improves the effectiveness of 2% chlorhexidine against 30-day-old *E. faecalis* biofilm in human mandibular root canal systems, and use of AFM to investigate the impact of different irrigation protocols on the topography of the surface by analyzing isthmus surface properties.

MATERIAL AND METHODS

Ethical approval (10-2022-488) was obtained from the Research and Ethics Committee of Institute of Laser for Postgraduate Studies, University of Baghdad.

Specimen selection and preparation

A total of forty-two extracted mandibular first and second molars without root canal fillings, root caries, or restorations were obtained and cleaned immediately after extraction. All samples were placed in a glass container containing distilled water with 0.1% thymol crystals (Lab Grade, Lab Alley, Texas, USA) until the day of the experiment.

A diamond disc (OSA-E28, Osakadent group ltd., Guangdong, China) was used for cutting off the teeth crowns. The mesial roots of all molar teeth were separated from the rest of the teeth to supply roots with a length of 12 mm from the apex. A stainless-steel K-type hand file #10 (Dentsply, Maillefer, Ballaigues, Switzerland) was used to locate the site of the apical foramen. After sighting the file tip through the apical foramen, 1 mm was subtracted from the file length that was measured, and this value was used as the working length (WL).

All root canals were prepared to this WL up to #25/.04 NiTi engine files (X3 Never Break Serious, Easyinsmile, New Jersey, USA) at speed and torque recommended by the manufacturer. During instrumentation, 1 mL of 5.25% NaOCl (Cerkamed, StalowaWola, Poland) was administered after each file size, using a 30-gauge irrigation needle with side vents (Endotop, Hang ZhouEndoTop Medi-Tech Co. ltd., Zhejiang, China). 1 ml of 17%

EDTA was used as the final irrigant (Cerkamed, StalowaWola, Poland). The fluid was left inside the canals for 3 minutes, within this period the solution was activated with an ultrasonic device (Guilin Woodpecker Medical Instrument Co. ltd, Guangxi, China) for 30 seconds.

At the last stage of the process, all the root samples were irrigated with 5 mL of distilled water (Pioneer Company, Baghdad, Iraq) to eliminate the remains irrigation solutions. The root canals and the outer surfaces of the teeth were dried, internally with paper points (Sure-endo, Sure Dent Corporation, Gyeonggi-do, Korea) and externally with paper towels. All roots were placed individually in Eppendorf tubes in an upright posture (Lab Serv, Thermo fisher Scientific, Gurugram, India) and autoclaved for 20 minutes at 121 °C and 15 psi pressure.

Bacterial inoculation

After sterilization, paper points were used to dry the root canals then all canals were inoculated with bacterial suspension of *E. faecalis* except for three roots which act as a negative control.

E. faecalis (ATCC 4083) taken from its frozen stock was streaked onto agar plate (Himedia, Mumbai, India) and cultured for 24 hours at 37 °C. Multiple *E. faecalis* colonies were taken from the agar plate and activated by being placed in brain heart infusion (BHI) broth (Himedia, Mumbai, India) a day before. Then 1 mL of bacterial infusion was diluted by adding 8 mL of normal saline to obtain a suspension equal to the McFarland standard 1.5×10^{8} colony forming units per milliliter (CFU/ml). The suspension of bacteria was injected into the cleaned root canals using a disposable syringe and a 30-gauge irrigation needle until they were filled completely. Each root specimen was immersed in 1.5 mL of BHI broth after introducing the bacteria into the canals. All sample tubes were stored in a warm environment at 37 °C under aerobic conditions for 30 days. Re-inoculation was conducted every three days to be sure of the presence of viable bacteria during the period of incubation, and the BHI was replaced daily with a new one. All procedures were carried out in a sterile environment.

Treatment groups

At the point of termination of the incubation period, the liquid medium was drawn out of the tubes, and the thirty-nine samples were irrigated with 5 mL of distilled water, then divided randomly into four groups: (A) Positive control group, which did not get any sort of therapy (n=3) (B) Samples were irrigated with 2% CHX delivered by irrigation needle (n=12). (C) Passive ultrasonic activation of 2% CHX (n=12), and (D) Er,Cr:YSGG laser at {60μs/pulse, 5 Hz, (0.25, 0.5, 0.75, 1) W using MZ6-6 mm length laser tip in PIPS protocol} agitate of 2% CHX (n=12).

Each root of the last three groups was subjected to several procedures: all samples' surfaces were cleaned with sterilized cotton pads immersed in 5.25% NaOCl, then they were mounted in plastic tubes filled with alginate impression material (Kromalgin, Vanninidental, Grassina, Italy) for easy handling of the samples.

For group B, a 30-gauge irrigation needle was used to irrigate the samples with 2% CHX and the fluid left inside the canals for 2 minutes, then washed with 5 mL of distilled water.

Group C samples were also irrigated with 2% CHX and left the fluid inside the canals for 2 min, then activated by a passive ultrasonic tip for 60 sec, then all canals washed with 5 mL of distilled water. The device tip was placed 1 mm shorter than the estimated WL.

Group D samples were irrigated with 2% CHX and left the fluid inside the canals for 2 min, within this time, the fluid was activated by 2780 nm Er,Cr:YSGG laser (Biolase, Waterlase, iPlus, CA, USA) for 60 sec. Infrared laser safety glasses (Innovative Optics, Hemlock Lane North Maple Grove, USA) were worn before laser activation. A newly designed water Lase iPlus /MD glass tip was used (MZ6 Zip tip diameter = 600 μm, length = 6 mm) and the laser unit's water, as well as air spray were both set to "off ". During laser work, the tip was put just into the canal opening, remained stationary, and didn't move apically into the root canal. Laser operation proceeded for thirty seconds of "on" time, followed by thirty seconds of "off" time, and this sequence was repeated twice (for a total of 60 seconds of activation). After that, the canal was irrigated with 5 mL of distilled water.

A properly fitting paper point cones were placed in the root canals to prevent tooth fragments from entering the endodontic canals and isthmus area. Without entering the root canals, all roots were grooved longitudinally on their outer surface using a diamond disc, and a chisel was used to cut specimens in half. A middle area of the

isthmus was marked about 6 mm from the apex for examination by the AFM (Nanosurf, Liestal, Switzerland) and SEM (Inspect F-50, FEI Electron Optics International B.V., Netherlands) tools.

Statistical analysis

The data has been interpreted using the Statistical Package for Social Sciences (SPSS) version 21 (IBM, Armonk, New York, USA). It was provided as a mean, while categorical data was displayed using the standard deviation. Data were expressed as a mean±SD. Analysis of variance (ANOVA) was utilized to compare the means of the tests. The least significant difference (LSD) test was used to compute the significant differences among the tested means. Results of $p>0.05$ were considered statistically non-significant, while $p\leq0.05$ was considered a significant value.

RESULTS

The efficacy of laser activation and other methods in the eradication of bacterial biofilm in the isthmus was assessed by using an AFM tool that analyzes surface roughness to determine the presence of the biofilm in this region.

The uncultivated isthmus area was illustrated in the two and three-dimensional images (Figure 1a and 1b), in addition to a histogram that showed the dominance of small particles with a low mean diameter value equal to 52.82 nm (Figure 1c). 2D & 3D images of not treated isthmus surface, about 6 mm from the apex were shown in Figure 2, and a mean diameter histogram displayed dominant small particles with a high mean diameter value of about 76.24 nm. In Figure 3, the isthmus surface after being treated with 2% CHX by irrigation syringe method without any agitation was shown in AFM images.

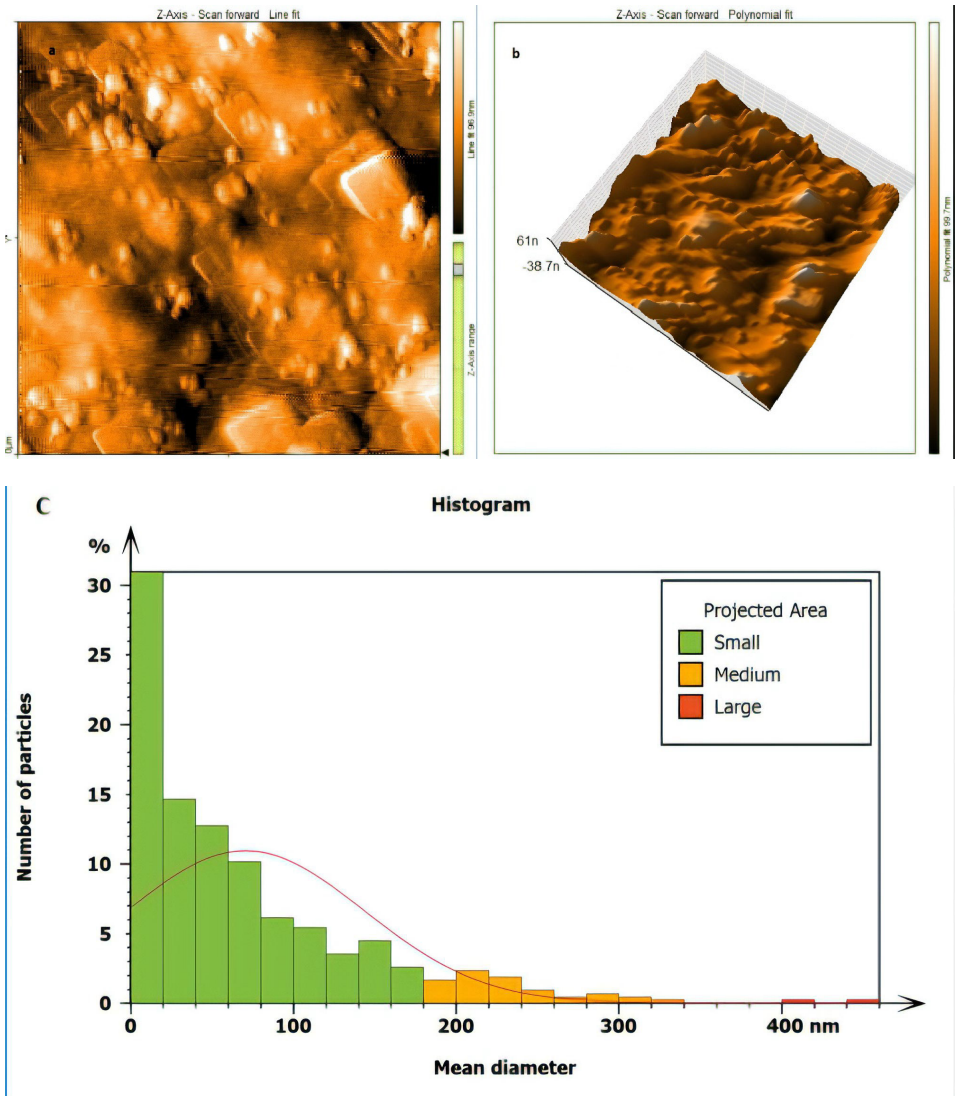

Figure 1 - AFM imaging of not inoculated isthmus surface about 6 mm from the apex (a) Two and, (b) Three dimensions (c) Histogram represents the number of particles for different particle's mean diameter, red columns for large particle, orange columns for medium particles, and green columns for small particles.

In addition, the mean diameter of surface small particles of about 57.42 nm was illustrated in Figure 3c.

The isthmus of the treated root with chlorhexidine gluconate activated with PUI was shown in Figure 4. In Figure 5, the images of the isthmus surface were exhibited after being treated with 2% CHX that agitated with Er,Cr:YSGG laser in PIPS at (60 μs, 5 Hz, 0.75 W), in addition to a histogram that showed low value of small particles mean diameter about 33.21 nm. Values of the root mean square of positive control and other test groups were statistically analysis as illustrated in a statistical columns chart (Figure 6). The lowest root mean square value was presented in the 2% CHX group that agitated by laser PIPS at (60 μs, 5 Hz, 0.75 W) (G D) and the highest root mean square value was presented in the positive control group (G A).To confirm the results obtained from an AFM test, the isthmus area was also evaluated by FE-SEM taken at 6 mm from the apex at (13000 and 50000 x) for more detailed view of the biofilm and bacterial cocci. After bacterial incubation for 30 days, a dense and heavy layer of bacterial biofilm formed on the isthmus surface occluding the dentinal tubules, as shown in Figure 7a and Figure 8a. The samples treated with the laser PIPS at (60 μs, 5 Hz, 0.75 W) and 2% CHX showed a clean surface with open tubules and few smashed bacterial biofilms and debris remained on the surface, as shown in Figures 7b and 8b.

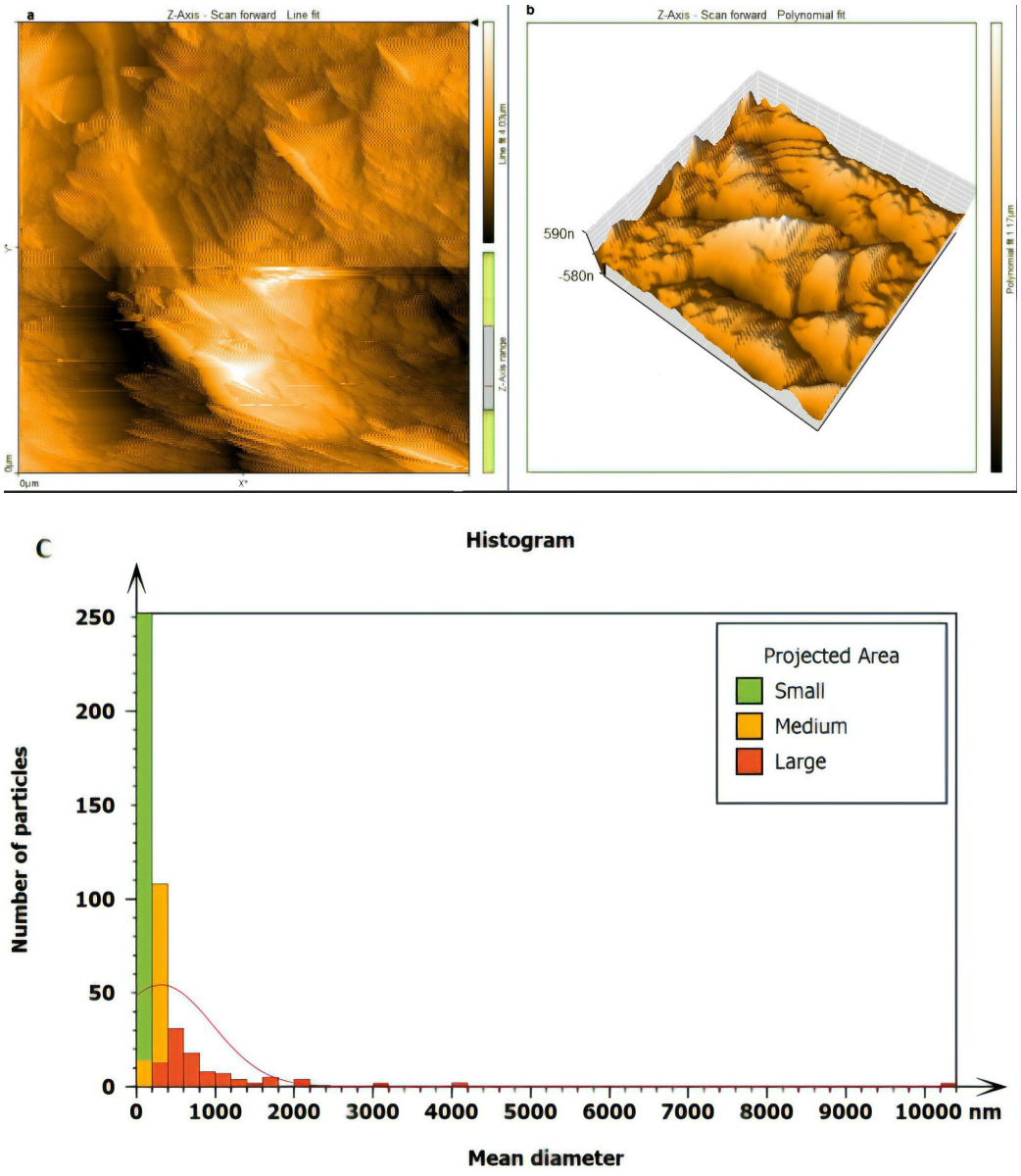

Figure 2 - AFM imaging of inoculated not treated isthmus surface about 6 mm from the apex (a) Two and, (b) Three dimensions (c) Histogram represents the number of particles for different particle's mean diameter, red columns for large particle, orange columns for medium particles, and green columns for small particles.

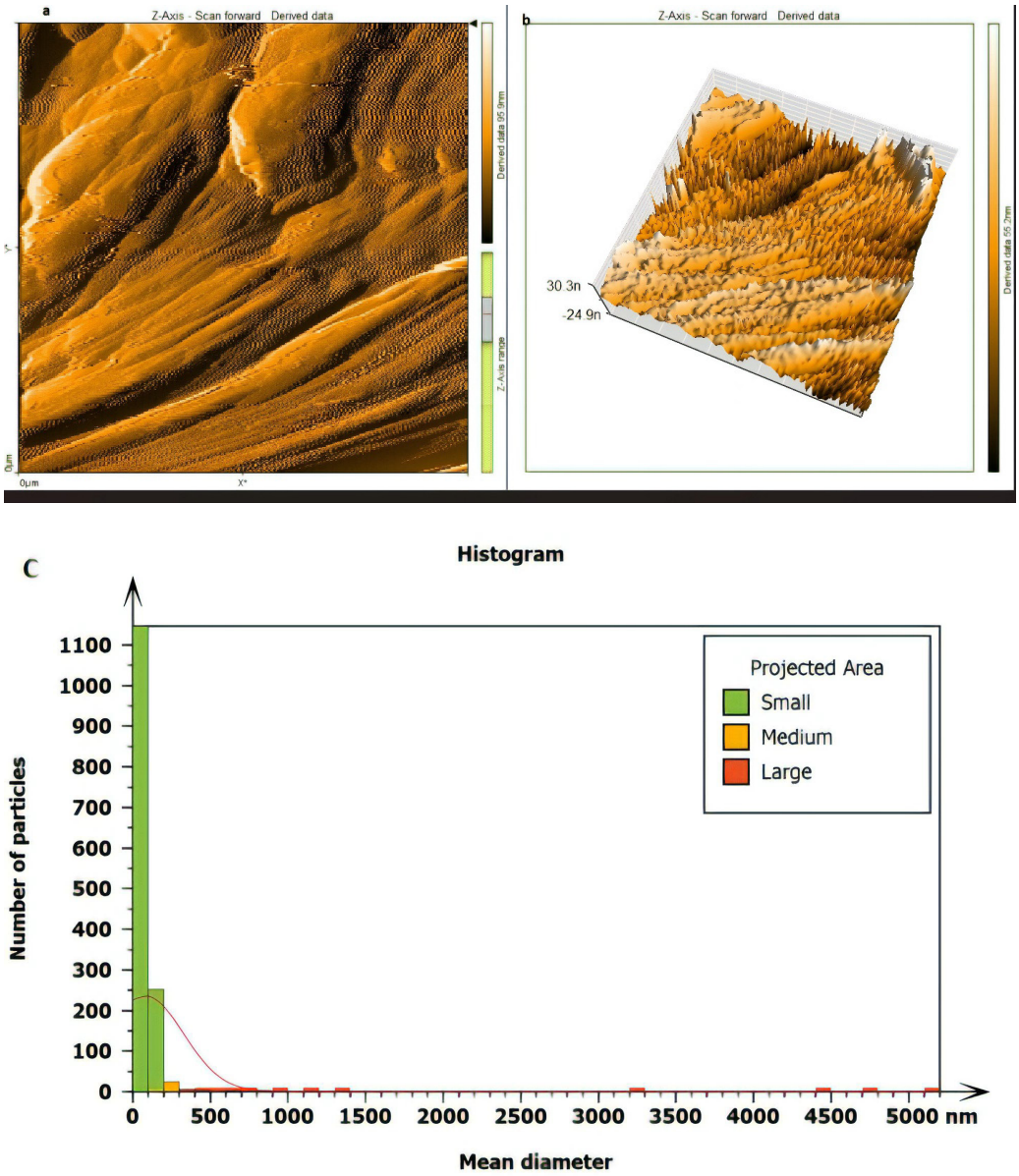

Figure 3 - AFM imaging of the isthmus surface after treatment with 2% CHX delivered by syringe irrigation (SI) (a) Two, and (b) Three dimensions (c) Histogram represents the number of particles for different particle's mean diameter, red columns for large particle, orange columns for medium particles, and green columns for small particles.

DISCUSSION

Endodontic therapy's main goal is to completely clean the root canal, although this is challenging due to the complicated structure of the canal system. The microbial community of teeth with persisting apical periodontitis is dominated by simple gram-positive microorganisms. *E. faecalis* was selected for this investigation because it is thought to be the most highly resistant microbe detected in infected root canals, having particular mechanisms for constructing a biofilm, significant virulence-related factors, adherence capacity to dentin collagen, survivability in harsh circumstances, capability to resist root canal treatment, and is easy to grow in vitro [17,18].

Various materials have been used in prior experiments to produce bacterial biofilm, such as nitrocellulose membrane filters [19], hydroxyapatite discs, or teeth previously extracted [20]. In this study, anatomically complicated extracted human teeth were inoculated with *E. faecalis* in a laboratory environment.

The time required for the development of biofilm differs between studies (15 minutes to 60 days) [21,22]. However, a prolonged period of incubation results in more mature biofilms. In the current work, infected teeth underwent incubation

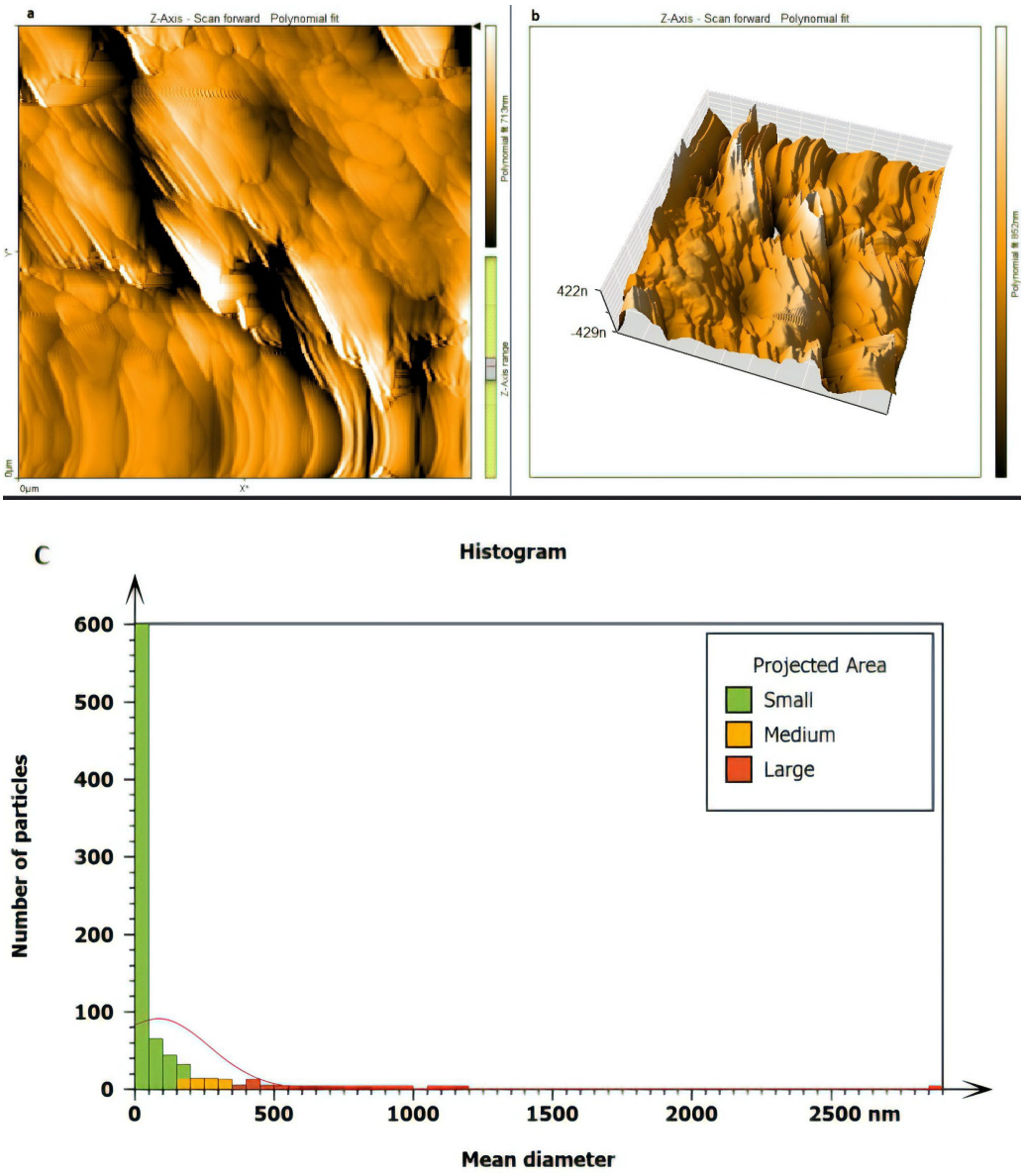

Figure 4 - AFM imaging of middle isthmus surface after treatment with 2% CHX + passive ultrasonic activation (a)Two, and (b) Three dimensions (c) Histogram represents the number of particles for different particle's mean diameter, red columns for large particle, orange columns for medium particles, and green columns for small particles.

at 37 °C for 30 days to allow mature *E. faecalis* biofilm to grow. Chlorhexidine gluconate (CHX) was chosen in this investigation because it has broad-spectrum antibacterial activity and destroys Enterococcus faecalis bacteria in the tubules of the dentin, as well as its biocompatibility [23]. Also, it has proven a bacteriostatic effect at low concentrations and a bactericidal effect at high concentrations owing to potassium and phosphorous leaking out and the coagulation of the cytoplasm [24]. As reported by Ercan et al. [25], both CHX and NaOCl were considerably effective in minimizing microorganisms in teeth with dead pulp, periapical disease, or both.

The effectiveness of irrigation is influenced by irrigation quantity, closeness to the apex, solution temperature, and the dynamics of fluids produced by activation methods [26]. The utilization of laser and ultrasonic devices has been proposed as complementary techniques for improving aqueous irrigation fluid dispersion and activation. In past experiments, both culture procedures and molecular methods have been used to determine the number of viable bacteria in the canals and dentine tubules [27]. A confocal laser microscope (CLSM) can also detect the viability of bacteria colonizing the root canal walls as well as the lateral canals and isthmus [28]. This study used an atomic force microscopy (AFM) tool to analyze the samples, which is easy to apply, precise, available, and more economical than CLSM. AFM is used as a supplementary tool for investigating

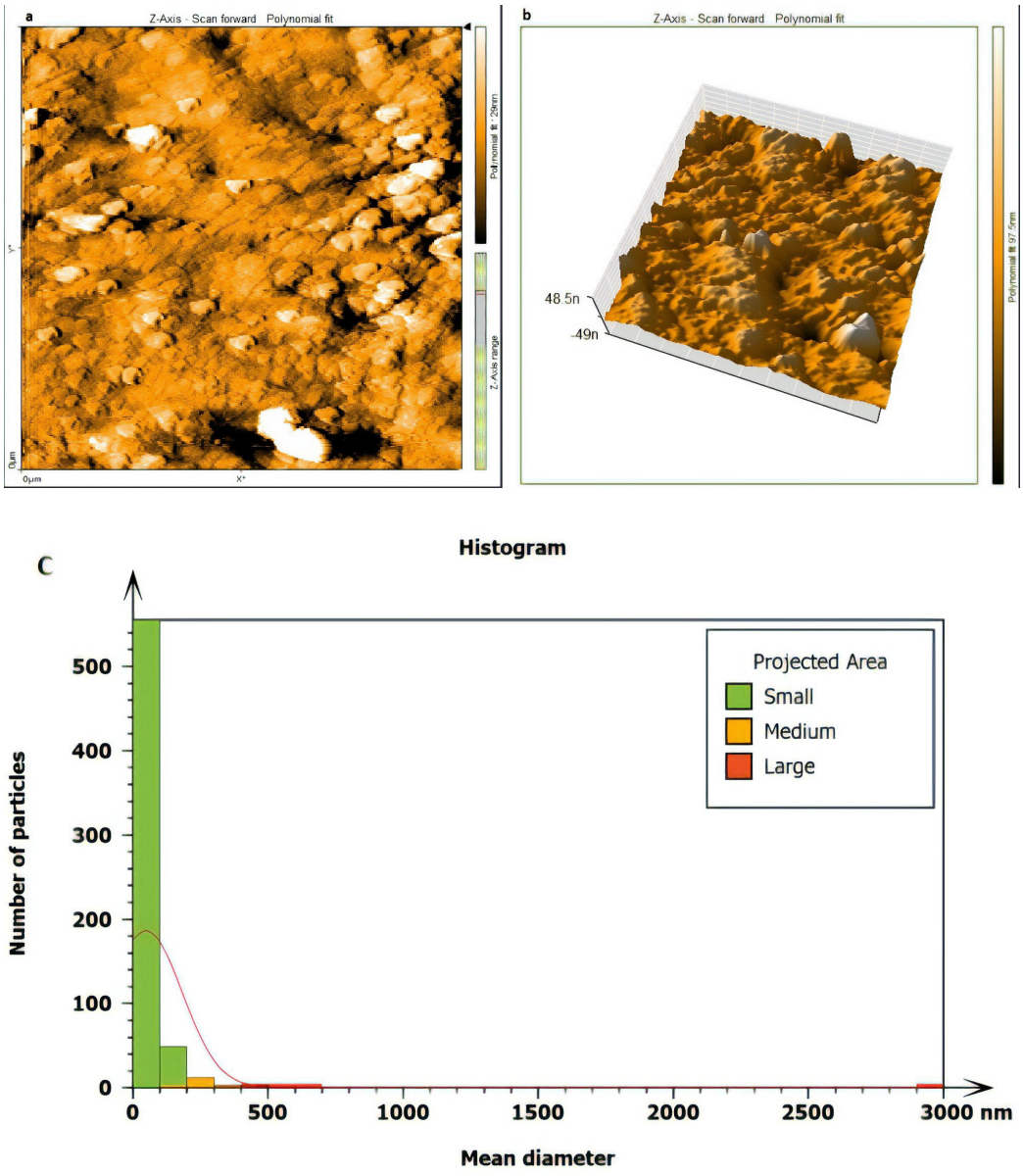

Figure 5 - AFM imaging of middle isthmus surface after treatment with 2% CHX agitated by Er, Cr: YSGG laser at (60 µs, 5 Hz, 0.75 W) in PIPS protocol (a)Two, and (b) Three dimensions (c) Histogram represents the number of particles for different particle's mean diameter, red columns for large particle, orange columns for medium particles, and green columns for small particles.

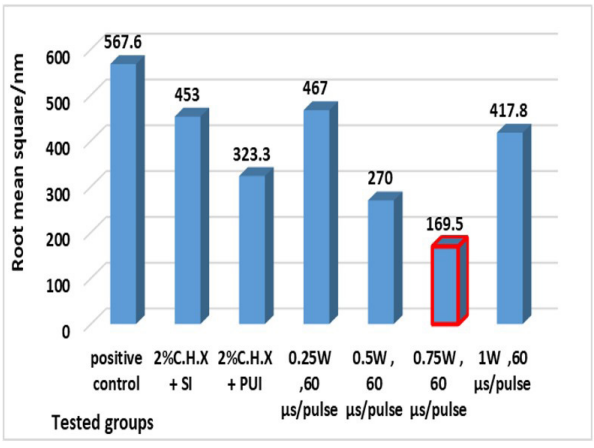

Figure 6 - Columns chart showing the root mean square roughness values of positive control and other test groups, the red border's column represents the best result.

antibacterial mechanisms by exposing the change in the roughness of the surface inoculated with *E. faecalis* biofilm. The topography of the surface of the isthmus was studied, and measurements of surface roughness, particle size, and particle analysis were taken. Surface roughness was determined by computing the parameters of the surface profile: root mean square (Sq.), average roughness (Sa), and maximum height (Sz.). The roughness parameters are determined by analyzing topographical scans of the sample's isthmus surface [29]. In this study, (sq.) was dependent, that is the root square of the surface height distribution and is thought to be more responsive to significant deviations from the mean line than average roughness [11].

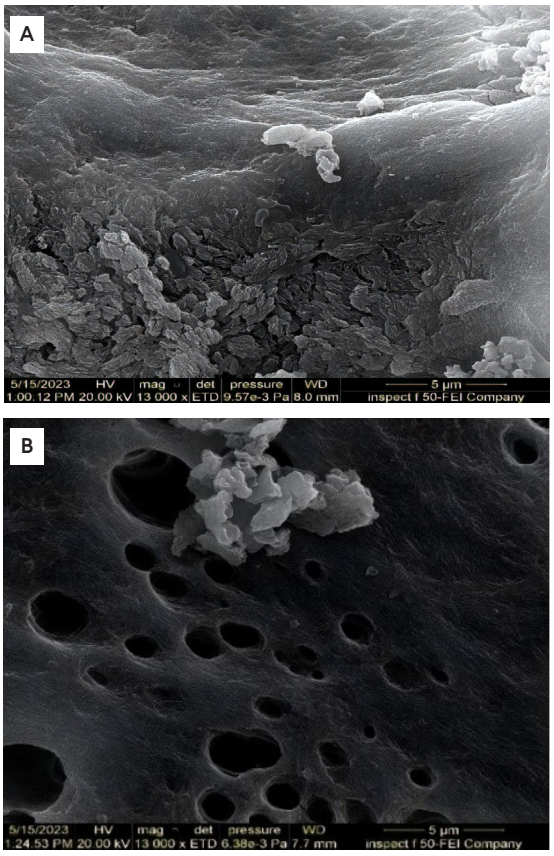

Figure 7 - FE-SEM (13000) magnification of the isthmus area about 6 mm from the apex (a) Positive control sample (b) The same area after being treated with 2% CHX+ Er, Cr: YSGG laser using PIPS.

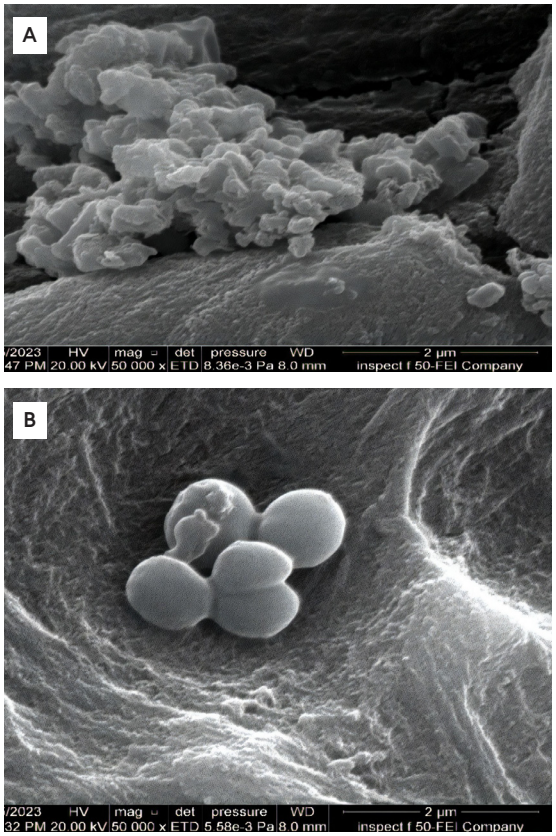

Figure 8 - FE-SEM (50 000) magnification of the isthmus area about 6 mm from the apex (a) The positive control sample (b) The same area after being treated with 2% CHX+ Er, Cr: YSGG laser in PIPS.

Previously, Arıcıoğlu et al. [30], determined the effectiveness of Er: YAG laser modalities (PIPS\SSP) and shock wave-enhanced (SWEEPS\ Auto SWEEPS) with NaOCl and CHX in the removal of *E. faecalis* incubated for 4 weeks, and concluded that using antimicrobial agents with laser activation is necessary for the best reduction of microbial deposits. While, the antibacterial activity of a 2% chlorhexidine irrigation fluid activated by a 2780 nm Er,Cr:YSGG laser in PIPS and a passive ultrasonic technique against *E. faecalis* biofilm in a complex root canal system was tested in this work.

The exterior of the isthmus surface after being treated with 2% CHX that is activated by a passive ultrasonic system contained a few prominent peaks and low troughs with a random or low bumpy surface. The root mean square (sq.) value of 323.3±10.7 nm was less than the value measured after the syringe irrigation method (453.0±34.1 nm), which means that the treatment mechanism of passive ultrasonic agitation to CHX was more effective than the conventional method, this result was reflected by the reduction in the roughness of the surface. Also, the mean diameter of small particles was about 31.63 nm, which is less than the mean diameter of surfaces that were treated with syringe irrigation alone (57.42 nm). Many studies concluded that ultrasonically stimulated irrigation was superior to traditional SI [31].

The extrinsic surface of the isthmus that was treated with 2% CHX activated with Er,Cr:YSGG laser was displayed in 2D & 3D AFM images and contained fewer high peaks and troughs as well as a random or reduced spiky surface. The sq. value was 169.5±8.0 nm, which is significantly less than the values measured after SI and PUI (453.0±34.1 and 323.3±10.7 nm), which means the reduction in *E. faecalis* biofilm on the isthmus surface by laser PIPS was more effective than traditional methods. Also, the mean diameter of small particles was 33.21 nm, which is noticeably less than the mean diameter of surface particles that were treated with the syringe irrigation protocol (57.42 nm).

Both PUI and PIPS were significantly more effective against bacterial biofilm than syringe irrigation. This might be attributed to the acoustic microstreaming generated by these mechanisms [32]. The second factor is cavitation which weakens the cell membrane

and makes cells of bacteria easier to penetrate by irrigants [33]. However, the extremely short pulse durations (60 μs) generate higher peak power than the lengthier pulse durations. This generates strong pressure and shockwaves that propagate three-dimensionally within the root canals that are filled with fluids, eliminating the need to put the tip near the morphologically thinning apical third [34]. Many studies tested the antimicrobial effect of Er,Cr:YSGG laser, and other lasers obtained similar results but at different laser settings and root canal systems. Aydin et al. [35] compared the antimicrobial effects of sodium hypochlorite and 2% CHX irrigation fluids agitated by Er,Cr:YSGG laser (LAI) or an ultrasonic device, and concluded that activating NaOCl and CHX irrigation fluids with Er,Cr:YSGG laser or an ultrasonic technique can be useful in the eradication of *E. faecalis* bacteria from the canals. Also, Sahar-Helft et al. examined the actions of SI with 2% CHX and Er: YAG using LAI on Enterococcus faecalis. They discovered that the total number of microorganisms dramatically decreased after being treated with LAI and CHX [36].

CONCLUSION

According to AFM and SEM results, it is possible to conclude that Er,Cr:YSGG pulsed laser and ultrasonic agitation of CHX provide greater biofilm removal than typical irrigation methods given by irrigation syringes without any activation. Finally, after testing many laser powers, it can be deduced that Er,Cr:YSGG laser in PIPS at (60 μs/pulse, 5 Hz, 0.75 W) was the best method to improve the antimicrobial effectiveness of 2% CHX against 30-day-old *E. faecalis* biofilm in the isthmus region of lower molars.

Author's Contributions

GII: Conceptualization, Methodology, Formal Analysis, Investigation, Resources, Writing – Original Draft Preparation, Writing – Review & Editing, Project Administration. HAJ: Conceptualization, Methodology, Formal Analysis, Resources, Supervision, Writing – Review & Editing.

Conflict of Interest

Declared none.

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Regulatory Statement

This study was conducted in accordance with all the provisions of the local human subjects oversight committee guidelines and policies of the Research and Ethics Committee of Institute of Laser for Postgraduate Studies, University of Baghdad. The approval code for this study is: 10-2022-488.

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