Original Article

Effect of ultrasonic and Er,Cr:YSGG laser-activated irrigation protocol on dual-species root canal biofilm removal: An *in vitro* study

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Abstract

Aim: The aim of the study was to investigate the disinfecting efficacy of a standardized irrigating solution activated by ultrasonics or laser irradiation on mature dual-species biofilms at different root levels *in vitro*.

Materials and Methods: Conventional access cavity preparations were done on 160 single-rooted mandibular premolar teeth with single canals. Freshly extracted oral microbial strains of *Staphylococcus aureus*, *Streptococcus mutans, Enterococcus faecalis,* and *Candida albicans* after biochemical confirmation were used to generate two discrete dual-species microbial inoculums. The sterilized tooth samples were randomly segregated into two groups (n = 80) and inoculated with a mixed inoculum of *S. aureus* + *E. faecalis* strains (Group 1) and *S. mutans* + *C. albicans* strains (Group 2), respectively. Following the 21-day incubation period under aerobic conditions, the infected specimens in each group were divided into four subgroups (n = 20) and subjected to experimental treatment protocols. This included a positive control (no treatment of biofilms), syringe irrigation alone with TruNatomy needle, passive ultrasonically activated irrigation with 20# Irrisafe tip, and laser agitation of irrigant with Er,Cr:YSGG laser using RFT 2 laser tip. Root canals of experimental specimens (except the control samples) are instrumented with TruNatomy rotary file system using 1:1 mixture of 3% NaOCl and 18% etidronic acid as irrigants. The quantitative assessment of reduction in viable biofilm microbes after treatment was done using colony-forming unit counts and confocal laser scanning microscopy image analysis. The obtained data were analyzed statistically with a significant level set at 0.05.

Results: Laser-assisted irrigation has shown a considerably higher mean percentage reduction of microbes compared to ultrasonic agitation and the syringe irrigation showed the least microbial reduction (P = 0.001). No significant difference was noted between the three root regions of ultrasonic and laser groups (P > 0.05), whereas in the syringe groups, apical portions showed higher microbial counts compared to cervical and mid-root regions (P = 0.001).

Conclusion: Erbium laser-assisted irrigation has performed superior to ultrasonic agitation against both the experimental dual-species biofilms, while the syringe irrigation showed the least microbial reduction specifically at apical root portions.

Keywords: Colony counts; confocal microscopy; dual-species biofilm; erbium laser activation; etidronic acid; sodium hypochlorite; ultrasonic activation

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INTRODUCTION

To avert persistent/recurrent root canal infections and for better prognosis of endodontically treated teeth, it is

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critical to adequately disinfect the root canal spaces to a level that encourages complete healing of periradicular tissues. Endodontic biofilms are therapeutically significant as they are regarded as one of the basic survival methods employed by bacteria in times of starvation and are resistant to clinical antimicrobial therapy.[1] Persistent endodontic infections are often predominated by bacterial genera, such as *Streptococcus, Staphylococcus, Prevotella, Lactobacillus, Neisseria, and Enterococcus*. Fungi such as *Candida albicans* also are noted in significantly higher frequencies in teeth with posttreatment disease when equated with primary infections.[2]

Microbial invasion within the dentinal tubules is commonly seen beneath biofilm structures that can be observed in dentin portions as deep as 300 µ.[1] Therefore, the antimicrobial irrigating agents used should have the ability to dissolve and disturb the biofilm matrix at deeper dentinal portions for successful endodontic therapeutic outcomes. However, the complex root canal microstructure limits the disinfecting capacity of these agents when used with traditional syringe irrigation protocol.[3] Thus, the form of irrigation protocol advocated along with mechanical preparation plays a pivotal role in determining the extent of intracanal microbial load reduction.

Several *in vitro* along with few *in vivo* studies had emphasized the use of ultrasonics and laser for activation of the irrigants and have shown considerable enhancement in the antimicrobial efficacy of these agents in comparison to other agitation techniques.[4-6]

Ultrasonic activation enhances the flow and velocity of irrigants that result in the movement of these disinfecting solutions into the complex anatomical areas of the root canal system which would otherwise be inaccessible by conventional syringe irrigation alone.[3] Lasers on the other hand, particularly the erbium group, working at wavelengths closer to the absorption peak of water have shown superior disruption of the biofilm matrix and removed debris from the intrinsic root structures 2.6 times more effectively than needle irrigation alone.[7]

Although better disinfection of root canal spaces was shown through activation of irrigants, none of these protocols tested to date have shown complete eradication of microbes from the intricacies of root canals.

Thus, the main objective of this *in vitro* study was to assess and compare the biofilm removal ability of three different irrigation protocols including syringe irrigation alone, passive ultrasonic irrigation, and Er,Cr:YSGG laser-assisted irrigation in removing mature intracanal dual species biofilms from the root canal spaces of mandibular premolar teeth at different portions, instrumented with TruNatomy rotary file system using a mixture of 3% NaOCl and 18% etidronic acid as irrigants.

MATERIALS AND METHODS

The research protocol was approved by Dr. NTR University of Health Sciences, Andhra Pradesh, and research was conducted following the PRILE 2021 guidelines for *in vitro* studies in endodontology.[8] The study included 160 intact noncarious, extracted single-rooted mandibular premolar teeth of humans with a single root canal and nearly similar buccolingual/mesiodistal dimensions. Based on the statistics obtained from earlier *in vitro* studies conducted by Neelakantan *et al*.[4] and Hoedke *et al*.,[9] the present study's effective sample size was estimated, with type 1 error as 1% and 95% power.

Sample preparation

Conventional access preparations were done on all samples, and the glide path was obtained. The root apices of samples were sealed using Filtek Z250 XT (3M ESPE, USA) nanohybrid composite. Each prepared tooth was then held in the sterile Eppendorf tube and disinfected for 40 min by autoclaving at 121°C and 15lb pressure. Sterility of the root spaces was confirmed through the absence of microbes on root canal surfaces using colony-forming units (CFUs) microbial count and confocal laser scanning microscopy (CLSM) imaging.

Preparation of dual-species broth suspension

The freshly extracted oral strains of experimental microbes including *Staphylococcus aureus, Streptococcus mutans, Enterococcus faecalis,* and *C. albicans* after biochemical confirmation were inoculated individually in 5 ml BHI broth, and these individual cultures were incubated at 37°C for 24 h. Equal volumes of these strains are mixed based on the predetermined experimental groups including *S. aureus* + *E. faecalis* (Group 1) and *S. mutans* + *C. albicans* (Group 2), respectively, and the turbidity of suspension was matched with 0.5 McFarland units using calorimeter (Nano, TA Instruments, USA).

Dual species biofilm generation

The sterilized tooth samples were randomly allocated into two experimental groups (n = 80) and inoculated with a mixed inoculum of *S. aureus* + *E. faecalis* strain and *S. mutans* + *C. albicans* strain, respectively. The root canals of teeth held within the closed Eppendorf tubes were filled with 10 µL of respective microbial suspension and then incubated at 37°C for 21 days under an aerobic environment. Canals of all teeth were replenished with 10 µL fresh BHI broth for every 2 days during the entire incubation period. After completing 21 days of incubation, the teeth samples were taken out of the Eppendorf tubes aseptically and rinsed gently using a sterile saline solution.

Experimental procedure

The infected teeth in the respective groups were equally and randomly divided (n = 20) based on the irrigant

activation protocol. The positive control specimens received no further treatment.

A volume of 5 ml of 1:1 mixture of 3% sodium hypochlorite (Prime Dental Products, Ahmedabad, India) and 18% etidronic acid (Tokyo chemical industry, Japan) was used as an experimental irrigant, and simultaneous instrumentation of all root canal spaces was carried 1 mm short of tooth length using TruNatomy rotary nickel–titanium single file system (Dentsply Sirona, Ballaigues, Switzerland) in a sequential order of 20/0.08, 17/0.02, 20/0.04, 26/0.04, and 36/0.03.

Syringe irrigation was carried out using an experimental irrigant with a 27-gauge TruNatomy irrigating needle (Dentsply Sirona, Switzerland) held 1 mm short of the working length with slight up and down movements during the irrigation process.

Ultrasonic activation of irrigant was done using the ultrasonic device (Satelec P5 Newtron, France) and an Irrisafe stainless steel ultrasound tip (Satelec/Acteon, Merignac, France) of size 20/0.2 at the power setting 3 with the tip held 2 mm short to the working length. Intermittent ultrasonic irrigant agitation was performed for 20 s after each chemomechanical instrumentation, and this process was repeated 3 times.

Laser activation of irrigant was performed using Er,Cr:YSGG laser (Waterlase iPlus, Biolase, California), with 2780 nm wavelength, 25 mJ pulse energy, 140-μs pulse duration, and 20 Hz repetition rate. The RFT 2 laser tip (Biolase, Inc. California, USA) was positioned within the irrigant-filled canals 3–4 mm apically from the canal orifice, and three cycles of laser-assisted irrigant activation was carried for a time interval of 20 s per each cycle.

Following chemomechanical disinfection, the samples were sectioned longitudinally into two equal halves using a microtome (IsoMet 1000). One-half section of the sample was used for the quantitative assessment of the viable biofilm microbes by determining the number of CFUs per milliliter and the other sectional half was observed under CLSM for microbial biofilms on dentinal surfaces including dentinal tubules.

Dentin powder analysis for the assessment of the viable biofilm microbes-colony-forming units

Dentin debris from apical, middle, and coronal portions of the root half was harvested up to 400 μ depth using no. 5 Gates Glidden drills (Mani Inc., Kiyohara, Japan) and collected in 1 ml of sterile BHI broth and then incubated at 37°C in an aerobic environment for 24 h. Each microcentrifuge tube content was serially diluted for three times using 100 μL of broth in 100 μL of normal saline. Ten microliters of this diluted solution collected from Group 1 specimens' root dentin was plated onto blood agar plates and the Group 2 specimens' root dentin on BHI agar plates, respectively. These plates were finally incubated for 24 h. The microbial count was obtained by the calculation of the CFUs per milliliter for each sample using the standard loop method.

The percentage reduction in the number of CFU/ml was calculated using the formula:[10]

$$\% \text{ reduction} = \frac{\text{Intial CFU count - Final CFU count}}{\text{Intial CFU count}} \times 100$$

Where the initial CFU count was taken from positive control samples.

Confocal laser scanning microscopic examination

The other half of root specimens were stained with Fluorophores SYTO 9 and propidium iodide stain (Live/Dead, Baclight, Invitrogen, Eugene, OR, USA) and then observed under CLSM (Leica DMi8, Leica Microsystems GmbH, Germany) at apical, middle, and coronal portions of roots. Simultaneous dual-channel imaging was used to display the green and red fluorescence indicating live cells and dead cells, respectively. Three-dimensional (3D) reconstruction was done using the ImageJ software. The quantification of the confocal microbial volumes was done using the BioimageL software, and the percentage of dead bacteria at each root portion per sample was calculated.

Statistical analysis was applied to the obtained data deploying software (SPSS Statistics for Windows, Version 22.0, IBM, Armonk, NY, USA). Intergroup comparison of percentage reduction in the number of CFUs/ml and percentage of dead bacteria, respectively, was calculated using an independent *t*-test, and intragroup comparisons were formulated using one-way analysis of variance. Pair-wise comparisons were done using Tukey's multiple *post hoc* procedures. Entire data were analyzed with 95% confidence interval where $P < 0.05$ was contemplated significant.

RESULTS

Among the two types of dual-species biofilm groups (*S. aureus* + *E. faecalis* and *S. mutans* + *C. albicans*), the three irrigant agitation protocols tested have produced no statistically considerable difference in biofilm removal efficacy ($P > 0.05$). None of the irrigation protocols tested were able to eliminate microbial load completely. However, laser-assisted irrigation has shown statistically higher mean reduction of microbes with CFU count and dead bacterial percentage by CLSM compared with ultrasonic and syringe protocols ($P = 0.0001$) [Table 1]. The lowest microbial

reduction was noted in the syringe irrigation group, and no significant effect against microbes in biofilm was noted in the control group.

Among the root regions, considerably higher mean scores of microbial reductions were observed in cervical and middle root regions, respectively, as compared to apical regions among all the disinfecting protocols tested. Moreover, the higher percentage of dead microbes was found in apical portions of both ultrasonic and laser-assisted irrigation protocols compared to the syringe ($P = 0.0001$) [Table 2], with the laser performing superior to ultrasonic irrigant agitation in all root portions [Figures 1 and 2].

DISCUSSION

The main role of irrigation while performing root canal therapy is to ensure superior cleaning and disinfection of intricate root spaces that remained untouched through mechanical instrumentation alone. Although the sequence of irrigation during this treatment is not clearly standardized, the commonly exercised irrigation sequence involves the use of NaOCl (1%–6%) and 17% ethylenediaminetetraacetic acid (EDTA). However, 17% EDTA has a higher risk of eroding peritubular dentin when used for a longer duration. In addition, the antibacterial effectiveness and tissue dissolving capacity of sodium hypochlorite are reduced by the chemical interaction with EDTA when used as a single mixture.[11] The concept of continuous chelation was developed to simplify endodontic irrigation regimes and simultaneously address the above issues. This considers the use of a noninteracting chelator and NaOCl together in one single solution.[12]

Current literature supports the use of 9%–18% Etidronic acid as a potential alternative to EDTA, which can be combined with NaOCl without interfering with the tissue dissolving or antimicrobial properties of hypochlorite. Based on the research done by Wright *et al*., the antibacterial and tissue-dissolving characteristics of 3% NaOCl are found to be uninterrupted by adding 18% etidronic acid as a chelating agent. This biocompatible agent was able to remove the hard-tissue debris and smear layer more effectively compared to 17% EDTA with no signs of root dentin erosions.[13] The superior smear layer removing the ability of 18% etidronic acid activated with ultrasonics was also highlighted in the study by Awati *et al*. that was based on the confocal imaging technique.[14] Similar findings were

Table 1: Comparison of microbial reduction (mean and standard deviation) between 2 microbial groups and intragroup comparison with different irrigation protocols by independent *t*-test

Disinfection/ irrigation protocols	CFU data				CLSM data			
	Group 1 (*S. aureus* + *E. faecalis*)		Group 2 (*S. mutans* + *C. albicans*)		Group 1 (*S. aureus* + *E. faecalis*)		Group 2 (*S. mutans* + *C. albicans*)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Syringe	79.80	5.98	81.16	5.97	71.48	2.19	72.21	1.98
Ultrasonic	91.05	4.04	91.76	3.78	89.56	1.71	90.62	1.57
Laser	99.92	0.05	99.93	0.05	93.98	1.13	94.53	1.16
F	117.3859		106.5414		16080.5934		19067.9818	
P	0.0001*		0.0001*		0.0001*		0.0001*	

*$P<0.05$ indicate significant difference. SD: Standard deviation, *S. aureus: Staphylococcus aureus, E. faecalis: Enterococcus faecalis, C. albicans: Candida albicans, S. mutans: Streptococcus mutans*, CFU: Colony forming units, CLSM: Confocal laser scanning microscopy

Table 2: Intra-group comparison of microbial reduction (mean and standard deviation) in different root regions by one-way ANOVA

Regions	CFU data											
	Group 1 (*S. aureus* + *E. faecalis*)						Group 2 (*S. mutans* + *C. albicans*)					
	Syringe		Ultrasonic		Laser		Syringe		Ultrasonic		Laser	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cervical	81.75	4.44	92.90	6.12	99.94	0.05	83.44	4.40	93.23	6.07	99.94	0.06
Middle	82.52	5.30	91.53	8.57	99.91	0.07	83.61	5.35	92.21	8.26	99.91	0.07
Apical	75.12	9.10	88.71	4.10	99.93	0.07	76.42	9.15	89.85	3.92	99.93	0.08
F	7.6042		2.1411		1.1430		7.6732		1.4961		0.7956	
P	0.0012*		0.1269		0.3261		0.0011*		0.2327		0.4563	
	CLSM data											
Cervical	73.23	3.50	91.48	1.83	94.72	1.07	74.33	2.44	92.44	1.85	95.48	1.25
Middle	73.72	2.36	91.48	1.83	94.72	1.14	73.67	2.86	92.39	1.74	95.64	1.14
Apical	67.50	2.64	85.73	2.00	92.49	1.81	68.62	2.47	87.04	1.76	92.49	2.13
F	28.9774		61.9592		17.3792		28.9114		60.6611		25.4943	
P	0.0001*		0.0001*		0.0001*		0.0001*		0.0001*		0.0001*	

*$P<0.05$ indicate significant difference. SD: Standard deviation, *S. aureus: Staphylococcus aureus, E. faecalis: Enterococcus faecalis, C. albicans: Candida albicans, S. mutans: Streptococcus mutans*, CFU: Colony-forming units, CLSM: Confocal laser scanning microscopy

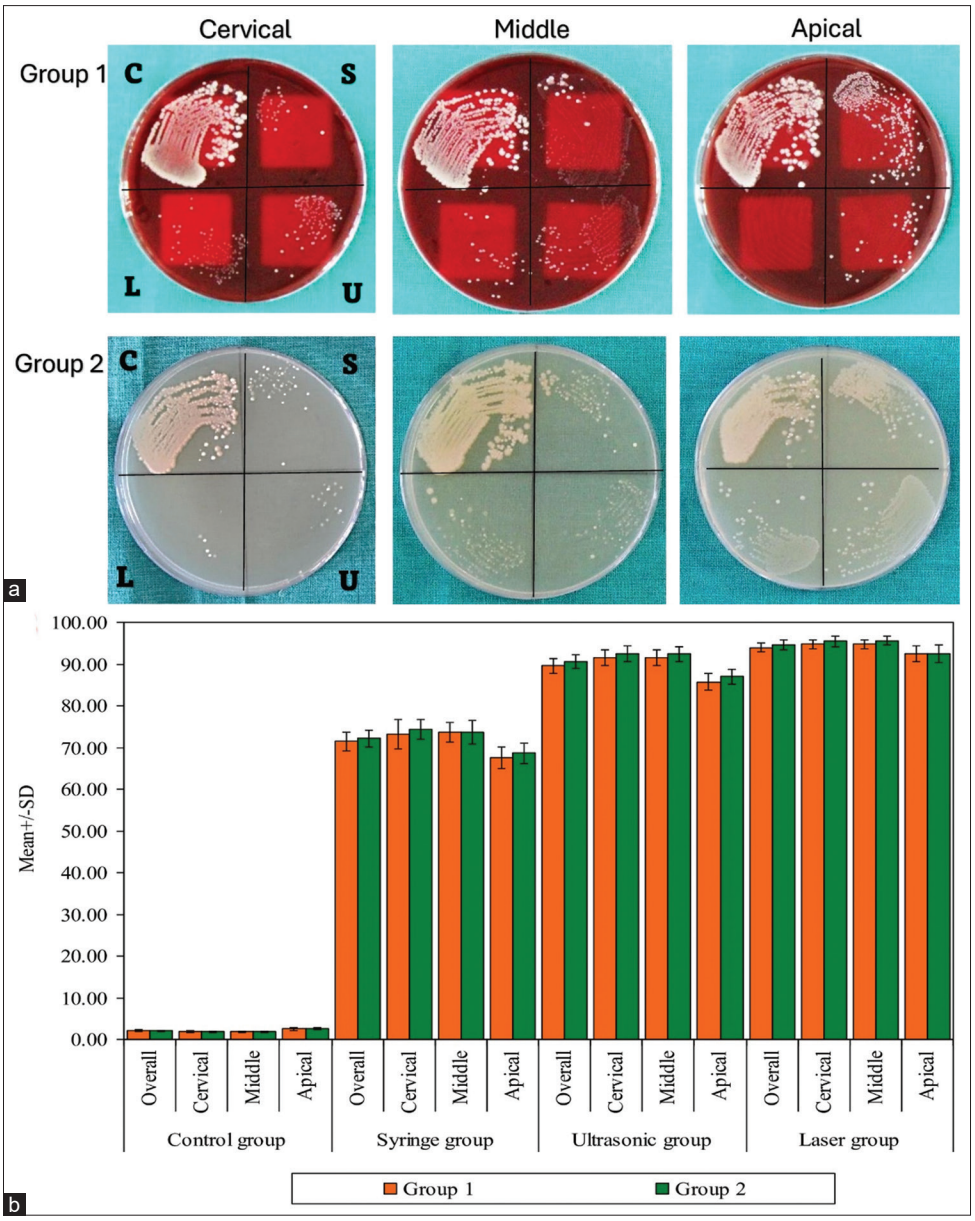

Figure 1: (a) Representative images of Group 1 dual species colonies (*Staphylococcus aureus* + *Enterococcus faecalis*) formed on blood agar medium, and Group 2 dual species colonies (*Streptococcus mutans* + *Candida albicans*) formed on BHI agar medium at different root portions of a randomly selected sample. C. *Control sample*, S. *Syringe sample*, U. *Ultrasonic activation sample*, L. *Laser irradiation sample*. (b) Bar diagrammatic representation of the mean percentage of dead microbes at different root regions with three different irrigation protocols

noted in a study by Neelakantan *et al.* where the significant microbial reduction was noted with the combined use of 6% NaOCl + 18% etidronic acid mixture as irrigant.[4] A freshly prepared 1:1 mixture of 3% NaOCl and 18% etidronic acid was thus used as the sole irrigant in this study.

Mechanical instrumentation carried with TruNatomy rotary nickel–titanium single files is known for its smaller flute diameter (0.8 mm) with a high degree of flexibility due to postgrind heat treatments while manufacturing. As reported in a study by Waleed *et al.,* TruNatomy files displayed superior smear layer removal and cleaning capacity than other tested file systems including Protaper Next, Wave One Gold, and S-One Plus.[15]

Syringe irrigation that is traditionally carried out using 27/30-gauge needle with various vent designs has shown a limited flow of irrigants in intricate root canal spaces.[3] The 27-gauge, double-vented TruNatomy irrigation needle made of flexible polypropylene used in this study could not be demonstrated to be very effective.

Laser-assisted or ultrasonic irrigant agitation techniques proved to optimize irrigation through earlier research on

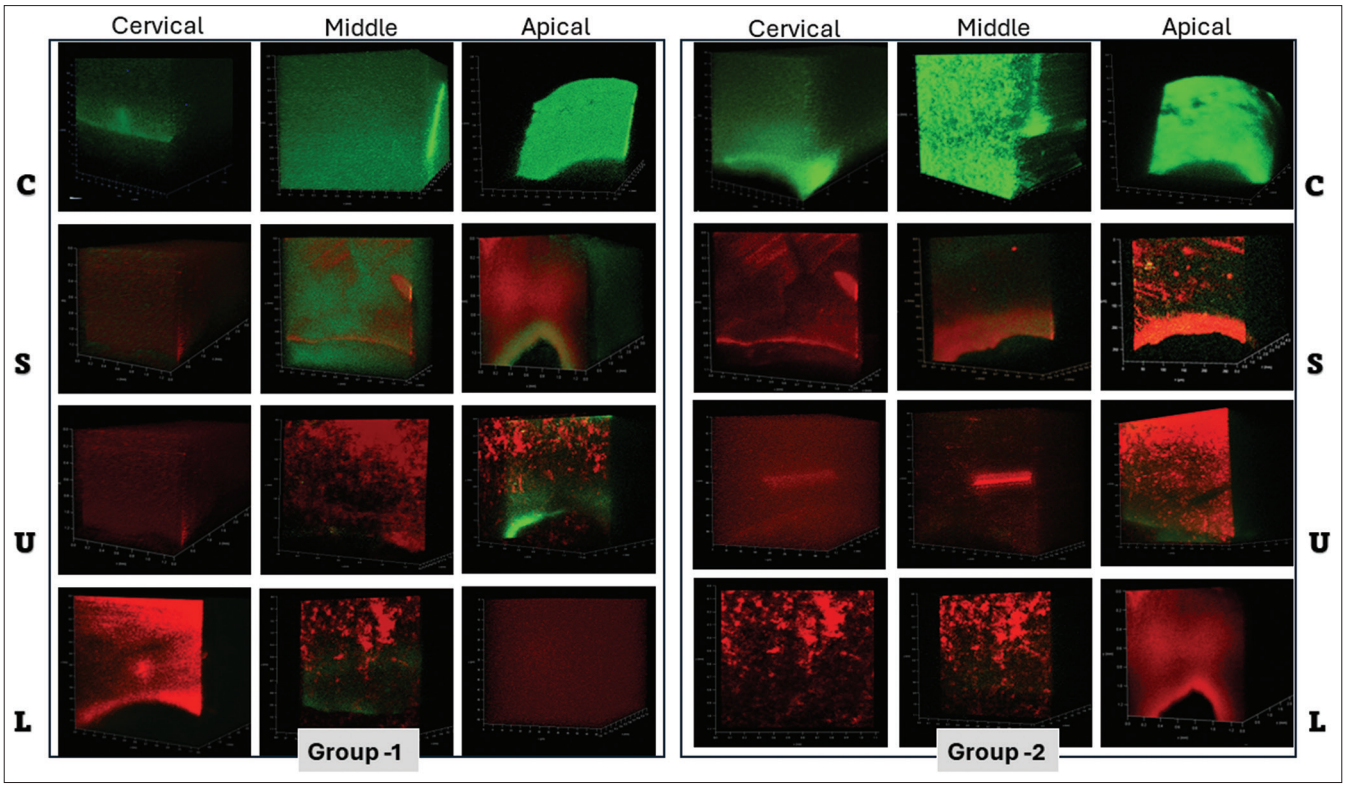

Figure 2: Representative three-dimensional confocal laser scanning microscope images of dentinal tubules showing live (green) and dead (red) bacteria at different root portions of group 1 and group 2 samples C. *Control sample*, S. *Syringe sample*, U. *Ultrasonic activation sample*, L. *Laser irradiation sample*

dentinal debris removal capabilities.[16] There is a lack of evidence comparing these methods of cleaning efficacy of biofilm-infected dentin.

Numerous studies showed that mature multispecies biofilms exhibit higher biofilm mass and enhanced resistance to antimicrobial treatment compared to monospecies counterparts. For instance, a study by Stojicic *et al.* found that 1–2-week-old monospecies biofilms were more susceptible to NaOCl treatment than 3 weeks old dual-species biofilms.[17] To mimic the biological burden of infected root canals, 21-day-old biofilm models of two different dual species were considered in this study. Based on the predominance of microflora in persistent endodontic infections,[2] the microbial strains considered in the present study were *E. faecalis, S. aureus, S. mutans,* and *C. albicans,* as they can be cultured with ease in an aerobic environment.

CLSM used in the study is a nondestructive microscopic technique that can provide a reconstructed 3D image of the complete biofilm. When combined with viability staining, CLSM images reflect the "true viability status" of microbes in the biofilm during starvation than a culture-based method. In this study, CFUs count was also done along with CLSM for comprehensive quantification of microbes as done by Kishen *et al.*[18]

According to the observations from CFUs data, both ultrasonic and laser-assisted irrigation can induce superior disinfection of the apical root portions compared to syringe irrigation alone. Based on the reduction in CFU microbial counts, Nair *et al.* and Kasić *et al.* concluded that Er,Cr:YSGG laser-assisted irrigation offered better biofilm elimination in the apical root portions compared to syringe and ultrasonic irrigant agitation techniques.[19,20]

The data of confocal image analysis in this study highlighted the superior disinfecting ability of tested irrigant agitation techniques compared to syringe, with the laser performing superior to ultrasonic irrigant agitation in all root portions. These findings are in accordance with Al Shahrani *et al.* study that stated that regardless of the irrigant solution utilized, erbium laser-assisted irrigation has higher biofilm removal effectiveness than syringe irrigation.[21]

Correspondingly by assessing treatment results using CFU counts and CLSM image analysis, Neelakantan *et al*.'s study stated that laser activation of 18% etidronic acid and 6% NaOCl mixed irrigating solution provided superior elimination of *E. faecalis* biofilm from root canals when compared to conventional syringe and ultrasonic techniques using similar irrigants.[4] Based on the same assessment methods, Choi *et al.* asserted that ultrasonic

activation of irrigants with Irrisafe files presented better removal of multispecies intracanal biofilms (*E. faecalis, Campylobacter rectus,* and *S. mitis*) from apical portions of teeth with curved canals in comparison to syringe irrigation using 1% NaOCl as irrigant.[22]

Based on a systematic review conducted by Josic *et al*. none of the activation methods including the sonic, ultrasonic, and Er:YAG laser-assisted irrigation protocols tested rendered the root spaces completely free of microbes, but laser irradiation performed superior to other activation protocols including syringe irrigation alone.[7]

The pulse energy of the erbium group of lasers is strongly absorbed by water and NaOCl, resulting in vaporization and creation of vapor bubbles inducing a secondary cavitation phenomenon.[23] The bactericidal capability of Er, Cr:YSGG laser used in this study is credited to the cellular water evaporation, which further expands rapidly through the laser pulse and causes disruption of the biofilm. This is further enhanced by improving the bactericidal effect of NaOCl irrigant ensuing the reduction in intracanal bacterial load.[24] Because of these merits, the highest reduction in the viable bacteria was noticed with laser-assisted irrigation in this study.

On the other hand, ultrasonic activation induces a rapid change in irrigant pressure within the root space giving rise to a transient acoustic cavitation effect and emitted shockwaves generate higher shear stress against root walls causing greater disruption of biofilms.[3] Furthermore, during activation, a portion of kinetic energy is converted to heat, which could also speed up the irrigant's chemical activity and maximize disinfection.[25]

Although this research was not conducted in a complex root canal anatomy which is regarded as a limitation of this laboratory study, the evaluated methods allow comparison of irrigant agitation protocols using standardized infected dentin.

CONCLUSION

Within the limitations of this *in vitro* study, the conclusions drawn are:

- Among the irrigation protocols tested, the microbial eradication ability of laser-assisted irrigation was superior against both *S. aureus* + *E. faecalis* and *S. mutans* + *C. albicans* dual-species biofilms
- The syringe irrigation showed significantly lower biofilm removal efficacy, especially in the apical root regions.

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Conflicts of interest
There are no conflicts of interest.

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