EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 69, 1659:1667, APRIL, 2023
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
CONSERVATIVE DENTISTRY AND ENDODONTICS
Submit Date : 15-02-2023 • Accept Date : 11-03-2023 • Available online: 10-04-2023 • DOI : 10.21608/edj.2023.193765.2441

EX VIVO STUDY OF THE EFFECT OF DIFFERENT ANTIMICROBIAL SOLUTIONS ON APICAL EXTRA-RADICULAR BIOFILM: A CLSM STUDY

Nelly Abdelsalam* *and* Mai Hamdy**

ABSTRACT

Introduction: The aim of this study was to investigate the anti-biofilm effect of silver citrate nanoparticles, Doxycycline, and a mixture of sodium hypochlorite-Etidronic acid on extra-radicular biofilm on the most apical part of the root of extracted teeth with persistent apical periodontitis.

Methods: The apical 2mm of the distal root of thirty freshly extracted mandibular teeth with persistent apical periodontitis was resected, the presence of mature extraradicular biofilm was confirmed with CLSM then samples were treated for 5 minutes with an antimicrobial solution according to their random distribution into three groups. Group I: Silver citrate nanoparticles, Group II: Doxycycline**,** and Group III: Mixture of 1% NaOCl with Etidronic acid (HEBP), then samples were scanned with CLSM to detect live/dead bacteria in the bacterial biofilm.

Results: There was no statistically significant difference between mean percentages of dead bacteria in the three groups (*P*-value = 0.597), experimental root end sections showed statistically significantly lower mean percentage of live bacteria than control root end sections (*P*-value <0.001).

Conclusions: The applied antimicrobial solutions in the diluted concentration and limited time of application failed to eradicate the mature extraradicular biofilm, therefore further investigations are needed to conclude a biocompatible chemical or mechanical method to be used during periradicular surgery for elimination of extra-radicular biofilm.

Clinical implications: Extra-radicular biofilm was present on the majority of the collected samples with persistent apical periodontitis; therefore, disinfection of the root end surface is a must when resection of the whole infected apical part of the root is not feasible.

* Assistant Professor of Endodontics, Faculty of Dentistry, Suez Canal University, Ismailia, Egypt
** Assistant Professor of Endodontics, Faculty of Dentistry, King Salman International University, El Tur, South Sinai, Suez Canal University, Ismailia, Egypt

INTRODUCTION

Persistent apical periodontitis is a common disease following root canal treatment, the main cause of this disease is microorganisms that are either hidden in the complex anatomy inside the root canal system or those present on the external surface of the apical part of the root [1]. These microorganisms occur in biofilms rather than in a planktonic form. Bacterial biofilms represent a barrier against complete eradication from the root canal system. While those residing on the external surface of the root

The drastic treatment option for persistent apical periodontitis related to the presence of extra-radicular biofilm is peri-radicular surgery. Conditioning of the resected root is an important step during the surgery because it eliminates remnants of bacteria and their toxins left after resection, enhances adhesion of periodontal tissue and cementum over the resected surface, and promotes the regeneration or repair, as demineralization of the dentin surface releases the sequestered growth factors. The commonly used conditioning agents in peri-radicular surgery are citric acid, Ethylenediaminetetraacetic acid (EDTA) and tetracycline [2].

Doxycycline is a semisynthetic derivative from the tetracycline group with a broad antimicrobial spectrum against most Gram-positive and Gram-negative bacteria as well as other unicellular micro-organisms [3]. It inhibits matrix metalloproteinase enzyme, enhances periodontal healing in replanted avulsed teeth and periodontal therapies [4]. It has a low acidic pH (2.2); therefore, it chelates with calcium ions with mild demineralization effect which enhances connective tissue healing [5]

Etidronic acid (1-hydroxyethane 1,1-diphosphonic acid (HEDP) is a weak chelator that that has the advantage of preserving the antimicrobial and proteolytic action of sodium hypochlorite, thus it was recommended to use this mixture for root canal irrigation for disinfection of the root canal system and smear layer removal throughout the preparation procedure instead of a final rinse [6,7].

Silver citrate nanoparticles is recently used as an endodontic irrigant for the effective antibacterial properties of silver nanoparticles combined with the chelating action of citrate rendering this combination an anti-biofilm and smear layer removal irrigant [8,9].

The aim of this study was to investigate using confocal scanning electron microscope (CLSM) the anti-biofilm effect of silver citrate nanoparticles, Doxycycline, and a mixture of sodium hypochlorite-Etidronic acid on extra-radicular biofilm on the most apical part of extracted teeth with persistent apical periodontitis.

MATERIALS AND METHODS

Preparation of samples:

All procedures performed were carried out in accordance with relevant guidelines, regulations, and ethical standards of the ethical committee of research of the Faculty of Dentistry (approval number for the study: 562/2022). Thirty-three mandibular molar teeth with persistent apical periodontitis suggested for extraction due to restorative reason were collected from the outpatient clinic were chosen for the current study.

The apical 2mm of the distal root of the freshly extracted teeth was resected, then each apex was cut into two equal halves with a precision sectioning saw (Isomet® 1000; Buehler Ltd, Lake Bluff, IL, USA), selecting the best complete cut half. The samples were scanned under Confocal Laser Microscopy (CLSM) for biofilm detection and confirmation of the presence or absence of multispecies bacterial biofilms. Three samples did not show the presence of biofilm were replaced by other samples, summing up the total number of samples to thirty apices.

These scanned samples represented the preoperative control group. A confocal (Carl Zeiss LSM 510 Meta; Carl Zeiss Mikroskopie, Jena, Germany), was used for the CLSM analytical microscopy imaging process (488 nm). Syto9 and PI were caught by two detection channels. Syto9 and PI were caught by two detection channels. Emission wavelengths of 505–550 nm (green, Syto9) and 650–750 nm (red, PI) were collected to visualize Syto 9 and PI, respectively.

Preparation of dentin conditioning agent:

The root apices were randomly distributed into three groups and each apex was immersed for 5 minutes in the root conditioning agent according to each group.

Group I: Silver citrate nanoparticles.

In order to create AgNps, a solution of 17 mg of AgNO3 (Sigma-Aldrich, MO, US) was mixed with 100 mL of boiling distilled deionized water (18 mS). After that, 10 mL of trisodium citrate (Sigma-Aldrich, MO, US) was infused into water that was 1 percent m/v deionized. The solution was kept warm at 37°C with constant stirring until, after 5 minutes, its color changed from a yellowish tone to a final color of green. This procedure allowed the AgNps-CM dispersion to develop, with a final concentration of100 µg/ml (10). By using a particle size analyzer Dynamic Light Scattering (DLS) (Zetasizer Nano ZN, Malvern Panalytical Ltd, United Kingdom) at a fixed angle of 173° at 25° C, the prepared particles were examined for their particle size and size distribution in terms of the average volume diameters and polydispersity index. Each sample was examined three times. Particle size of the silver citrate nanoparticles was 32.93 0.24 nm, and their Zeta potential was -39.8 0.8 mV.

Group II: Doxycycline

In order to create a doxycycline HCl solution with a concentration of 10% (500mg/5ml), 5 capsules of vibramycin 100mg from Pfizer Egypt were opened, their contents emptied, and 5ml of distilled water were added and stirred well (11).

Group III: Mixture of 1% NaOCl with Etidronic acid (HEBP)

For the 1% NaOCl /9% HEBP combination, both irrigants were prepared at double concentration and mixed at a 1:1 ratio (12).

The samples were taken out of the antimicrobial solutions for the analysis of antimicrobial activity using CLSM, washed for 1 minute in deionized water to remove any antimicrobial solution residues, stained with florocine diacetate FDA and propidium iodide PI, covered with a cover slide, and examined under a confocal microscope to detect live/dead bacteria in the bacterial biofilm. The data were then gathered, tabulated, and statistical

Statistical Analysis

By examining the distribution of the data and utilizing tests for normalcy, quantitative data were examined for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests). All data showed normal by examining the distribution of the data and utilizing tests for normalcy, quantitative data were examined for normality. ONE way ANOVA test was used to compare between percentages of dead bacterial in the three groups. Repeated measures ANOVA test was used to compare between percentages of live bacterial in experimental and control root sections within each group when the ANOVA test is significant, the Bonferroni post-hoc test was applied for pair-wise comparisons. The significance level was set at $P \leq 0.05$. Statistical IBM SPSS Statistics for Windows, Version 23.0, was used for the analysis. IBM Corp., Armonk, New York.

RESULTS

Percentage of dead bacteria

There was no statistically significant difference between mean percentages of dead bacteria in the three groups (*P*-value = 0.597, Effect size = 0.038). (Table 1, Figure 1)

TABLE (1): Descriptive statistics and results of one-way ANOVA test for comparison between percentages of dead bacteria in the three groups

Group I (n = 10)		Group II (n = 10)		Group III (n = 10)		P-value	Effect size (Eta Squared)
Mean	SD	Mean	SD	Mean	SD		
27.7	8.1	31.6	9.9	28.9	7.9	0.597	0.038

**: Significant at P ≤ 0.05*

TABLE (2): Descriptive statistics and results of repeated measures ANOVA test for comparison between percentages of live bacteria in experimental and control root sections

Group	Experimental (n = 10)		Control (n = 10)		*P*-value	*Effect size (Partial Eta Squared)*
	Mean	SD	Mean	SD		
Group I	72.3	8.1	91.1	7.1	<0.001*	0.544
Group II	68.4	9.9	92.4	4.6	<0.001*	0.66
Group III	71.1	7.9	93.6	3.2	<0.001*	0.631

**: Significant at P ≤ 0.05*

Percentage of live bacteria in experimental and control root sections

In all groups, experimental root end sections showed statistically significantly lower mean percentage of live bacteria than control root section (*P*-value <0.001, Effect size = 0.544), (*P*-value <0.001, Effect size = 0.66) and (*P*-value <0.001, Effect size = 0.631), respectively. (Table 2, Figure 2, 3, 4, and 5).

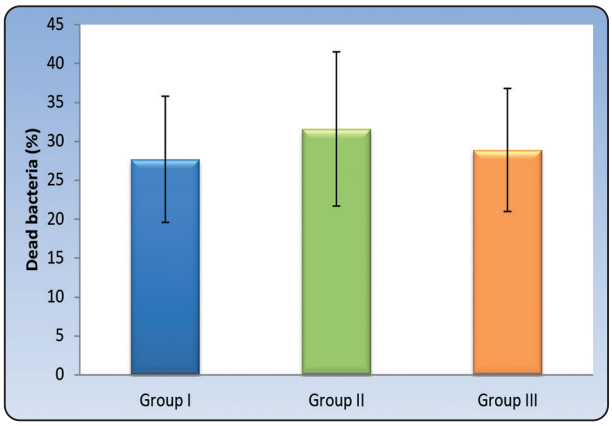

Fig. (1). Bar chart representing mean and standard deviation vlaues for percentages of dead bacteria in the three groups

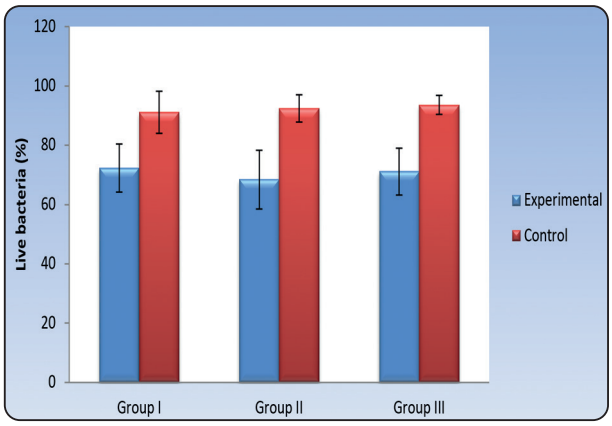

Fig. (2). Bar chart representing mean and standard deviation vlaues for percentages of live bacteria in in experimental and control root sections

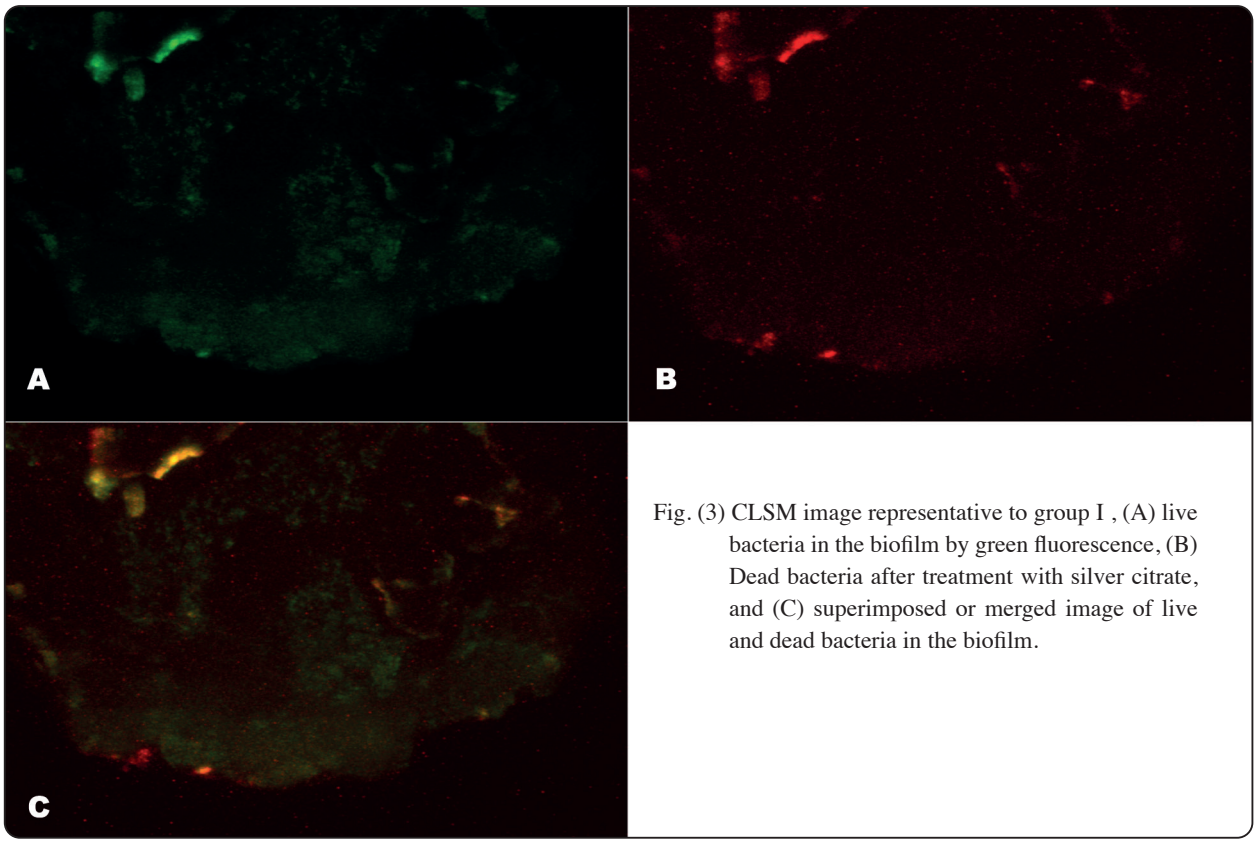

Fig. (3) CLSM image representative to group I , (A) live bacteria in the biofilm by green fluorescence, (B) Dead bacteria after treatment with silver citrate, and (C) superimposed or merged image of live and dead bacteria in the biofilm.

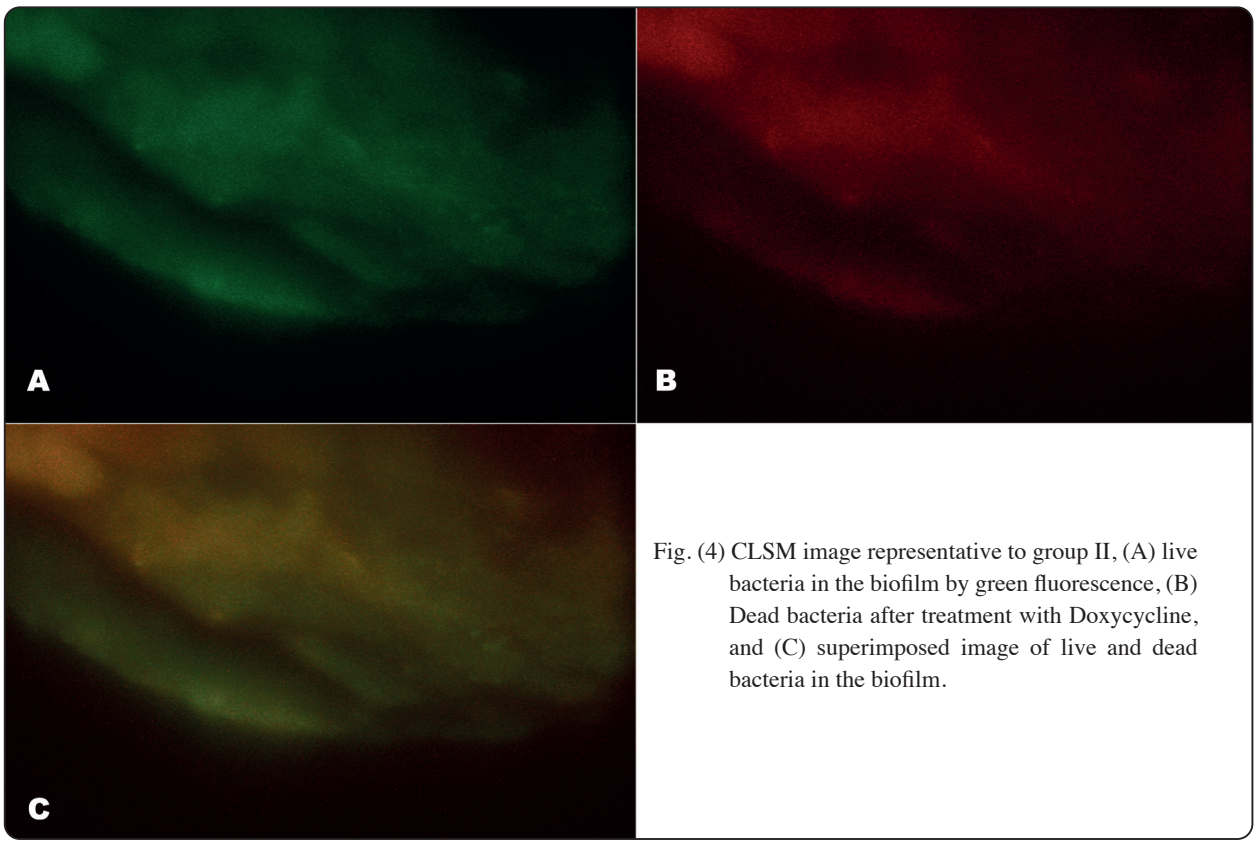

Fig. (4) CLSM image representative to group II, (A) live bacteria in the biofilm by green fluorescence, (B) Dead bacteria after treatment with Doxycycline, and (C) superimposed image of live and dead bacteria in the biofilm.

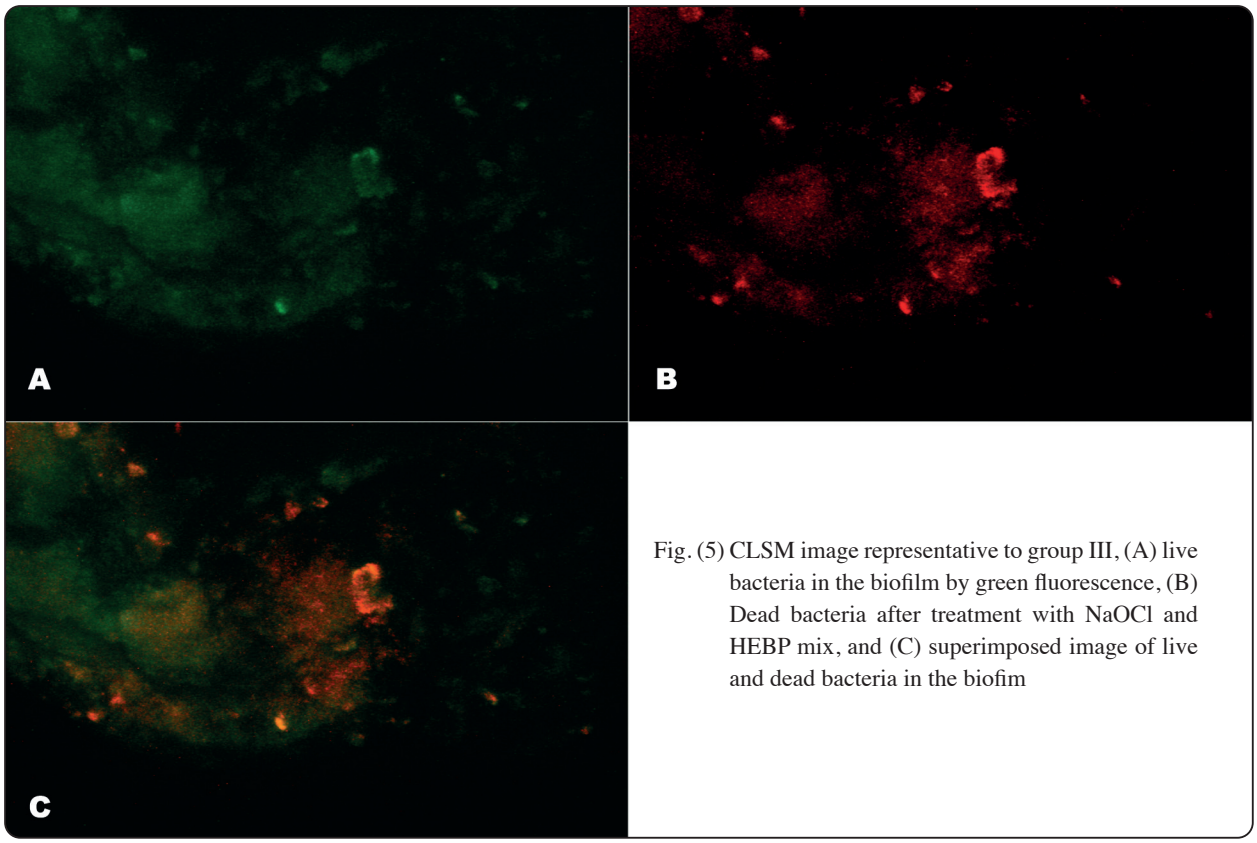

Fig. (5) CLSM image representative to group III, (A) live bacteria in the biofilm by green fluorescence, (B) Dead bacteria after treatment with NaOCl and HEBP mix, and (C) superimposed image of live and dead bacteria in the biofim

DISCUSSION

Microbial biofilm related to pulpal and periapical diseases are classified as intracanal, extra-radicular and periapical biofilm. Extra-radicular biofilm is a multispecies bacterial community adherent to the apex of the root and protected by an extracellular polymeric substance [13].

Extra-radicular biofilm was mainly identified in roots associated with asymptomatic apical periodontitis, chronic apical abscess, post-treatment apical periodontitis and persistent or refractory cases [14–16]. The last resort for those refractory cases is peri-radicular surgery. The aim of apical root resection in peri-radicular surgery is the removal of infected diseased tissue with its harbored bacteria. However, there are limitations for resection of the whole infected part of the root such as: compromised crown-root ratio, excessive apical root resorption, presence of long post and revision surgery [17]. Thus, disinfection of the external root surface in those cases with limited resection is crucial not only for resolution of the persistent apical periodontitis but also for healing, regeneration, and attachment of the resorbed dental tissue (cementum and periodontal ligament). This step is usually called Root conditioning as it involves the use of chelating agents to demineralize dentin and remove the created smear layer after resection to provide a surface conductive to tissue growth and adhesion [18], however the use of chelating conditioning agents is not enough to eliminate remnants micro-organisms on the root end surface. Therefore, the present study evaluated the effect of antimicrobial chelating solutions on extra-radicular biofilm. CLSM was used to evaluate the efficacy of the antimicrobial solutions because it accurately measures the bacterial biofilm viability quantitatively (19).

To mimic the clinical situation where various bacteria, interact in the intra-radicular or extra-radicular biofilm. The biofilm model used in this study was not created in the lab but rather existed pathologically on the apex of the collected teeth, which we have confirmed by the CLSM, so it is considered a mature pathologic biofilm that occurs on the external surface o teeth in relation to persistent apical periodontitis.

Samples treated with Doxycycline revealed the highest non-significant percentage of dead bacteria (31.9%) followed by sodium hypochlorite-Etidronic acid mixture (28.9%) followed by silver citrate nanoparticles (27.8%).

Doxycycline is effective antibacterial agent against anerobic bacteria which predominates in persistent endodontic infection, furthermore it has been demonstrated that doxycycline inhibits Gram-negative biofilm through interference with the quorum sensing ability of the biofilm and inhibition of the secretion of the extracellular polysaccharide matrix [20]. This derivative of tetracycline can act as a chelating agent to remove smear layer and debris either inside root canal or on the root surface [21].

The effect of 1% sodium hypochlorite-Etidronic acid mixture on the biofilm was comparable to other studies that investigated only sodium hypochlorite with different concentration 2% & 6% on a mature biofilm 25% & 54% correspondingly [1]. Obviously, the higher the concentration the higher the percentage of dead bacteria but we could not use higher concentration in this study, as the biofilm is extra-radicular; in a clinical situation this would result in tissue and bone necrosis surrounding the root apex due to the toxicity and tissue dissolution ability of the higher concentration sodium hypochlorite. On the contrary, Cha´vez de Paz et al 2010 [22], recorded higher percentage of dead bacteria in a biofilm (78%-89%) for 1% sodium hypochlorite, however the 24h incubation period is not enough to form a mature biofilm in their study; therefore, they revealed contradictory results as 1% NaOCl was able to penetrate and eliminates higher percentage of bacteria of this young biofilm which is easier to eliminate than a mature biofilm.

Nano silver citrate showed the least non-significant percentage of dead bacteria, this could be attributed to the inability of silver nanoparticles to penetrate the biofilm and the time of application as longer time of application is needed to disrupt the structural integrity of the biofilm [23,24], although many studies proved the effective antibiofilm effect of silver nanoparticle-based irrigant [25,26]. Moreover, the combination between silver nanoparticles and chelating agent should have enhanced the stability and the antimicrobial effect of AgNps [27].

None of the experimental antimicrobial solution could eradicate the extra-radicular biofilm in this study, this could be attributed to the absence of agitation or mechanical method, which was obviously important to detach this mature biofilm (28), another important factor is the concentration of the solutions as increasing the concentration is directly related to the antimicrobial efficacy of the used solutions. However, increasing the concentration will increase the toxicity of the antimicrobial solutions [29], which in clinical situation will be applied on the external apical part of the root during periradicular surgery in close proximity to periradicular tissue. Furthermore, the time of contact is another influential factor on eradication of biofilm, more than five minutes were needed to enhance the antibiofilm activity of the solutions. However, this extra-radicular biofilm is located on the external surface of the apex of the root in close contact with the peri-radicular tissue of refractory cases, and it should be removed during peri-radicular surgery and the remnants of this biofilm should be disinfected with those experimental solution considered as a conditioning agents for the resected root in surgery, however the time of application is critical for wound closure that's why we limited the

contact time for five minutes although longer time would have revealed better results.

Based on the results of this study, it can be concluded that, antimicrobial solutions in the diluted concentration and limited time of application failed to eradicate the mature extraradicular biofilm, therefore further investigations are needed to conclude a biocompatible chemical or mechanical method to be used during periradicular surgery for elimination of extra-radicular biofilm when resection of the whole infected apical part of the root is not feasible.

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