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Antimicrobial Efficacy of Chlorhexidine-Loaded Silver Nanoparticles as an Endodontic Irrigation on Enterococcus Faecalis Biofilm: An in-vitro study

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ABSTRACT

Objectives: to evaluate the antimicrobial efficacy of chlorhexidine-loaded silver nanoparticles (CHX-loaded AgNPs), silver nanoparticles (AgNPs), and 2% chlorhexidine (CHX) solutions on Enterococcus faecalis biofilm.

Methods: Forty-five root specimens were chemo-mechanically prepared using rotary files, cleaned in ultrasonic bath and autoclaved. The specimens were then inoculated with a pure strain of *E faecalis* to develop a 6 weeks old biofilm. A fresh broth adjusted to No. 1 MacFarland turbidity standard was replenished every 72 hours. After 6 weeks, 5 specimens were examined with scanning electron microscope (SEM) to confirm biofilm formation. The rest were divided into 4 groups (n=10), group I: CHX-loaded AgNPs, group II: 100 ppm AgNPs, group III: 2% CHX, and group IV: sterile saline (negative control). The antimicrobial effectiveness was determined using colony forming units (CFU) method and verified via confocal microscopy, via calculating bacterial reduction (Cr%) and red intensity percentages (%R) for both tests respectively. Statistical analysis was done using one-way ANOVA, followed by Tukey's and Bonferroni post hoc tests.

Results: The highest C_(r%) was seen after 10 minutes of application (46.21%) and in group I (65.30%), followed by groups II (43.30%) and III (38.37%), and the least in group IV (10.36%). The highest %R was observed after 10 minutes (40.93%) of application and in groups I (48.54%) and II (42.15%), while groups III (31.09%) and IV (27.78%) were significantly lowest.

Conclusions: The combination between CHX and AgNPs showed a synergistic activity against E faecalis biofilm compared to either of them alone.

INTRODUCTION

Biofilms of pathogenic microorganisms, both aerobes and anaerobes, are the main cause of chronic, persistent infections. Biofilms are heterogeneous, multispecies, microbial communities embedded in a self-produced, viscoelastic extracellular polymeric substance (EPS) matrix which is responsible for various antibiotic resistance mechanisms[(1)]. *Enterococcus faecalis* is the most frequently isolated endodontic pathogen from secondary and persistent endodontic infections, though it is not the predominant one [(2)]. *E faecalis* exhibits many virulence factors; including cytolysins and proteolytic

enzymes, adhesins and capsular and cellular wall polysaccharides [3]. It can survive extreme environments, prolonged starvation periods, and in the presence of several antibacterial irrigants and medicaments; as it can enter the viable but non-cultivable (VBNC) state, and leave persisters that later recolonize the surfaces. It can invade dentinal tubules, penetrate the cementum, attach to collagen, and metabolize the fluids within the dentinal tubule [4].

Chemical antimicrobial agents are pivotal in endodontic biofilms removal. They should have sufficient penetration ability and high antimicrobial efficiency, in order to reach the deep microbial biofilms within the root canal system and eliminate them. CHX is a commonly used antimicrobial in endodontic disinfection owing to its substantivity, ability to inhibit adherence of some bacteria, and bactericidal effect. In a study, CHX, in both gel and liquid forms, maintained a low CFU count 7-days post-treatment when compared to sodium hypochlorite (NaOCl) [5]. Yet, CHX is incapable of dissolving organic matter, and is deactivated by dentin and bacterial spores. Furthermore, *E faecalis* is resistant to a number of other antimicrobials and cannot be completely eradicated from root canals using the current intracanal irrigants and medicaments. Thus, the need for innovative antimicrobial agents arises.

Non-antibiotic-based treatments arise as a growing field in order to better eradicate complex infections and improve the clinical outcomes, particularly with the emergence of multi-drug resistant (MDR) bacteria, costly and difficult antibiotics development, and the enhanced knowledge of microbiota [6]. Biological processes occur basically at the nanoscale. Accordingly, nanoparticles are able to easily interact with the cellular biomolecules. AgNPs gained considerable attention due to its effectiveness against bacterial antibiotic resistance, as well as its anti-fungal and anti-viral abilities [7]. In a study that evaluated the bactericidal effect of AgNPs as a final irrigating agent on *E faecalis* biofilm, no statistical difference was found between AgNPs and 2.25% NaOCl [8].

Combining AgNPs with different antimicrobials increases their efficiency, while decreasing bacterial tolerance to them along with their cytotoxicity[9–11]. One of the most tested combinations is the combination of CHX and AgNPs which showed synergistic, antimicrobial effect [12,13]. CHX disrupts the bacterial cell membrane, which increases its permeability and facilitates the uptake of the released Ag^+ into the bacterial cell; this in turn enhance AgNPs effectiveness [14]. However, most of these studies were conducted as pilot studies, on young biofilms, or in an environment that does not simulate the clinical endodontic settings [15]. The purpose of the current study was to evaluate the antimicrobial efficacy of AgNPs and CHX-loaded AgNPs solutions on mature, intracanal *E faecalis* biofilm, in a simulated clinical endodontic setting. The null hypothesis is that no true difference exists between the aforementioned solutions.

MATERIALS AND METHODS

Selection of specimens

Sixty freshly extracted human single-rooted teeth were collected, cleaned with 0.1 % NaOCl solution for 15 minutes and placed in ultrasonic bath for 4 minutes before they were washed and stored in sterile saline until used. G*Power 3.1 software (University Düsseldorf, Germany) was used for sample size calculation, accordingly, a minimum of 8 teeth per group would be required to show a difference with effect size=.4, power (1-β error) =.95, and α-type error=.05. The teeth were examined under 4.5X magnification using EyeMag Pro S loupes (Carl Zeiss, Oberkochen, Germany), combined with buccolingual and proximal periapical radiographs to reach a final sample of 45 teeth. The inclusion criteria was maxillary incisors or mandibular premolars, with straight roots, Vertucci type 1 root canal, without caries, cracks or other anatomical variations, and extracted for orthodontic or periodontal reasons [16]. The study protocol was approved by the

Ethics Committee of the Faculty of Dentistry, Ain Shams University (FDASU_Rec_m071808).

Preparation of specimens

The teeth were decoronated to obtain a standardized root segment of 15 mm and the working length was determined 1 mm shorter than the root segment. Instrumentation was done to a MTaper rotary file #30/.06 (IMD, Shanghai, China) using crown down technique, and irrigated with 5.25% NaOCl and 17% EDTA. Prepared root segments were then placed in ultrasonic bath of 5.25% NaOCl, 17% EDTA and sterile saline (4 minutes each respectively) for smear layer removal and complete removal of the used chemicals. After this, the specimens were sterilized in an autoclave for 20 minutes at 121˚C and then incubated in sterile BHI broth at 37˚C for 24 hours to ensure their sterility.

Biofilm development

A pure strain of E faecalis was incubated in BHI broth at 37˚C for 24 hours. Then the bacterial suspension was taken and inoculated on BHI agar plate at 37˚C for 24 hours. The bacterial cells were resuspended in sterile saline to reach a final concentration of 3 x 10^8 cells/ml adjusted to No. 1 MacFarland turbidity standard.

After discarding the sterile broth, 3 ml of the bacterial suspension mixed with BHI broth was used to infect the prepared canals under anaerobic conditions. The specimens were incubated in BHI broth at 37˚C for 6 weeks; a fresh broth adjusted to No. 1 MacFarland turbidity standard was replenished every 72 hours to ensure cell viability, continuous supply of nutrients and wash out of unattached bacterial cells and bacterial by-products [17].

Preparation of nanoparticles

A solution of 100 ppm AgNPs (NanoTech Egypt for Photo-Electronics, Cairo, Egypt) was prepared using the chemical reduction method as reported by Turkevich [18] and Lee and Meisel [19]. CHX-loaded AgNPs solution was prepared via electrostatic attraction method by functionalizing the silver nanoparticles to gain a positive charge and become electrostatically attached to the positively-charged CHX. Dispersion of 100 ml of 100 ppm AgNPs in 100 ml of CHX to give a final concentration of 100mg/L, followed by sonication/mixing overnight to allow electrostatic binding between CHX and AgNPs [13].

To verify the formation of AgNPs, two tests were performed [14]; the Ultraviolet-visible (UV-Vis) absorption spectra and transmission electron microscopy (TEM) at an accelerating voltage of 200 kV. AgNPs in the form of a grayish-yellow, suspension were spherical, non-aggregated and well separated in shape with an average size of 20±5nm. The UV-Vis light absorption spectra showed a maximum peak around 410nm, i.e., λ_{max} = 410 nm (Figure 1).

Classification of specimens

Five specimens were randomly selected as biofilm control, and were scanned via SEM to confirm biofilm formation. The remaining 40 specimens were randomly divided into 4 groups (n=10);

Group I: CHX-loaded AgNPs,

Group II: 100 ppm AgNPs,

Group III: 2% CHX (Chemajet Chemical Company, Alexandria, Egypt),

Group IV: Sterile saline (negative control).

A total of 3 ml/time of each irrigant was introduced into each canal, using a side-vented, 27-gauge, sterile needle that was placed 1 mm shorter than the working length; with a flow rate of 3mm/min. Each canal was flooded with the designated irrigant and left untouched for 1, 3 or 10 minutes. After each time period, the canals were gently washed with sterile saline as part of CFU sampling [20].

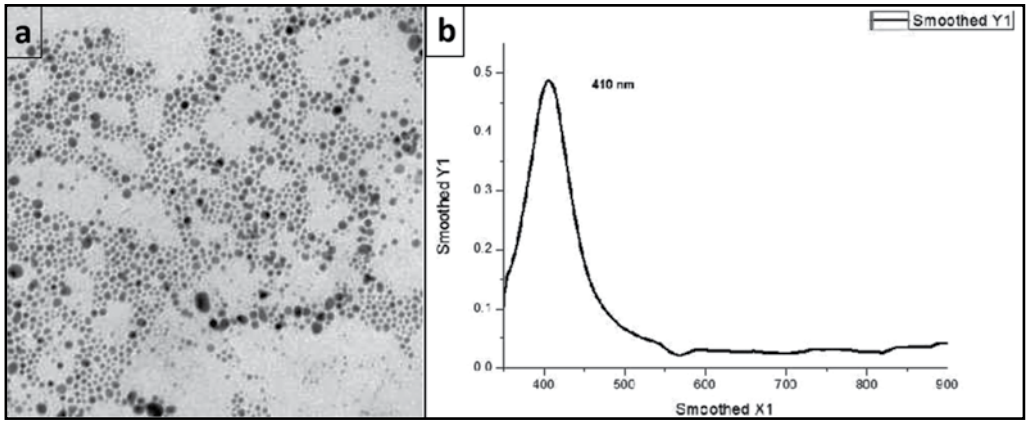

Fig. (1) a: TEM image of Ag nanoparticles at different scale (20±5nm), b: UV-Vis light (maximum peak around 410nm)

Microbiological CFU sampling

All 45 specimens were sampled to establish the level of contamination prior to antimicrobials application (S_1). S_2, post irrigation samples were collected at all 3 time points for each canal (N=120). The sampling technique was done as described by Afkhami et al [10], briefly the canal were gently washed with 3 ml of sterile saline, then a #15 K-file was introduced inside the wet canal to within 1 mm from the working length and was used circumferentially for 10 seconds. Two sterile paper points were passively inserted in each canal and transferred into a sterile microtube containing 1 ml of sterile saline. Then 10-fold dilutions (1:10, 1:100 and 1:1000) were prepared, and 0.1ml from each dilution was smeared to be incubated at 37°C for 48 hours. CFU per 1 ml was counted[11]. The antimicrobial effectiveness was evaluated by determining the percentage reduction in colony count (C_r%) at the end of each time period using the following formula (10),

$$C_r\% = \frac{S_1 - S_2}{S_1} \times 100$$

Confocal Laser Scanning Microscope (CLSM) evaluation

For this section, 12 representative samples were used (n=3/group) to examine the effect of irrigants on the *E faecalis* biofilm after 1, 3, and 10 minutes. The samples were horizontally sectioned using Isomet saw (Buehler, Leinfelden-Echterdingen, Germany) under constant cooling with sterile saline to a thickness of 2 mm to obtain two sections for each specimen, middle and apical ones

All samples were stained with Live/Dead viability kit [21] and they were then examined using LSM 710 and ZEN 2009 (Carl Zeiss, Jena, Germany). The utilized objective lens of the CLSM was EC Plan-Neofluar 40x/1.30 Oil DIC M27. The Live/Dead stain was used in a final concentration of 1 μL/mL. The acridine orange (AO) stain emits green fluorescence indicating viable bacteria, its excitation wavelength was 514 nm, its emission wavelength was 558 nm and the master gain was 677. The propidium iodide (PI) stain emits red fluorescence indicating nonviable bacteria, its excitation wavelength was 458 nm, its emission wavelength was 679 nm and its master gain was 698. Analysis of CLSM photos was done to calculate the percentage of red intensity (%R) as described by Azim et al (2016) using the following formula (22) (Figure 2),

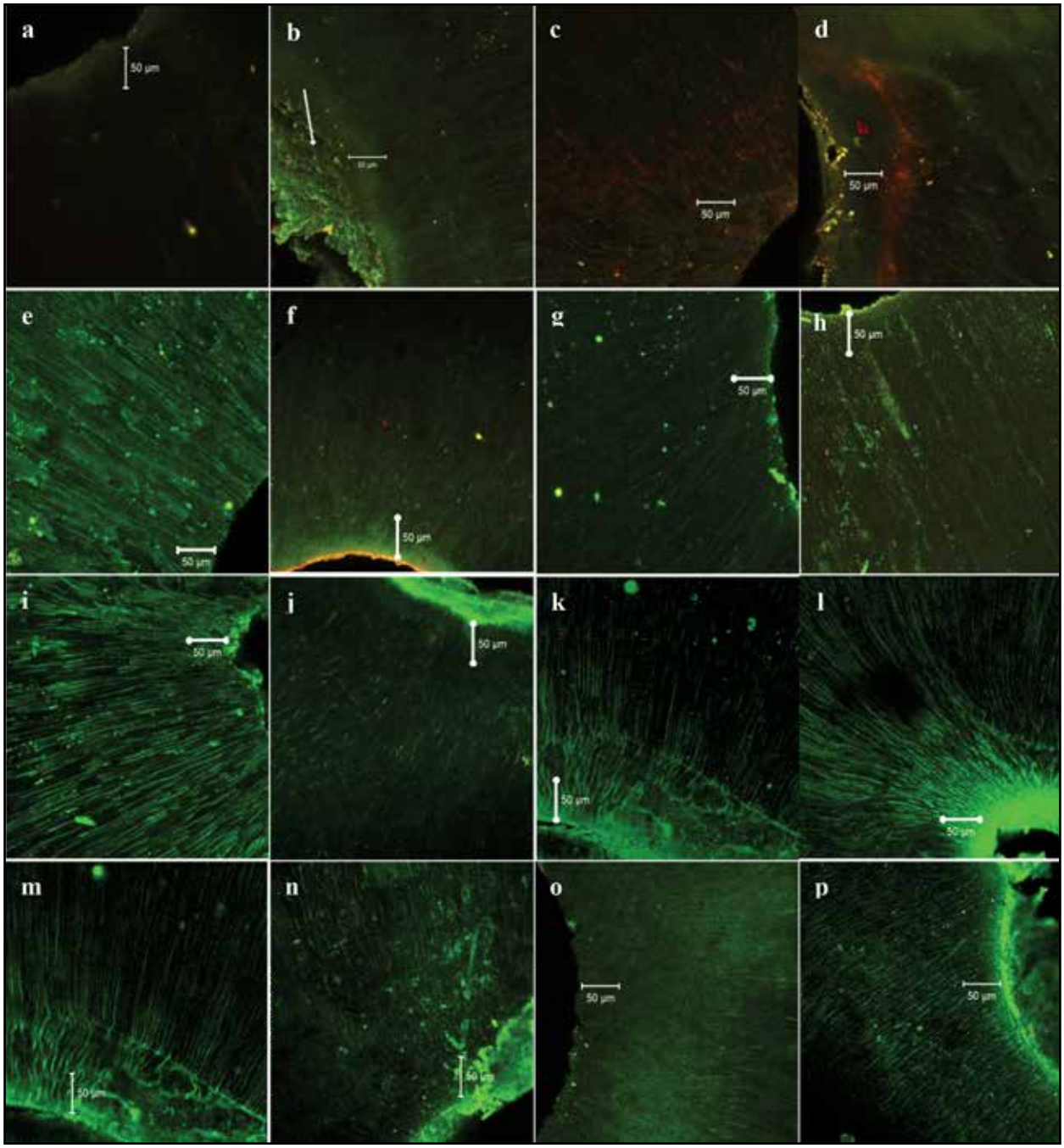

Fig. (2) CLSM scans of Group I, **a:** middle/1 minute, **b:** apical/1 minute (arrow referring to biofilm), **c:** middle/10 minutes, **d:** apical/10 minutes. Group II, **e:** middle/1 minute, **f:** apical/1 minute, **g:** middle/10 minutes, **h:** apical/10 minutes. Group III, **i:** middle/1 minute, **j:** apical/1 minute, **k:** middle/10 minutes, **l:** apical/10 minutes. Group IV, **m:** middle/1 minute, **n:** apical/1 minute, **o:** middle/10 minutes, **p:** apical/10 minutes.

$$\% R = \frac{\text{red intensity}}{\text{red intensity + green intensity}} \times 100$$

Statistical analysis

Numerical data were explored for normality. Data showed parametric distribution so; it was represented by mean and standard deviation (SD) values. Intergroup comparisons were done using one-way ANOVA followed by Tukey's post hoc test, while intragroup comparisons were done using one-way repeated measures ANOVA followed by Bonferroni post hoc test. The significance level was set at P≤0.05 within all tests. Statistical analysis was performed with IBM® (IBM Corporation, NY, USA) SPSS® Statistics Version 25 for Windows (SPSS, Inc., an IBM Company).

RESULTS

The C_r% results, detailed in tables 1 and 2, revealed that group I (65.30%) showed the significantly highest reduction in bacteria count, followed by groups II (43.30%) and III (38.37%); then group IV (10.36%). Considering the time factor, the highest C_r% was detected after 10 minutes (46.21%), followed by after 3 (38.54%) and 1 minute (33.88%) of application. F (2,33)= 2.13, p≤.002, η_p^2= .44.

The results of %R, detailed in table 3, revealed that no statistical significance was found between (a) different timings of application within each group, except for apical section in group II where the highest %R was after 10 minutes (42.43%); (b) nor between middle and apical sections; F (2,24) = 1.92, p<.05, η_p^2=.132. However, collectively, after 10 minutes (40.93%) was significantly the highest compared to after 1 (34.56%) and 3 minutes (36.39%) of application. Equally, a statistically significant difference was found between different groups within different timings of application; F (3,22) = 1.50, p<.05, η_p^2=.49, (Figure 3).

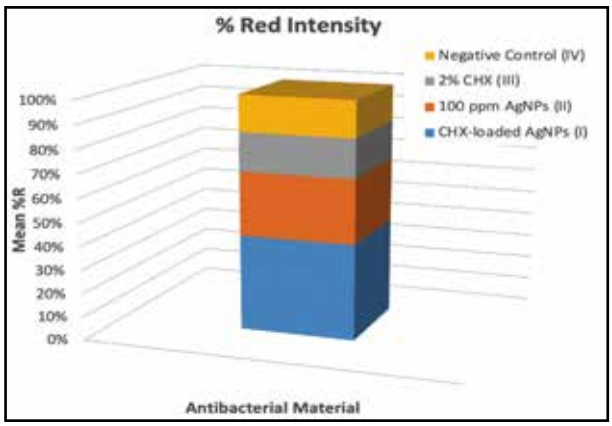

Fig. (3) 100% Stacked Column showing the mean %R for different groups regardless of the time factor

Table (1): *Mean ± standard deviation (SD) of $C_{r\%}$ for different groups within each time frame*

Timing of application	Groups (mean±SD)				p-value
	CHX-loaded AgNPs (I)	100 ppm AgNPs (II)	2% CHX (III)	Negative Control (IV)	
After 1 minute	52.52±4.39^{A}	41.04±1.63^{B}	32.29±3.76^{B}	5.66±3.10^{C}	**<0.001***
After 3 minutes	63.32±15.13^{A}	42.67±1.60^{B}	38.78±9.66^{B}	9.38±2.89^{C}	**<0.001***
After 10 minutes	79.37±22.12^{A}	46.56±1.94^{B}	41.07±2.18^{B}	17.83±4.25^{C}	**<0.001***

*Different superscript letters indicate a statistically significant difference within the same horizontal row *; significant (p ≤ 0.05) ns; non-significant (p>0.05)*

Table (2): *Mean ± standard deviation (SD) of $C_{r\%}$ for different timings of application within each group*

Groups	Timing of application (mean±SD)			p-value
	After 1 minute	After 3 minutes	After 10 minutes	
CHX-loaded AgNPs (I)	52.52±4.39^{C}	63.32±15.13^{B}	79.37±22.12^{A}	**0.002***
100 ppm AgNPs (II)	41.04±1.63^{A}	42.67±1.60^{A}	46.56±1.94^{A}	**0.76**^{ns}
2% CHX (III)	32.29±3.76^{A}	38.78±9.66^{A}	41.07±2.18^{A}	**0.90**^{ns}
Negative Control (IV)	5.66±3.10^{B}	9.38±2.89^{B}	17.83±4.25^{A}	**<0.001***

*Different superscript letters indicate a statistically significant difference within the same horizontal row *; significant ($p \leq 0.05$) ns; non-significant ($p>0.05$)*

Table (3): *Mean ± standard deviation (SD) of %R for different groups and timings of application*

Timing of application	Section	Groups (mean±SD)				p-value
		CHX-loaded AgNPs (I)	100 ppm AgNPs (II)	2% CHX (III)	Negative Control (IV)	
After 1 minute	**Middle**	47.73±5.21^{A}	47.47±5.24^{A}	29.78±16.88^{B}	25.93±18.08^{B}	**0.03***
	Apical	42.43±4.70^{A}	32.52±13.45^{B}	26.74±16.66^{B}	23.89±15.44^{B}	**0.02***
After 3 minutes	**Middle**	50.02±2.10^{A}	45.91±2.75^{A}	31.17±15.59^{B}	28.09±17.90^{B}	**≤0.009***
	Apical	44.92±3.10^{A}	32.62±14.14^{B}	30.69±15.72^{B}	27.73±15.63^{B}	**0.02***
After 10 minutes	**Middle**	53.00±4.31^{A}	51.95±3.75^{A}	35.05±9.32^{B}	32.31±8.38^{B}	**0.02***
	Apical	50.90±2.84^{A}	42.43±4.70^{B}	33.12±12.66^{C}	28.66±15.58^{C}	**<0.001***

*Different superscript letters indicate a statistically significant difference within the same horizontal row *; significant ($p \leq 0.05$) ns; non-significant ($p>0.05$)*

DISCUSSION

Endodontic microbiota invades the root canal system in areas unreachable via mechanical preparation alone and penetrates deep within the dentinal tubules. *E faecalis* is the most commonly isolated endodontic pathogen, and it can exist in mono-, bi- or poly-microbial biofilms [23]. Accordingly, the incorporation of chemotherapeutic agents is vital to combat endodontic infections. The aim of this study was to compare the efficacy of CHX-loaded AgNPs, 100 ppm AgNPs, and 2% CHX regarding the eradication of 6-weeks old, intraradicular *E faecalis* biofilm. For the purpose of standardization, the teeth were either maxillary incisors or mandibular premolars [22], they were sectioned to obtain 15 mm root segments, and were mechanically prepared to rotary file #30/.06. During the incubation period, fresh broth was replenished to avoid biofilm starvation. Additionally, in order to mimic the clinical endodontic setting, during the application of different irrigants, fresh irrigant was

introduced inside the canal; after washing out the previously applied amount, to avoid any cumulative effect of repeated exposure to the irrigant.

The specimens were examined by two methods, CFU and CLSM. CLSM results confirmed the CFU results that CHX-AgNPs combination had a synergistic antimicrobial effect and showed the highest reduction in bacterial load, followed by 100 ppm AgNPs, then 2% CHX, while saline statistically showed the least bacterial reduction. Therefore, the null hypothesis was rejected and a true difference was proven to exist between the tested irrigants. Charannya et al. [12] evaluated the efficacy of 2% CHX and AgNPs; both separately and combined in a pilot study, against *E faecalis, Candia albicans,* and *Klebsiella pneumonaie*. They first identified the minimum inhibitory concentration of AgNPs to be 15 ppm, before using the agar diffusion method to evaluate the antimicrobial efficacy of the tested solutions. Their results showed CHX-AgNPs combination to have the highest efficacy against all three pathogens suggesting a synergistic effect of that combination. Likewise, Lu et al. [13] revealed that CHX-loaded, silver-decorated mesoporous silica nanoparticles could fully inhibit the *S aures* and *E coli* colonies, while equivalent concentrations of CHX, Ag-mesoporous silica, $AgNO_3$, and mesoporous silica did not inhibit their growth. Conversely, Wu et al. [24] reported that 2% NaOCl was more effective than 0.1% AgNPs solution against *E faecalis* biofilm. Likewise, Halkai et al. [25] reported that no statistical significance was found between fungal-derived AgNPs, 0.2% and 2% CHX against 2 weeks old *E faecalis* biofilm. However, Nabavizadeh et al. (16) showed positively-charged imidazolium AgNPs to have a comparable antibacterial to 2.5% NaOCl against *E faecalis* biofilm

The discrepancy in results may be attributed to the varying study protocols; where some studies were concerned with MIC, and others investigated 21-days old biofilm and/or added an extra mechanical preparation step. Furthermore, this difference may be attributed to the usage of different *E faecalis* strains [26], or to the difference in AgNPs preparation, which differed in particle size and solution concentration. Additionally, some preparations were based on carriers, chemicals, or leaf extracts, whereas others were fungal-derived.

In this study, all groups showed the highest bacterial reduction after 10 minutes, which gradually increased from 1 to 3 to 10 minutes. This may be attributed to the increased uptake of AgNPs by bacterial cells and/or CHX substantivity. AgNPs antibacterial mechanisms are not specific at a single level, but they influence many bacterial structures and metabolic processes at the same time as described by Tang and Zheng [27]. The main antibacterial mechanisms governing the action of AgNPs are based on direct binding to cellular components, generation of reactive oxygen species (ROS), and release of Ag^+ ions. The release of Ag^+ ions is mediated by AgNPs oxidative dissolution. Ag^+ ions enter the bacterial cell, bind to electron-donating groups, and consequently alter the 3D protein structure forming insoluble compounds and blocking active binding sites. This impairs cell function, energy production and division. Moreover, increased production of ROS and free radicals, as singlet oxygen and superoxides, with the concurrent reduction of glutathione concentrations; a ROS scavenger, results in direct mitochondrial damage, DNA breakage, and hyper-oxidation of proteins and lipids. All three mechanisms work simultaneously and initiates a vicious circle resulting in ultimate bacterial cell death. This explains the lack of resistance against AgNPs. Additionally, it was reported that the combination of some antibiotics and AgNPs can overcome the bacterial resistance to these antibiotics and increase the bacterial cell sensitivity to them [14].

CHX was demonstrated to exert bactericidal effects on Gram-positive and Gram-negative bacteria, facultative and strict anaerobes, fungi, and some

viruses, and it positively affects *E faecalis* adherence to dentin [28]. CHX retains its activity in the presence of blood and organic matter. Also, it could be more effective in improving the antibacterial activities of alkaline root canal medicaments [29]. Despite that, CHX is unable to dissolve organic matter, its antimicrobial activity is inhibited by dentin, and is inactive in the presence of bacterial spores. Some studies showed increased resistance of biofilms to CHX after 3 and 10 minutes, however, the results in this study indicated otherwise, which was probably due to CHX substantivity [30]. This could be related to adequate irrigant flow, and little or no bacterial resistance.

Kitagawa et al. [31] and Kuang et al. [32] demonstrated that repeated exposure of *E faecalis* to CHX resulted in resistance. Conversely, Kayaoglu et al. [33] specified that *E faecalis* resistance was associated with the expression of certain genes and presence of certain proteins, collagen. Additionally, Kheljan et al. [34] stated that CHX was the most effective tested biocide against *E faecalis*. A study about antimicrobial substantivity reported that 2% CHX was more effective against 14-days old, intraradicular biofilms of *E faecalis* and *C albicans*, compared to calcium hydroxide, AgNPs, AgNPs-CHX, and AgNPs-calcium hydroxide combinations [11]. This was supported by Alabdulmohsen and Saad [35] who reported that calcium hydroxide showed the highest percentage of bacterial reduction in 1 and 2 weeks post-treatment intervals, compared to AgNPs alone and in combination with calcium hydroxide. However, Samiei et al. [36] contradicted these studies when the silver cross-linked hydrogel nanocomposite maintained its antibacterial activity against E faecalis biofilm, while2% CHX and 2.5% NaOCl lost theirs.

CLSM analysis showed that CHX-loaded AgNPs, AgNPs and CHX were statistically different from each other, while CHX and saline were not. This may be attributed to the fact that bacteria in older biofilms are more resistant to antimicrobials compared to those in younger ones [7]. Furthermore, CLSM is based on double staining with nucleic acid-binding fluorescent dyes to analyze cellular viability. It couples the use of cell permeant, green dye; as AO stain, and a cell impermeant red dye, as PI stain; which renders the bacteria with damaged cell membrane a yellow or red color, while the viable ones appear green. Thus, CLSM is able to detect bacteria in VBNC [21]. Moreover, this confirms the results of other studies that complete bacterial elimination cannot be achieved and further investigation is needed to assess biofilm regrowth [20,30].

The gradual increase in bacterial reduction in the saline group can be the result of the solution flow and pressure; accordingly, biofilm regrowth is inevitable [5,37,38]. The difference between the middle and apical sections was reported in some studies. This may be due to difference in irrigants penetration, their flow, and/or wettability, which affects their intracanal advancement. However, our study detected no statistical difference between middle and apical sections. This may be due to the relatively small sample size for the discretion between these two sections, therefore, larger sample size is required for the comparison of the antibacterial efficacies of these irrigants at different canal levels. Therefore, it is recommended to have further studies concerning the incorporation of carrier molecules, substantivity, biofilm regrowth, and toxicity of CHX-AgNPs combination before its translation to endodontic clinics.

CONCLUSIONS

The combination between CHX and AgNPs showed a synergistic activity against *E faecalis* biofilm compared to either of them alone. One minute of application provided rapid bacterial reduction, while 10 minutes of application provided the highest reduction. No statistical difference was detected between different canal levels.

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