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# IMPACT OF INTEGRATING TWO ACTIVATION TECHNIQUES WITH NANO CHITOSAN IRRIGATION ON ANTIBACTERIAL EFFICACY

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## *ABSTRACT*

**Introduction:** The purpose of this study was to compare in vitro study the impact of activation of nano chitosan irrigation with two different techniques on antibacterial efficacy on root canals infected with two different specimens.

**Aim of study:** to asses and contrast impact of integrating passive ultrasonic irrigation or laser activation with nanochitosan against double species biofilm. **Methods:** A total of 50 extracted single-rooted teeth were sectioned at the cementoenamel junction, instrumented, sterilized using autoclave, then divided into five equal groups (n = 10) at random based on the designated antimicrobial protocols. All groups were inoculated with Enterococcus faecalis and staphylococcus aureus except Group I: (Negative control group) Autoclaved specimens in this group received no additional procedures, ensuring that any microbial growth could be attributed to contamination rather than procedural variables. Group II: No additional procedures were performed in the positive control group, enabling comparison against treated groups. Group III: specimens were irrigated with 0.2% chitosan nanoparticles (CNPs) with conventional needle irrigation (CNI). Group IV: specimens received irrigation with 0.2% CNPs and passive ultrasonic irrigation (PUI). Group V: specimens were irrigated with 0.2 chitosan nanoparticles and laser activation (LAI). The effectiveness of the suggested therapies was assessed and demonstrated, respectively, by microbiological testing (colony forming unit counts). The One-Way ANOVA test was applied to the data. for comparison of quantitative data between the five groups followed by post hoc LSD analysis.

**Results:** Group 1 showed complete absence of bacterial growth (0±0 $x10^4$), while group 2 exhibited the highest bacterial proliferation with a mean of 164±12.6 $x10^4$ (range: 144-176). Group 3 showed moderate bacterial growth with 82.3±13.5 $x10^4$ (range: 62-96), while both group 4 and group 5 demonstrated significantly lower bacterial counts at 32±10 $x10^4$ (range: 16-40) and 32±15.1 $x10^4$ (range: 16-48) respectively.

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**Conclusions:** Both PUI and LAI groups by using 0.2% nano-chitosan demonstrated success in reducing root canal infection compared with conventional irrigation group.

**KEYWORDS:** Nano-chitosan, Root canal irrigation, Laser activation, Passive ultrasonic activation, Enterococcus faecalis, Staphylococcus aureus, Antibacterial efficacy, CFU counting.

## INTRODUCTION

Bacteria have been known as the primary cause of development of pulp and periapical lesions. The objective of root canal treatment is to eliminate microbial biofilms, remove all the vital and necrotic tissues, microorganisms and their byproducts from root canal system so we can achieve successful root canal therapy by chemo-mechanical cleaning and shaping. [1] Nevertheless, even after chemo-mechanical preparation biofilms and microorganisms remain in root canal. [2] Enterococcus faecalis is a Gram-positive facultative anaerobe frequently implicated in endodontic failure. It has been recovered from 18% of primary root canal infections and 67% of retreatment cases. [3]

Staphylococcus aureus is among the most resilient microorganisms encountered in failed endodontic treatments and has been implicated in the pathogenesis of both initial and persistent root canal infections. [4]

Irrigation considers the only method to impact areas of root canal wall that are not touched by mechanical instrumentation such as isthmuses and lateral canals, Biofilms and tissue remains may be present in these regions. Traditional mechanical preparation approximately 150-micrometers depth, which insufficient for eliminating infections, as bacteria can proliferate up to 4 hundred micrometer or more within the tubular structure of dentin. [5]

Chitosan been developed as the last irrigant during root canal therapy, it has many advantageous properties, for example, biocompatible, biodegradation, harmless to human cells, bio-adhesive and antibacterial which considers the most important property. [6]

Nanoparticles possess distinct surface properties including high charge density and large surface area which, together with their polycationic or polyanionic nature, enhance their antimicrobial potential. [7]

Chitosan nanoparticles; are hydrophilic polymers; which provide close interaction with the dentin of the root canal and further possesses ability to penetrate deep into the dentinal tubules of the root canal system. It has a lot of free hydroxyl and amino groups, which cause the chelating agent and dentin calcium ions to interact ionically. [6] Chitosan nanoparticles can suppress bacterial enzymatic activity, which in turn minimizes the potential for bacterial infiltration. [8]

Activating irrigants via PUI, sonic, or laser techniques improves their cleaning efficacy, dynamic flow, antibacterial properties, and therapeutic outcomes. [5]

The Goal of this investigation was to investigate as well as contrast effect of integrating passive ultrasonic activation or laser activation with nanochitosan against double species biofilm.

The null hypothesis states that there is no significant difference among the tested groups regarding antibacterial effect.

## MATERIAL AND METHODS

### Ethical Approval

This study was approved by the Ethics Commission of the school of dentistry of Minia University **(approval number 103/881/2024)**.

Informed consent was obtained from the individual whose teeth were employed for the study prior to extraction.

Upon completion of the study, all extracted teeth were properly disposed of in regulated medical waste containers.

**Sample Collection and preparation**

50 extracted single rooted teeth were used, they were extracted due to orthodontic or periodontal reason. Teeth were prepared by mechanically removing soft tissue and calculus from the root surfaces and to verify complete root development, the lack of root fillings, and if their internal calcifications or resorption, radiographies were taken in the buccolingual and mesiodistal directions. Every sample was decoronated. to obtain uniform specimens, the crowns were sectioned using a diamond disc (Diatech, Coltene, Switzerland), resulting in a standardized root length of 17 mm, endodontic access was then established and apical patency was verified with a size A size 10 K-file (MANI) was advanced through the root canal until its tip became visible at the apical foramen, indicating canal patency. The root was then adjusted to a standardized length of 17 mm using a stopper on the file. Subsequently, the working length (WL) was set at 16 mm, maintaining a 1 mm distance short of the apical foramen, which is 1 mm shorter than the apical foramen. All samples were instrumented using the conventional sequence of the E-Flex Blue rotary system, following the manufacturer's instructions, with a final apical preparation size of 40/0.04 at a rotational speed of 300 rpm. Coronal third flaring was initially performed using an orifice opener, followed by Sequential preparation with rotary files until reaching the final apical size of 40/0.04, designated as the master file.at the established working length (WL). During preparation, each canal was irrigated with 1 mL of 2.5% sodium hypochlorite (NaOCl) between instruments using a disposable 2 mL syringe fitted with a 27-gauge side-vented needle positioned 2 mm short of the WL. Following the final instrument, the canals were irrigated with 2 mL of 5.25% sodium hypochlorite solution, 2 mL of 17% ethylenediamine tetra acetic acid solution, and 2 mL of saline as a concluding rinse. The irrigation period for each solution was 1 minute (1 mL per 30 seconds). Subsequent to processing, the roots were desiccated with paper points and each apical foramen was sealed with composite resin then the roots were placed in envelopes and sterilized in an autoclave at 121°C and 15 MPa for a period of thirty minutes.

**Culture and Inoculum Preparation**

E. faecalis and staphylococcus aureus were kindly provided by the Department of Microbiology, Faculty of Pharmacy Deraya University.

E. aecalis (ATCC 29212) was cultivated at 37 °C in (BHI) agar until log phase (A time of exponential growth of bacteria). The microbial suspension was adjusted to optical density of 0.6 at 600 nm for inoculation into the root canals. [9]

Staphylococcus aureus (ATCC 25175) was incubated at 37°C for 24 hours, the bacterial suspension was then adjusted to a turbidity equivalent to the 0.5 McFarland standard using a KristalSPEC™ device, corresponding to approximately $1.5\times 10^4$ CFU/mL. This standardized suspension was subsequently used to inoculate blood agar plates to ensure uniform and reproducible bacterial growth [5].

**Preparation of irrigation solutions**

Chitosan nanoparticles (CSNPs) were synthesized following the ionotropic gelation technique as described in previous studies [10,11]. To create a homogeneous solution, 200 milliliters of 1% (v/v) acetic acid (pH 4) was mixed with one gram of chitosan powder and constantly agitated for six hours [12]. Thereafter, 150 mL of 0.2% (w/v) sodium tripolyphosphate (TPP) solution was added dropwise under magnetic stirring. The transformation of the clear solution into a turbid suspension indicated nanoparticle formation. The suspension was subsequently centrifuged at 15,000

rpm for 30 minutes (Hermle Z32 HK, Germany) and washed three times with deionized water ($DH_2O$) to remove unreacted components. [13]

**Division of samples based on treatment protocols**

After the period of incubation, the 50 samples will be randomly divided into 5 equal groups n=10

1. ***Group I:*** Negative control group: Specimens in this group were sterilized by autoclaving and received no subsequent treatment. (This group served to confirm the absence of contamination, ensuring that the autoclaving process effectively maintained sterility throughout the experimental procedures).
2. ***Group II:*** after autoclaving, specimens were deliberately infected and received no additional treatment. (This group functioned to ensure that viable bacterial contamination was present in all infected samples).
3. ***Group III:*** Samples were sterilized by autoclaving, subsequently infected, and then irrigated with 0.2 %nano-chitosan with conventional needle irrigation, utilizing a side-vented needle of 27 gauge. For 30 seconds, each canal received a steady flow of 1 mL 0.2% nano-chitosan (1 mL every 30 seconds) while being kept 2 mm away from the WL, using an up-and-down motion and allowing a 30-second soaking period, and this irrigation cycle was conducted twice, resulting in an overall volume of 3 mL of 0.2 %nano-chitosan was used during this procedure.
4. ***Group IV:*** Samples were sterilized then infected; after that were passively irrigated with 0.2% nano-chitosan and passive ultrasonic activation, in order to enable irrigation, a side-vented needle of 27 gauge was applied at the orifice level. The ultrasonic file was then placed 2 mm from the working length and activated using a PUI system set to 45 kHz. Each canal received irrigation for 30 seconds with 1 milliliter 0.2% nano-chitosan using an Ultra X device, then accompanied by a 30-second soaking period. This irrigation cycle was carried out a couple of times resulting in a total of three rounds.
5. ***Group V:*** specimens will be autoclaved and infected; then irrigated with 0.2% nano-chitosan and laser activation. In the beginning, the canal was passively charged with 0.2% nano-chitosan A 200-µm fiber tip was positioned 2 mm from the WL for activation with a circumferential motion for 4 seconds, utilizing a 27-gauge side-vented irrigation needle that was positioned at the orifice level for irrigation. The laser tip was triggered at 280 mJ, 5 Hz, and 1.4 W. After that, 1 ml of 0.2% nano-chitosan was used to irrigate the canal, and a laser tip was applied at 2mm from WL then was shortened by another 2mm with the same motion for 4 seconds, this procedure was repeated until achieving the first 2ml of the coronal third of the root canal.

Aseptic techniques were strictly maintained during irrigation procedures, which were performed in a laminar flow hood using sterile gloves and a dedicated sterile syringe for every specimen.

**Microbiologic Analysis**

Following the irrigation procedure, three sterile paper points were consecutively inserted into each root canal up to the full working length and left in place for 60 seconds per point to absorb intracanal contents. After that, the paper points were aseptically placed into Eppendorf tubes with individual labels and 1 milliliter of sterile phosphate-saline buffer (PSB). Each vial was vortexed for 1 minute to disperse the adherent bacteria into suspension. Subsequently, standard tenfold serial dilutions were prepared, and to quantify bacteria, the number of colony forming units (CFU) was determined per milliliter. [14]

### Statistical Analysis

One Way ANOVA test for comparison of quantitative data between the five groups was applied followed by post hoc LSD analysis between each group at significant level P value < 0.05.

## RESULTS

The statistical analysis of bacterial growth demonstrates highly significant differences among the five experimental groups ($p<0.001$) **Table 1**. The negative control group showed complete absence of bacterial growth (0±0 $x10^4$), while the positive control exhibited the highest bacterial proliferation with a mean of 164±12.6 $x10^4$ (range: 144-176). Among the treatment groups, CNI showed moderate bacterial growth with 82.3±13.5 $x10^4$ (range: 62-96), while both PUT and LAI groups demonstrated significantly lower bacterial counts at 32±10 $x10^4$ (range: 16-40) and 32±15.1 $x10^4$ (range: 16-48) respectively **Figure 1.**

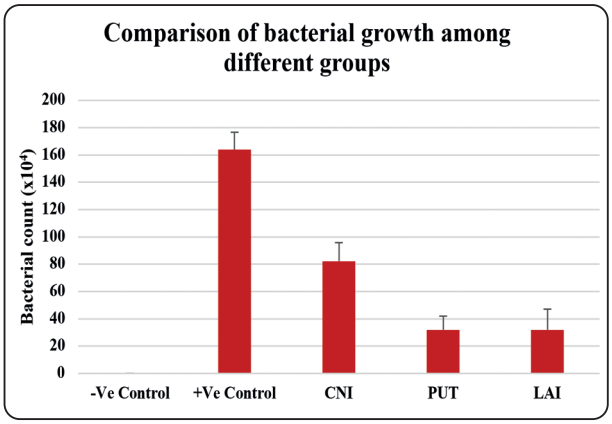


Fig. (1). Microbiological analysis: bar chart illustrating the mean and standard deviation of colony-forming units (CFUs) of Enterococcus faecalis and Staphylococcus aureus across each treatment group.

The post-hoc LSD analysis revealed distinct statistical patterns, with different superscript letters indicating significant pairwise differences between groups.

TABLE (1). Comparison of bacterial growth among different groups

| Group | -Ve Control | +Ve Control | CNI | PUI | LAI | P value |
|---|---|---|---|---|---|---|
| N | 10 | 10 | 10 | 10 | 10 | |
| **Bacterial growth ($x10^4$)** | | | | | | |
| Range | (0-0) [a] | (144-176) [b] | (62-96) [c] | (16-40) [d] | (16-48) [d] | *<0.001** |
| Mean ± SD | 0±0 | 164±12.6 | 82.3±13.5 | 32±10 | 32±15.1 | |

## DISCUSSION

Endodontic treatment aims to eradicate microbial biofilms and eliminate both vital and necrotic tissues, along with associated microorganisms and their metabolic byproducts from the root canal system. [1] Nevertheless, even after chemo-mechanical preparation biofilms and microorganisms remain in root canal. [2] Enterococcus faecalis is a gram-positive anaerobic cocci responsible for most cases of endodontic treatment failures. [3] Irrigation considers the exclusive method aiming to influence areas of the root that didn't be reached with conventional preparation. [5] Chitosan been created for root canal therapy's last irrigation, it possesses numerous advantageous properties as antibacterial which considers the most important property. Chitosan nanoparticles make close contact with the root canal dentin and have the capacity to delve deeply into the root canal system's dentinal tubules. [6] Enhancing irrigant activation leads to improved performance, greater penetration through dynamic flow, and superior antibacterial action and clinical

outcomes. [5] The objective of the current study was to assess and contrast the influence of integrating passive ultrasonic activation or laser activation with Nano-chitosan against double species biofilm.

Fifty human single-rooted teeth that had been extracted had straight canals and mature apices had been utilized to establish the bacterial growth model, ensuring standardization and minimizing anatomical variability. [15]

The crown of each tooth was removed at the level of the cementoenamel junction and kept at 17 mm to assure standardization of specimens as it eliminated some variables, such as the anatomy of the coronal area. [16]

All decoronized teeth were instrumented using E-flex Blue rotary system up to (40/04) file to allow adequate cleaning and irrigant penetration in apical third and allowing creation of a large reservoir for the irrigant solution. [17]

A 27-gauge needle with lateral vents connected to a disposable plastic syringe was used to irrigate each canal with 1 ml. of 2.5% NaOCI between each instrument. After mechanical instrumentation, the canals were irrigated with 3ml of 5.25% (NaOCl) within 2 mm from the WL to remove organic debris and 17% (EDTA) into the canal for one minute to remove the smear layer from dentinal tubules. [18] Finally, 2 mL of the distilled water to irrigate each channel to eliminate any remaining traces of prior irrigants. [19] then the samples were packed in sterilization pouches to prevent any form of contamination during and after sterilization process and autoclaved for 30 min at 121 °C and 15 MPa. using steam air autoclave to eliminate all microorganisms from the teeth. [20] The samples were then infected with E faecalis and staphylococcus aureus suspension as they were chosen because of their ability to penetrate dentinal tubules, colonize the root canal system and they demonstrated effectiveness for evaluation of disinfecting root canals. [21, 5] The rationale behind establishing a multi-species biofilm model was the observed decreased susceptibility of E. faecalis to NaOCl in mixed-species environments compared to monospecies biofilms. [22] The bacterial suspension was introduced using sterile micropipettes, and its distribution to the working length was confirmed using a sterile #15 K-file. [23]

Several endodontic irrigants have been used in endodontic treatment for effective decontamination of the root canal system. Sodium hypochlorite (NaOCl) remains the gold standard due to its well-documented antimicrobial properties [24,25] and its capacity to dissolve organic tissue [26,27]. However, the search continues for alternative irrigants that can achieve comparable decontamination efficacy without causing harm to surrounding periapical tissues.

Chitosan was developed for the last irrigation during root canal therapy, it shows many advantageous properties and it was used due to its antibacterial property. Chitosan was used in form of nanoparticles to provide close interaction with the root canal moreover, it has the capacity to deeply enter the root canal system's dentinal tubules. [6]

Five equal groups were randomly selected from among the 50 single-rooted teeth, each consisting of ten specimens. Group I functioned as the negative control, wherein specimens were sterilized by autoclaving without receiving any subsequent treatment. This was conducted to verify the absence of microbial contamination post-sterilization. Group II served as the positive control, in which specimens were intentionally contaminated following autoclaving to simulate clinical infection. No further intervention was performed, thereby confirming the establishment of viable infection across all specimens, Group III samples were sterilized and contaminated to simulate clinical infection; then irrigated with 0.2 %nano-chitosan with conventional needle irrigation to allow comparisons with activated irrigation groups (Group IV & Group V) and evaluate whether mechanical activation enhances its antibacterial effect. Group

IV specimens were autoclaved and infected; then were passively irrigated with 0.2% nano-chitosan and activated using a PUI device (Ultra X device) to evaluate whether mechanical activation could enhance its antibacterial efficacy, ultrasonic file was placed 2 mm from the WL to ensure activation within the apical third without risking damage or extrusion, Group V specimens were autoclaved and infected; then rinsed by 0.2% nano-chitosan then agitated using laser; a 200-μm fiber tip was positioned 2 mm away from the working length to avoid periapical tissue irritation. or extrusion of irrigant with a circumferential motion to ensure uniform energy distribution along the canal walls for 4 seconds this procedure was repeated in segments by shortening the fiber tip position by 2 mm at each cycle, enabling activation from apical to first 2ml of the coronal third of the root canal. Then The number of colony-forming units (CFUs), expressed as log CFU/mL, was quantified to assess the impact of each treatment in this study. This method was chosen based on previous studies [28, 29] as this technique allows for bacterial assessment within the root canal system [30], microbiologic samples in this study were limited to the main canal. The use of passive ultrasonic and laser for activation of irrigant in endodontic therapy has been suggested as an alternative to increase cleaning and disinfection of the root canal system. [31,32]

Numerous studies have reported superior microbial reduction when endodontic irrigants are used in conjunction with passive ultrasonic irrigation or laser activation [33–36]. These results align with the outcomes of the current study, where passive ultrasonic activation and laser activation proved effective in enhancing disinfection when combined with chitosan nanoparticles and improving the potential for decontamination in root canals infected with E. faecalis and Staph aureus.

Contrary to current results, earlier studies found no statistically significant improvement when endodontic irrigants were used in combination with PUI [37, 38] or LAI [39, 40] compared to their use without activation.

According to the present study the statistical analysis of bacterial growth demonstrates highly significant differences among the five experimental groups ($p<0.001$). group IV (CNPs+ PUI) and group V (CNPs+ LAI) showed the most significant suppression of Enterococcus faecalis and Staphylococcus aureus colonization $32\pm10$ x$10^4$(range: 16-40) and $32\pm15.1$ x$10^4$ (range: 16-48) respectively. followed by group III (CNPs+ CNI) $82.3\pm13.5$ x$10^4$ (range: 62-96) which was the highest with contamination among the treatment groups.

The positive control exhibited the highest bacterial proliferation with a mean of $164\pm12.6$ x$10^4$ (range: 144-176), while conversely, findings in the negative control group indicated complete absence of bacterial growth ($0\pm0$ x$10^4$)

According to the current study's findings, chitosan nanoparticle activation (CNPs) irrigants using ultrasonic or laser techniques can enhance their antimicrobial efficacy opposing Staphylococcus aureus and Enterococcus faecalis within the root canal space. This is in agreement with previous reports indicating that passive ultrasonic irrigation (PUI) and laser-activated irrigation (LAI) contribute to the disruption of bacterial biofilms [34, 35] and improve debridement in anatomically complex areas of the root canal system [36, 41].

Nonetheless, complete decontamination of the root canal system was not achieved by any of the tested approaches. Thus, the null hypothesis was rejected.

## CONCLUSION

Based on the current results, it is reasonable to conclude that 0.2% nano-chitosan activated with passive ultrasonic or laser can play a substantial role in decreasing the microbial load during endodontic therapy compared with conventional irrigation group.

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