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ORIGINAL ARTICLE

# Assessment of antibacterial activity of 2.5% NaOCl, chitosan nano-particles against *Enterococcus faecalis* contaminating root canals with and without diode laser irradiation: an *in vitro* study

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**ABSTRACT**

**Objective:** This study was done to evaluate the antibacterial effect of chitosan nano-particles (CNPs) root canal irrigant as a new alternative to Sodium hypochlorite (NaOCl) for disinfection of root canals inoculated with *Enterococcus faecalis*, with and without laser activation.
**Methodology:** Sixty single rooted human premolars were decoronated, prepared and had their apical foramina sealed. *E. faecalis* were incubated in the root canals for 15 days. The teeth were then randomly divided into two experimental groups ($n = 30$) according to the disinfection protocol used. In Group I: disinfection was performed using the irrigant solutions only (Saline, 2.5% NaOCl, CNPs). Whereas in Group II, disinfection was done using the same irrigants followed by Diode laser at (980-nm) at 2 W output for $5 \times 5$ s. Intra-canal bacterial samples were taken before and after canal disinfection to determine the CFU count.
**Results:** In group I, 2.5% NaOCl was as effective as CNP in eradication and significantly more effective than Saline ($p = 0.008$) in eradication of *E. faecalis*. In Group II, either 2.5% NaOCl or CNP in combination with diode laser irradiation showed a similarly high effect in bacterial eradication.
**Conclusions:** Within the parameters used in this study, a combination therapy consisting of irrigation followed by diode laser irradiation should be utilized as an effective treatment modality for eliminating E. faecalis from root canal systems.

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## Introduction

Mechanical debridement of root canals, the use of irrigants and effective microbial control are the main factors influencing the long-term outcome of endodontic treatment. Mechanical debridement alone, can lead to reduction of the bacterial count without completely eradicating it [1]. Several root canal irrigants and disinfection techniques have been introduced to further decrease the intra-radicular bacterial count. However, evidence of their complete elimination has not been recorded in literature. Insufficient eradication of intra-radicular bacteria could be attributed to the complex root canal morphology and the organization of intra-canal bacteria into biofilms. Moreover, the protective layer formed by dentine matrix and dentine powder inhibits the antimicrobial activity of the root canal irrigants [2].

*Enterococcus faecalis* (*E. faecalis*) is a gram-positive anaerobic coccus responsible for most cases of endodontic treatment failures. It can survive under inadequate nutritional conditions and stay viable as a single microorganism. *E. faecalis* can penetrate the dentinal tubules and capable of biofilm formation. Bacterial biofilm is highly resistant to conventional irrigants because of the extracellular polymeric matrix formation. Moreover, bacterial biofilm provides nutrition and protects the bacteria from the immune system thus; it increases the resistance of microorganisms [3].

Due to the organic tissue dissolving ability and antimicrobial effect of NaOCl, it remains the most commonly used irrigant. Despite of its advantages, NaOCl has extensive drawbacks; it is cytotoxic if accidently injected into periapical tissues, has a foul taste and smell, bleaches clothes and has a potentiality to corrode metallic objects [4]. Over and above it doesn't eradicate all bacteria [5–7], or does it completely remove the smear layer [8]. NaOCl also changes dentin characteristics [9,10]. Knowing this information, an ongoing search for a safe irrigant with good antibacterial effect is recommended.

Chitosan is a natural polysaccharide found in shells of shrimps and crabs. It is biocompatible, biodegradable, shows bio-adhesion and lacks toxicity. It also shows a broad-spectrum antimicrobial property and is associated with high chelating characteristics; therefor its use in endodontics is of interest [11]. Due to the broad-spectrum activity and biocompatibility of numerous nano-particles, they achieved wide popularity as antimicrobial agents. Nanoparticles show better antibacterial activity because of their polycationic/polyanionic nature with their high surface area and charge density, showing more interaction with the bacterial cell [12].

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Because irrigating solutions act by directly contacting the target, their narrow depth of penetration into canal wall irregularities, makes them incapable of eliminating microorganisms in the deeper dentine layers [13]. Correspondingly, several researchers attempted to disinfect the complicated root canal anatomy with laser devices. Lasers seemed effective against different microorganisms; despite that, there are debates concerning the antibacterial efficiency of various lasers when used to disinfect the root canal; complete eradication of endodontic bacterial species, which grow to form biofilms, couldn't be accomplished [14]. Hence, combining irrigation together or in succession with laser has been reported to be efficient in root canal disinfection.

To date, none of the studies has consistently investigated the antibacterial effect of chitosan nano-particles (CNPs) as a root canal irrigant against *E. faecalis*. So, in this study we aimed at evaluating the antibacterial efficiency of CNP when used as an irrigant for root canals when compared to NaOCl with and without laser activation.

## Materials and methods

### Sample size calculation

This power analysis is for a 3 $\times$ 2 fixed effects analysis of variance; the first factor (Irrigant) includes three levels and the second factor (laser application) includes two levels. Based upon the results of Mehrvarzfar et al. [15], using alpha ($\alpha$) level of 0.05 (5%) and Beta ($\beta$) level of 0.20 (20%) i.e. power = 80%. The minimum estimated sample size will include five specimens per cell for a total of 30 specimens.

Sample size calculation was performed using IBM® SPSS® SamplePower® Release 3.0.1.

### Preparation of teeth

This study was conducted on 60 single-rooted human premolars. The teeth were radiographically confirmed to have a single canal and were decoronated to obtain standardized lengths of 15 mm using a diamond disc. The canal patency was checked by #10 K-file (Mani, Japan) and the working length was considered 1 mm short of the root length (14 mm). Root canals were prepared using ProTaper rotary instruments (Maillefer-Dentsply, Baillagues, Switzerland) till size F4. In between the rotary files, canals were rinsed with 2 ml of 2.5% NaOCl (Clorox, Cairo, Egypt). After completion of instrumentation, the canals were rinsed with 1ml of 17% EDTA, 5 ml saline and 1 ml of NaOCl, respectively, for 3 min each to remove the smear layer. Then, all the canals were rinsed with 5 ml Saline as a final flush. The apical foramen was sealed with self-cure glass ionomer cement (Tokyo, Japan) and the root surfaces were covered with two layers of nail varnish. Each sample was transferred to a 2-ml microtube and autoclaved at 121 °C under a pressure of 15 psi for 30 min.

### Bacterial inoculation of the root canals

A suspension of *E. faecalis* (American type culture collection [ATCC] 29212) in 2 ml thioglycate broth (Merck KGaA, Darmstadt, Germany) was prepared. The concentration of inoculation was adjusted to 1 McFarland scale. The root canals were filled with 30 µL of *E. faecalis* suspension and were incubated for 15 days at 37 °C degrees. Samples of the inoculated broth suspension were transferred to nutrient agar (37 g/1L, Merck KGaA, Darmstadt, Germany) for 48 h at 37 °C aerobically. The colony forming units of *E. faecalis* were counted prior to intervention and then converted into actual numbers.

### Experimental procedures

#### Samples grouping

After the incubation period, the specimens were randomly divided into two main groups (30 each). Each group was then subdivided into three subgroups according to the disinfection protocol used; ($n$ = 10).

In Group I; Root canal disinfection was performed using the irrigant solutions only, it was classified as follow; *Subgroup A:* (Control group*)* Canals were irrigated with 5 ml of normal saline for 5 min, *subgroup B:* Canals were irrigated with 5 ml of 2.5% NaOCl for 5 min and *subgroup C:* Canals were irrigated with 5ml of 3% CNPs (Nanostreams-Egypt; NS0115) for 5 min in accordance with the manufacturer's instructions. All the samples were irrigated using a 30 G needle (NaviTip, Ultradent, South Jordan, UT, USA) inserted short of the working length by 1 mm.

In Group II; Root canal disinfection was done using the irrigant solutions followed by the application of diode laser. Where, *Subgroup A: (*Control group) Canals were irrigated with 5 ml of normal saline for 5 min, *subgroup B:* Canals were irrigated with 5 ml of 2.5% NaOCl for 5 min and *subgroup C:* canals were irrigated with 5 ml of 3% CNPs. After irrigation, laser treatment was carried out in each subgroup with a diode laser (Wiser, doctor smile, LAMBADA spa, Italy), at a wavelength of 980 nm and output power of 2 W with the repeated pulse mode. Laser irradiation was performed five times for 5 s each time, with a 5-s interval between irradiations. The laser irradiation was delivered into the canal up to 1 mm short of the working length via a fibre tip 320 µm in diameter. The handpiece was held to form an angle of approximately 10 degrees between the fibre and the root canal wall. The protocol for diode laser was performed according to the manufacturer's instructions by a clinician proficient with the protocol. Irradiation was performed with circling movements from the apical part towards the coronal part (step-back technique) without any water spray or air-cooling. After laser irradiation, samples in the second group were treated in a similar way to the first group.

### Microbiological procedures

#### Primary sampling

A baseline microbial sample was taken from each specimen just before canal disinfection. For primary sampling. Then,

the root canals were filled with sterile saline solution using a 30-G syringe, and dentin was scraped from inside the canals using a #40 Hedstrom file (Mani, Tochigi, Japan). Then, a #40 sterile paper point (Gapadent Co, Hamburg, Germany) was placed inside the canals for 60 s and then immersed in thioglycate broth (Merck KGaA, Darmstadt, Germany) in 1.5 ml Eppendorf tubes then vortex for 30 s and incubated for 24 h at 37 degrees aerobically. Samples of the inoculated broth suspension were transferred using 0.01 μl calibrated loop to the nutrient agar (37 g/1L; Merck KGaA, Darmstadt, Germany) and incubated for 48 h at 37 °C aerobically to check the colony forming units of *E. faecalis* prior to the intervention.

### *Final sampling*

To standardize all groups, root canals were irrigated with 5 mL sterile saline, which remained in the root canals for 30 s. Sampling from inside the canals was done using a sterile #30 H-file and circumferential filing was performed for 20 seconds to collect dentin chips, mostly from the coronal and midparts of the canal. A sterile #40 K-file (Mani Inc.) was used for sampling from the apical part by reaming for 20 s. Then, sterile paper points were used in the same procedures as mentioned in primary sampling.

All procedures were carried out under sterile and aseptic conditions.

### *Statistical analysis*

The mean and standard deviation of CFU values were calculated for the samples. Viable counts of antibacterial activity were transformed to their log10 values. Degrees of disinfection in the experimental subgroups were calculated in relation to the positive controls. Data were explored for normality using Kolmogorov–Smirnov and Shapiro–Wilk tests and showed non-parametric (not normal) distribution. The CFU values were analysed by Kruskal–Wallis test. Mann–Whitney *U*-test was used for subgroup comparisons. The significance level was set at $p \leq 0.05$.

## Results

In *Group I*, significant difference was noted between subgroups A (Saline), B and C (NaOCl and CNPs) ($p = 0.008$). The lowest mean CFU/ml ($4.3 \times 10^4 \pm 1.3 \times 10^4$ and $4.6 \times 10^4 \pm 1.14 \times 10^4$) was detected in subgroups B and C, respectively, without statistically significant difference between them ($p = 0.667$) (Table 1).

In *Group II*, massive reduction in the bacterial counts was noted in subgroups B and C (NaOCl plus Laser and chitosan with laser respectively) being significantly different from subgroup A (saline with laser) ($p = 0.005$, respectively). There was no statistically significant difference between subgroups B and C ($p = 0.221$).

On comparing the two groups, a significant reduction in the bacterial counts were noted in all the subgroups of group II. A significant difference was noted between Saline and Saline plus laser ($p = 0.005$), NaOCl and NaOCl plus Laser ($p = 0.007$) and CNP and CNP plus Laser ($p = 0.008$).

## Discussion

Success of endodontic treatment requires complete disinfection and elimination of the pathogenic microorganisms from the root canal system. Eradication of bacteria from the root canals is difficult, causing current endodontic techniques to be unable to completely disinfect the root canals [16]. Endodontic therapy attempts to eliminate bacteria by utilizing protocols that combine mechanical instrumentation in conjugation with chemical irrigation using antimicrobial agents [17]. Thus, the aim of this study was the assessment of the antibacterial potency of CNP when used for irrigating the infected root canals and to clarify whether intracanal irradiation using high power diode laser in combination with 2.5% NaOCl and CNPs would be capable of eradicating canals contaminated with *E. faecalis*.

Selection of *E. faecalis* in this study was based on a belief that it is one of the most resistant microorganisms found in the infected root canals showing higher prevalence in secondary infections compared with primary infections [3]. Where, *E. faecalis* has the ability of forming biofilm, which confers resistance against phagocytosis, antibodies, and antimicrobial agents [18,19]. Also, it has long been used for the evaluation of the anti-bacterial effects of different irrigants and various laser devices [14,20].

Instrumentation of single rooted premolar teeth was performed to size F4 (Protaper system) to obtain easy access and adequate size for the fibre tip. The fibre tip was introduced short of the working length by 1 mm as to address the apical portion of the roots. Considering the fact that most of the lateral canals and ramifications are at this area, undoubtedly many portions of the canals remained out of reach of both irrigants [21].

Spangberg and Langeland conducted several *in vitro* and *in vivo* studies on a variety of irrigants [22]. They postulated that together with being highly toxic and irritating, 5% sodium hypochlorite was markedly stronger than needed to

**Table 1.** Mean, standard deviation of CFU/ml and percentage of disinfection performed by the irrigants with and without the use of diode laser.

| Groups | Subgroups | Disinfection (%) | CFU/ml (±SD) |
|---|---|---|---|
| First group (without diode laser) | Saline | 8 | $9.2 \times 10^4$ ($\pm 1.09 \times 10^4$) |
| | 2.5% NaOCl | 57 | $4.3 \times 10^4$ ($\pm 1.3 \times 10^4$) |
| | Chitosan NPs | 54 | $4.6 \times 10^4$ ($\pm 1.14 \times 10^4$) |
| Second group (with diode laser) | Saline | 88.6 | $1.14 \times 10^4$ ($\pm 2.19 \times 10^3$) |
| | 2.5% NaOCl | 99.8 | $2 \times 10^2$ ($\pm 4.47 \times 10^2$) |
| | Chitosan NPs | 99.4 | $6 \times 10^2$ ($\pm 5.47 \times 10^2$) |

Bacterial base line count was: $10^5$ CFU/ml.

kill root canal bacteria, while 0.5% concentration dissolves necrotic tissue but has no effect on *Staphylococcus aureus*. They then recommended that a solution combining maximum antimicrobial effect with minimal toxicity is the ideal concentration to be used. For this reason 2.5% NaOCl was the concentration used in this study.

In the first group, our results were in agreement with Shretha et al. [18] and Carpio-Perochena et al. [23] who stated that CNPs can be used as a final irrigant due to its potentiality to act as an anti-biofilm agent. In our study, there was no statistically significant difference between 2.5% NaOCl and CNPs in the eradication of *E. faecalis* from the root canals. Where, CNPs showed potentiality in completely eliminating *E. faecalis* found in the planktonic state, and also were capable of causing a heavy reduction in the number of bacteria in the biofilm state as stated by Kishen et al. [12] and Shertha et al. [18]. Several hypotheses have been postulated to explain the antibacterial mechanism of chitosan. One is based on its cationic nature that interacts with the negatively charged bacterial cell membranes, increasing its permeability, resulting in leakage of the cytoplasmic contents and bacterial cell death [24]. Another hypothesis is based on the ability of chitosan to chelate metals, thus inhibiting the microbial growth by reducing enzyme activity through metal chelation. In addition, CNPs can penetrate the bacterial cell membrane, bond to its DNA and inhibit its transcription and mRNA synthesis together with its capacity to prevent bacterial enzymatic degradation reducing the likeliness of penetration of bacteria and dentinal micro-fractures [23,25].

In general, the antibacterial efficacy of the nanoparticles is attributed to two particular mechanisms; the first concerns binding of the nanoparticles to bacterial cell membrane by electrostatic forces, leading to a change in the membrane potential and loss of membrane integrity, causing disturbance of major bacterial cell functions such as respiration and disturbance of energy transduction, causing bacterial cell death [26]. The second involves producing free oxygen radicals, which influence bacterial cell survival by blocking the protein function, destroying DNA and resulting in excess radical production [27].

Since, the introduction of diode laser to the field of laser-aided endodontics by Moritz et al. [28] it has provided a safe and effective means for endodontic treatment, although a few studies declared that diode laser was incapable of eliminating *E. faecalis* completely [14,29,30]. However, Moritz et al. [28] revealed that total bacterial eradication could only be accomplished if teeth were disinfected using higher-power irradiation that produced high temperatures on root surfaces. Many authors demonstrated the effectiveness of Diode laser in eradicating diverse microorganisms [28,31].

The wavelength of 980-nm in diode lasers had the strongest water absorption, so accordingly it's greatly absorbed in superficial dentinal tubules, which are fortified with water [30]. Therefore, superficial dentin layers get the majority of the antibacterial effect while the deeper layers gain less. Correspondingly, bacteria that penetrate deeper into the tubules can be secured from laser irradiation; hence, the disinfection capacity of the 980-nm diode laser is decreased. The use of optic fibres with fine diameters (320 μm) enables effective delivery of laser to the root canal, reducing the bacterial contamination. It can reach over 1 mm deep into the dentin, [14] surpassing the effective range of chemical disinfectants, as NaOCl.

In the present study, the results of the second group showed that laser treatment after sterile saline irrigation was only able to disinfect root canals up to 88.6%. In accordance with Sohrabi et al. [32] who stated that the efficacy of the 980-nm diode laser in reducing bacterial counts was acceptable. The high-power diode laser has shown promising results for the disinfection of root canals [33,34] due to its ability to reach parts of the tissue and areas where classical techniques and instruments cannot [35–37]. Though other studies reported that the antibacterial effect of diode laser on dentin slices with a thickness of 100 μm is greater than 95% [14,29], which is contradicting to the results of this study. The variations may be attributed to that they used dentin slices and performed sampling for microbiological analysis immediately after irradiation, while in this study we used human teeth. Also, Gutknecht et al. [37] stated that the high-power laser eliminated 97% of the bacteria. The difference in results could be due to the difference in lasing parameters such as pulse length, fluency and irradiance, which affect the anti-bacterial effect [38].

Our results showed that the combined use of diode laser irradiation with 2.5% NaOCl or with CNPs was able to eliminate *E. faecalis* from the canals at a very high efficient rate (99.8% and 99.4%, respectively), which was in accordance with Mehrvarzfar et al. [15], who recommended the combination therapy; which includes chemical irrigation and laser irradiation as a potent treatment regimen for eliminating *E. faecalis* from root canals. Also, Rahimi et al. [39] reported that lasers have limited effect in root canal disinfection compared to the combination of laser with NaOCl. In a corresponding study, de Souza et al. [40] concluded that laser irradiation following chemo-mechanical irrigation was more effective than NaOCl irrigation alone in terms of root canal disinfection and elimination of *E. faecalis*.

## Conclusions

Chitosan nano-particle can be utilized as an antibacterial irrigant. Based on the laser parameters utilized in this *in vitro* study, it can be declared that a combined therapy composed of irrigation followed by laser irradiation should be utilized as an efficient treatment modality in elimination of *E. faecalis* from the root canal system. Laser irradiation is a potential integration to an existing protocol for root canal disinfection rather that being an alternative.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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