

EVALUATION OF ANTIBACTERIAL EFFECT OF DIODE LASER 980 NM, TRIPLE ANTIBIOTIC PAST, AND CALCIUM HYDROXIDE ON *ENTEROCOCCUS FAECALIS* BIOFILM

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ABSTRACT

Aim: The purpose of this study is to evaluate the anti-biofilm capacity of Diode laser 980 nm, triple antibiotic mixture and calcium hydroxide.

Methods: Eighty five single-rooted teeth with mature apices were prepared using Protaper Universal rotary nickel titanium system till size # F4 then contaminated with *E.faecalis*. Irrigation done using 2.5% sodium hypochlorite followed by 17% EDTA. Samples were divided into 4 groups (n= 20) according to the irrigant activation method. Laser group, TAP group, Ca(OH)₂ group and the control group. Residual bacteria were plated onto Brain Heart Infusion media and determined as colony-forming units (CFU mL⁻¹). Data were analyzed using one way ANOVA followed by performance of Tukey post hoc tests. Significance was set at p < 0.05.

Results: There was a statistically significant reduction in the mean numbers of colony-forming units among all groups. However, none of the activation methods was able to kill *E. faecalis* biofilm completely. The Laser group behaved most effectively among all groups.

Conclusion: The adjunctive use of 980nm laser is an effective method for bacterial reduction after chemo-mechanical instrumentation of the root canal.

KEYWORDS: Diode laser, TAP, antibiofilm activity.

INTRODUCTION

Microorganisms found in the infected root canal space are colonizing either as free-floating planktonic cells or attached to each other or to the root canal walls to form biofilms⁽¹⁾. Though planktonic microorganisms can be eradicated by different methods, the elimination of biofilm

bacteria from the root canal remains a foremost task⁽²⁾.

A biofilm is a community of microorganisms embedded in a matrix of extracellular polymeric substance and attached to a solid surface. It has been believed that within this community the biofilm bacteria have different phenotypes, with different

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characteristics, than do the same bacteria in their planktonic state. Notable among these differences is the increased resistance to antimicrobial agents⁽³⁾. Since the bacteria in the necrotic root canal grow frequently in biofilm forms, the success of endodontic treatment will depend on the effective elimination of such biofilms⁽⁴⁾.

Facultative gram-positive *Enterococcus faecalis* is one of the most resistant bacteria discovered in infected root canals, especially in cases with persistent apical periodontitis forming intraradicular biofilms⁽⁵⁾. Numerous researches have described their low susceptibility to irrigant solutions⁽⁶⁾ and to intracanal medicaments such as calcium hydroxide^(7,8,9).

Consequently, efforts to eradicate microorganisms could link with achieving effective disinfection. Placement of intracanal medicaments such as calcium hydroxide is frequently suggested as it has high pH that alters the bacterial lipopolysaccharides in the cell wall⁽¹³⁾. However, it has been shown that this high pH is not maintained⁽¹⁴⁾. Furthermore, the increase in pH, can enhance the bacterial attachment to collagen fibers of dentine thus protecting them from the disinfection processes⁽¹⁵⁾. Also, it can decrease the strength of dentine, even with short span use⁽¹⁶⁾ and it has low ability to penetrate dentinal tubules⁽¹⁷⁾.

Antibiotics are used as an adjunct to endodontic treatment but its ineffectiveness in systemic route of administration has led to the intracanal application to increase its efficacy [18]. Hoshino et al in 1996⁽¹⁹⁾ suggested sterilizing infected root canals by topical use of mixture of ciprofloxacin, metronidazole and minocycline. After that, several studies reported the antimicrobial efficacy of this mixture against the pathogens commonly found inside the root canal system including *E. faecalis*^(20,21).

Recently, disinfection of root canals using Diode Laser has received focus as an alternate antibacterial disinfection protocol for drug-resistant microorganisms⁽²²⁾. Diode lasers emitting at 980 nm transmit energy through thin flexible fibers that are

compatible with the dimensions and curved shapes of root canals. The power output of these lasers ranges from 0.5 to 7 W and is delivered in two operating modes: continuous wave and pulsed mode. The use of modern laser technology has the great help in reaching areas that are not accessible⁽²³⁾. However, very few studies have examined the efficacy of Diode laser in total eradication of intracanal biofilms, and literature needs further investigations. Hence, this study was undertaken to compare the antibacterial efficacy of diode laser, triple antibiotic mixture, and calcium hydroxide against intraradicular *E. faecalis* biofilm.

MATERIALS AND METHODS

Sample preparation:

After approval of the local Ethics committee, eighty five single-rooted teeth with mature apices extracted for periodontal reasons, and free from wear facets or apical resorption, were collected from 45-55 year old patients attending the outpatient clinic at the Oral Surgery Department, Faculty of Dentistry, Ain Shams University, Cairo, Egypt and stored in 0.9% physiological saline at room temperature. The patients were informed about the use of their teeth for scientific purposes.

The teeth were decoronated using a safe sided diamond disc (NTI diamond disc, Axis Dental, USA) mounted on a high-speed contra-angle with water coolant and roots standardized to a length of 10 mm.

All samples were prepared with Protaper Universal rotary nickel titanium system (Dentsply Maillefer, Ballaigues, Switzerland) till #F4 6%. The root canals were irrigated with 3 ml of 2.5% NaOCl at each change of file followed by 17% EDTA solution for 1 minute for smear layer removal; and, later rinsed with saline and dried using paper points. The roots were then waterproofed externally using cyanoacrylate and all apical foramina were sealed with composite resin. Finally, the roots were steam autoclaved at 134°C for 15 minutes.

Cultivation of *E. Faecalis* Biofilm

A clinical reference isolate of *E. faecalis* from the Microbiology Laboratory (Microbiology Department, Faculty of Medicine, Ain Shams University, Egypt) was cultured on brain-heart infusion agar (BHI) (Land Bridge Technology Co, Ltd, Beijing, China) (BHI) and incubated anaerobically at 37°C for 24 hr. A single colony was collected and suspended in sterile BHI broth at 37°C. The root canals were placed into 1.5-mL Eppendorf tubes with 1mL BHI broth containing 10⁸ colony-forming units (CFU)/mL *E. faecalis* and then incubated anaerobically at 37°C for 3 weeks. The sterile BHI broth was refreshed every other day to ensure bacteria viability. Five specimens were randomly selected and examined by SEM to ensure the presence of *E. faecalis* biofilm⁽²⁴⁾.

Classification of Samples

Eighty contaminated roots were randomly divided into 4 groups (n = 20) according to the disinfection protocol as follow

Ca(OH)₂ group

Calcium hydroxide and iodoform paste (Metapex®, Meta Biomed Co., Ltd, Korea) was introduced into canals. The paste was vertically compacted using an endodontic plugger size 4 (Dentsply, Tulsa Dental Specialties, Tulsa, OK) till the entire canal was filled.

TAP group

The triple antibiotic paste was prepared using metronidazole (500-mg tablets [Flagyl 500 mg; Aventis, Cairo, Egypt]), ciprofloxacin (250-mg tablets [Ciprocin 250 mg; EPICO, Cairo, Egypt]) and doxycycline (100-mg capsules [Vibramycin; Pfizer, Cairo, Egypt]). The doxycycline capsule content was evacuated in a sterile mortar; a tablet of metronidazole and a tablet of ciprofloxacin were crushed and ground into homogenous powder in the same mortar using a pestle. Saline drops were added and mixed using the pestle until a creamy paste was

achieved. The canal was filled with the paste using the same procedure as Ca(OH)₂.

Laser group;

Intracanal irradiation was performed using a high power 980 nm diode laser (Fotona, EU) and set at a power of 7 W and 50–60 Hz frequency. It was divided into 3 sessions in which the total irrigation volume and time were standardized as 5mL and 2 min but the irrigant was different. 2.5% NaOCl, 17% EDTA and finally sterile saline were the irrigants used in each session respectively.

Using an oscillatory technique, the diode fiber (200µm fibre optic tip) was introduced 1 mm short of the apex and recessed in helicoidal movements at a speed of approximately 2 mm/sec for 5 seconds, and repeated 8 times at intervals of 10 seconds. This laser activation procedure was repeated 3 times.

Control group; The samples were left empty.

All samples were incubated for a week at 37°C under humid conditions.

Assessment of Antibacterial Activity

After one week, the samples were irrigated with 20 ml sterile saline solution to remove the root canal contents. Bacterial sampling and CFU counting protocols were used to determine the antimicrobial efficacy in each group (25).

Briefly, following all treatments, canals were filled with sterile 0.85% normal saline solution, #40 H-file was introduced in the canal and churned for 1 minute, and then two #40 sterile paper points were inserted into the canal to collect the bacteria for 1 minute. The paper points were transferred into 1 mL normal saline, and the process was repeated 3 times. All samples were vortexed for twenty seconds and 10-fold dilutions were prepared in saline. Aliquots of 0.1 ml were spread plated onto BHI agar plates, incubated at 37°C for 48 hours, and colony-forming units (CFU) per 1 mL were enumerated.

Statistical analysis

CFU/mL values were analysed by using ANOVA. Statistical analysis was performed using the SPSS program for Windows 10.0 (spss Inc., Chicago, IL, USA) ($P < 0.05$).

RESULTS:

Results are demonstrated in (Table 1). The bacterial concentration in the control group had the highest number of microorganisms that revealed that bacteria survived the test period confirming the competence of the methodology.

For all experimental groups, there was a statistically significant reduction in the mean numbers of colony-forming units. However, none of them resulted in complete elimination of biofilm bacteria.

Overall, calcium hydroxide showed the weakest antimicrobial activity, and the tri antibiotic paste

and laser were higher. However, tri antibiotic paste and laser did not show statistical significance differences ($P < .05$).

SEM was done as an additional method to confirm the presence/absence of biofilm. Overall, a significant amount of bacteria revealed as biofilm was observed after 3 weeks of *E. faecalis* inoculation covering the entire dentinal surface (Fig. 1). In $\text{Ca}(\text{OH})_2$ group, SEM images showed similar results when compared with the CFU/mL data. Canals treated with Laser and TAP displayed a relevant bacteria-free surface

TABLE (1) Mean and standard deviations of CFU($\times 10^4$)/mL in all groups

| Group | Control | $\text{Ca}(\text{OH})_2$ | Laser | TAP |
|-------------------|-------------------|--------------------------|-------------------|-------------------|
| CFU $\times 10^4$ | 10.28 \pm | 4.39 \pm | 0.55 \pm | 1.15 \pm |
| Mean \pm SD | 1.49 ^a | 0.69 ^b | 0.12 ^c | 0.24 ^c |

Mean values followed by different lower case letters represent statistically significant differences ($P < .05$).

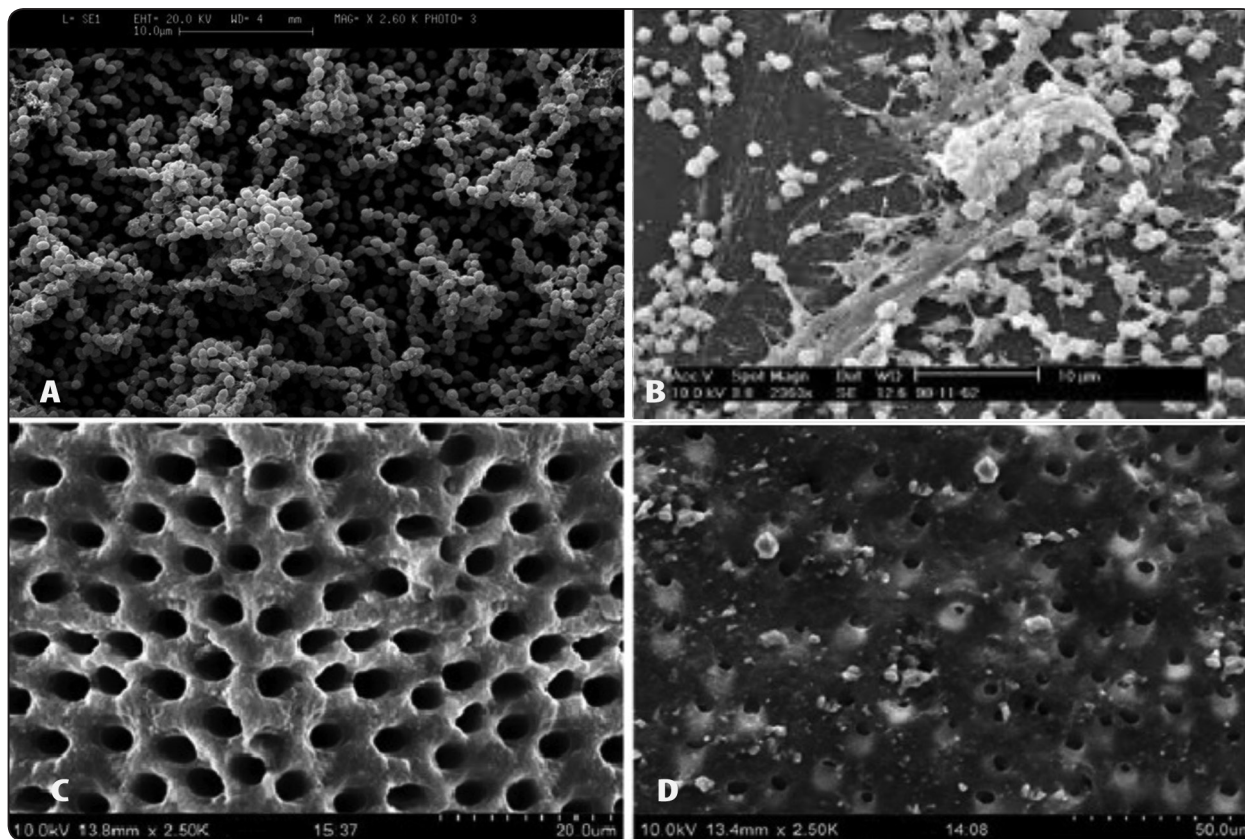


Fig. (1) SEM (X1500) showing : A. *E. faecalis* biofilm after 3 weeks of inoculation covering the entire dentinal surface. B: $\text{Ca}(\text{OH})_2$ treated group with fewer adhering bacteria. C: laser treated sample and D: TAP treated sample.

DISCUSSION

The aim of this study was addressed to assess the difference between laser-activated irrigation and the use of two different root canal medications in microbial reduction in infected root canals. Yet, under clinical conditions, those are only one of the steps to treat endodontic infections. The antimicrobial control stage also includes the use of irrigant solutions and mechanical instrumentation, which were both standardized.

E. faecalis was selected as the test species since its proved microbial role in persistent root canal infection. Human permanent teeth were used as the samples to mimic the clinical scenario. Studies evaluated the efficacy of a protocol in removing biofilms grown in wells⁽²⁶⁾, on membrane filters⁽²⁷⁾ and on dentin samples⁽²⁸⁾. However, the bacterial colonization structure on dentine with dentinal tubules and considerable amount of unmineralized type I collagen that serves as an adhesion substrate to oral streptococci represents more logic way^(29,30).

Despite the high initial bacterial levels, all protocols were efficient in reducing significantly the levels of *E. faecalis*.

It had been proved that $\text{Ca}(\text{OH})_2$ is an effective intracanal medicament⁽¹⁷⁾ and it is the dressing most recommended for the treatment of infected root canals. Unfortunately, new concern has grown up about the limited antimicrobial efficiency of $\text{Ca}(\text{OH})_2$ against some microorganisms commonly found in infected root canals including *E. faecalis* that can withstand its high pH^(31,32). In the current study, $\text{Ca}(\text{OH})_2$ failed to effectively eliminate *E. faecalis* from infected root canals in human teeth. The findings of the current study are consistent with recent researches⁽³³⁻³⁵⁾ but contradict the findings of others⁽³⁶⁻³⁸⁾. According to previous studies, the reasons for this were attributed to improper amount of hydroxyl ions reached after 1 week^(39,40), or the buffering action of dentin⁽¹⁴⁾.

Tri antibiotic paste use has been previously reported for the treatment of necrotic teeth with open apexes^(41,42). It was shown to have inhibitory

effect on *E. faecalis* biofilm due to the components of this paste. It is made from metronidazole, which has a wide bactericidal effect against obligate anaerobes that are common in infected root canals. Certain bacteria are resistant to metronidazole; hence, ciprofloxacin and minocycline were added to achieve better antimicrobial action^(19,43). Our results showed a good ability of this paste to kill bacteria inside the biofilms in contrast to calcium hydroxide. However, this paste could not kill 100% of the bacteria inside the biofilm. This was in consistent with previous works in which the antimicrobial activity of these medications against oral bacteria, and their ability to sterilize infected dentin has been confirmed^(19,20,44). Unfortunately, Minocycline binds to calcium ions by chelation to form an insoluble complex causing tooth discoloration. Hence, TAP should be limited to the root canal^(45,46). In addition, an adequate antibiotic concentration that avoids toxicity to host stem cells has not been completely addressed yet⁽⁴⁷⁾.

Consequently, there is a need to search for other antimicrobial method with comparable properties to the tested tri antibiotic paste deprived of its accompanied detrimental side effects.

Despite the proved efficacy of various lasers against microorganisms, studies in regards to laser's antimicrobial ability in root canal system are controversial. Our results showed reduction in CFU in laser group. This can be attributed to the direct delivery of laser energy by the used fine diameter optic fiber (200-320 μm) that enabled effective delivery of laser light deeply into the root canal. The laser energy absorbed by the bacteria caused a photo-thermal interaction resulting in a bactericidal effect⁽⁴⁸⁾. Diode laser has high permeability and low interference with dentin, which allows the laser to be effective on microorganisms that have penetrated into the dentinal tubules⁽⁴⁹⁾. Coluzzi⁽⁵⁰⁾ reported that diode lasers have a depth of penetration per pulse that is 10,000 times greater than that of the Er:YAG laser and may act more deeply within the dentinal tubules. Gutknecht et al.⁽⁵¹⁾ reported that irradiation with a 980-nm diode laser can eliminate bacteria

that have migrated deep into the dentine (up to 500 μm), whereas chemical solutions can only reach 100 μm .

In this study, single straight root canals were selected to facilitate comparison of the bactericidal effect among different agitation techniques under a standard condition. However, in clinical practice, there are many teeth with curved root canals. On this occasion, the bactericidal effect of laser systems might be affected if the optical fiber could not easily advanced in the canals. Therefore, evaluation of the bactericidal effect of these systems in curved root canals and naturally infected root canals (*ex vivo*) would be performed in our future investigations.

In the present study, microorganisms were collected with sterile paper points from the root canal system as done in previous studies^(24,25). This sampling method has limitations because the paper points are only able to detect planktonic bacteria. Moreover, the paper points cannot access irregularities and other regions of the root canal system. Consequently, this approach might fail to harvest viable bacteria in biofilms and in some areas of the root canal system⁽⁵²⁾. Thus, we accomplished the bacterial sampling by instrumenting the canal wall with sterile #40 to remove dentine, permitting for a more predictable sampling.

Additionally, some cells of *E. faecalis* in biofilms can enter a stationary phase, which makes them undetectable with conventional culture methods⁽⁵³⁾. Thus, the data obtained from CFU counts must be interpreted with caution⁽⁵⁴⁾.

CONCLUSION

The diode laser systems, at the current settings had comparable properties to the tested tri antibiotic paste in reducing *E. faecalis* in the infected tooth model *in vitro* deprived of accompanied detrimental side effects of tri biotic paste. The search for laser applications in endodontics should however be continued and different laser wave lengths and settings should be examined.

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