ORIGINAL ARTICLE

Antibacterial Efficacy of Sodium Hypochlorite, Ozonated Water, and 980 nm Diode Laser Used for Disinfection of Root Canal against *Enterococcus faecalis*: A Microbiological Study

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Abstract

Aim: Evaluation and comparison of the antibacterial efficacy of sodium hypochlorite, ozonated water, diode laser, and diode laser in combination with ozonated water against *Enterococcus faecalis*.

Materials and methods: One hundred and twenty extracted premolar teeth were sectioned at the cementoenamel junction and root canals were prepared using step-back technique with K-file up to #40. The teeth were arbitrarily allocated to four groups and stowed into brain heart infusion broth containing a microbial suspension of *E. faecalis*. Group I samples were irrigated with 3% sodium hypochlorite. Group II samples were irrigated with ozonated water. Group III samples were irrigated firstly by distilled water followed by laser irradiation. Group IV samples were irrigated by ozonated water followed by laser irradiation. After the treatment, the teeth were kept in vials containing 2 mL of nutrient broth. The vials were then incubated at 37°C for 24 h. Standard methods were then used to identify grown colonies.

Statistical analysis: Kruskal–Wallis test, Mann–Whitney test, Chi-square test, and Wilcoxon signed-rank test were used to measure the colony-forming units (CFUs) obtained at the end of the incubation period among the various groups for the evaluation of antibacterial efficacy of various disinfection protocols.

Results: The highest mean CFU/mL is recorded in group II (with ozonated water) followed by group I (with sodium hypochlorite), group III (with a laser), and the least mean CFU/mL is seen in group IV (with laser and ozonated water). The variation in CFU/mL among the three groups is statistically significant ($p < 0.05$).

Conclusion: The outcome of this study reveals that 980 nm diode laser along with ozonated water when used together can eliminate and disinfect the root canals with *E. faecalis*.

Keywords: 980 nm diode laser, *Enterococcus faecalis*, Ozonated water, Sodium hypochlorite.

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Introduction

Endodontic therapy includes removal of the microflora and irritants from the canal and periapical tissues and thorough debridement is essential for the treatment to be successful.[1] Progression of dental caries to pulpal and periapical diseases is mainly due to the involvement of different microbial species.[2] Thus, endodontic treatment aims to acquire a sterile environment in the root canal to reduce chances of failure of the treatment,[3] as the root canal system is very complex; various irrigants are used as an adjunct along with mechanical preparation for the disinfection of root canals.

Enterococcus faecalis is a microorganism that can withstand severe challenges and is considered to be a crucial organism for the collapse of endodontic treatment. It accounts for 4–40% of primary endodontic infections; in fact, it is nine times more likely to exist in failed root canal treatment than primary endodontic infections.[4] Sodium hypochlorite and hydrogen peroxide, or the combination of both, are the most frequently used endodontic irrigants. Sodium hypochlorite (sodium hypochlorite) is widely used as an irrigant at concentrations ranging from 0.5 to 6%. Sodium hypochlorite when mixed with water forms hypochlorous acid that contains active chlorine, a strong oxidizing agent that contributes to its prophylactic properties. It leads to invariable oxidation reaction, thus disrupting the metabolic actions of the bacterial cell.[5] However, studies of the interaction mechanism of sodium hypochlorite and hydrogen peroxide suggested that the fear of toxicity of sodium hypochlorite and hydrogen peroxide as an irritant to periapical tissue has tended to discourage its use. Thus, the need for newer irrigants with less toxicity and more biocompatibility arise. With the institution of lasers in the field of dentistry, endodontic treatment is enhanced by a myriad of treatment options that raise the scope of favorable therapy outcomes.

Laser therapy has been efficient in sterilizing the root canal walls and specifically lateral dentinal tubules, which are not quite reachable in conventional treatment and can be considered as cisternae for disease-causing pathogens.[6] Diode laser use in root canal therapy surged in the contemporary era, particularly due to the antimicrobial properties of lasers.

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Historically "Ozone" was known as "ozein" in the Greek era signifying "to smell". It is an inorganic and allotropic molecule of oxygen with the chemical formula (O_3) also known as trioxygen or triatomic oxygen. It is a light blue gas with a strong odor and half-life of 40 minutes at 20°C. It is readily formed in the upper atmosphere when ultraviolet (UV) rays cause oxygen (O_2) atoms to temporarily recombine in groups of three; it can also be formed by the action of electrical discharge on O_2 and can absorb harmful UV rays. In 1839, German chemist Christian Frederick Schonbein was successful in separating the gaseous chemical and called it "ozone", on this basis, he is acreddited for discovering ozone. In 1932, Dr E A Fisch, a Swiss dentist used ozonated water as a disinfectant.[7,8] Ozone is used in medicine for many years; the properties of ozone contribute to being a non-stable gas and it rapidly releases nascent O_2 molecule to form oxygen gas, this nascent oxygen is effective in oxidizing any biological entity and function as a strong germicide which can extinguish microorganisms of all entity. Its action has been demonstrated against *E. faecalis*.[9] According to the reports, it was found that ozone at concentration, as low as 0.1 ppm, is adequate to render inactivity bacterial cell walls and their spores.[10] It occurs naturally in the atmosphere and can be simply generated by an ozone generator. When introduced in water, ozone dissolves rapidly and dissociates quickly. In this context, ozone is considered a feasible antimicrobial agent in dentistry due to its high antimicrobial potency and low probability of drug resistance. Thus, the purpose of the present research was to assess and compare the antibacterial efficacy of sodium hypochlorite, ozonated water, diode laser, and diode laser in combination with ozonated water against *E. faecalis*.

Materials and Methods

The study was carried out at the Department of Pedodontics and Preventive Dentistry, K.D. Dental College and Hospital, Mathura, and Department of Microbiology, College of Life Sciences, Gwalior (M.P). The study protocol was assessed, analyzed, and accepted by the review committee of K.D. Dental College and Hospital, Mathura (U.P).

Sample Preparation

One hundred and twenty human premolar teeth were used for this study; the teeth were extracted for orthodontic and periodontal reasons. The extracted teeth were then placed in saline solution, followed by thorough ultrasonic cleaning of the tooth surfaces, and were then sectioned at a cementoenamel junction with the help of a high-speed water-cooled diamond disk. The root canal length was finalized using 15#K file up to the apical foramen followed by pulp extirpation. The working length was established up to the apex of the apical foramen. Root canal preparation was done up to ISO size 50 using the crown-down/step-back technique. The coronal third of the canals were enlarged with #2, #3, and #4 Gates Glidden drills (Dentsply Corp.). The prepared teeth were air-dried overnight at room temperature and the apices of foramen were sealed externally with temporary cement (Temp RS-Prime Dental Products) and waterproofed with transparent nail varnish. The teeth were then sterilized in an autoclave at 121°C for 15 minutes at 15 lbs pressure and thereafter all teeth were stored in a sterile environment until further use.

Media Preparation for *E. faecalis*

The microorganism strain used for the study was obtained from Microbial Type Culture Collection (MTCC), Chandigarh and were cultured according to the supplier's instructions. An uncontaminated, single colony of *E. faecalis* was isolated from the cultured plate and Gram's staining was done to validate its growth, which was observed under the microscope, and then a loop full of the bacterial colony was extracted from the cultured plate with the help of inoculum loop and was inoculated in a test tube containing 10 mL of prepared brain heart infusion (BHI) broth. The broth underwent an incubation cycle at 37°C for a duration of 24 hours and changes in turbidity were recorded to observe bacterial growth. A drop of BHI containing *E. faecalis* was mixed into the saline solution and a spectrophotometer was used to check correct bacterial concentration. The density of the bacterial suspension is standardized by comparing the broth at a density equivalent to the barium sulfate standard of 0.5 McFarland units, which is equivalent to 1.5×10^8 colony-forming units per milliliter (CFU/mL). Before the procedure, a micropipette with a sterile needle was used to inoculate the canals with 10 mL of the bacterial suspension in BHI broth. The samples were then vaguely divided into four groups.

Group I: Samples were irrigated with 3% sodium hypochlorite.
Group II: Samples were irrigated with ozonated water.
Group III: Samples were irrigated firstly by distilled water followed by laser irradiation.
Group IV: Samples were irrigated by ozonated water followed by laser irradiation.

Thirty teeth specimens in each group before the commencement of treatment procedure were placed in an upright position in autoclaved microtip holder box and then their canals were inoculated with 0.2–0.4 mL of bacterial suspension in BHI broth. After that, the box was sealed with paraffin wax and wrapped in sterile aluminum foil, and placed in the incubator at 37°C for 24 hours.

Procedure

Group I

After 24 hours, the teeth samples were treated with 3% sodium hypochlorite, for this, the canals were thoroughly irrigated 3–4 times with sodium hypochlorite solution. The treated teeth samples were then placed in sterile vials containing 2 mL of nutrient broth and incubated at 37°C for 24 hours.

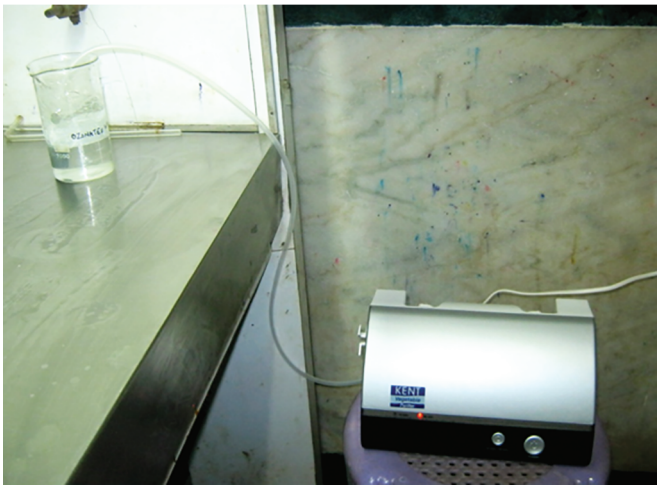


Fig. 1: Preparation of ozonated water using a Kent ozone generator

Group II

After 24 hours, the teeth samples were treated with ozonated water which was prepared by Kent Ozone water purifier (Fig. 1). The canals were thoroughly irrigated with ozonated water 3–4 times with a 5 mL syringe and the treated samples were transferred into sterile vials.

Group III

After 24 hours, the teeth specimens were irradiated with a diode laser (Denlase) with energy set at 3 W using an oscillatory technique as suggested by Gutknecht et al. The optical fiber is introduced 1 mm short of the apex and is recessed in helicoidal movements at a speed of approximately 2 mm/second for 5 seconds, repeated 4 times at intervals of 10 seconds, between each one. This rest period between irradiation avoided temperature change. Sterile saline solution was used as an intermediate irrigant. The teeth were then transferred into sterile vials (Fig. 2).

Group IV

After 24 hours, the teeth specimens were treated with a diode laser (Denlase) with energy set at 3 W using an oscillatory technique as suggested by Gutknecht et al. The optical fiber is introduced 1 mm short of the apex and is recessed in helicoidal movements at a speed of approximately 2 mm/s for 5 seconds, repeated 4 times at intervals of 10 seconds, between each one. This rest period between irradiation avoided temperature change. Ozonated water was used as an intermediate irrigant. The teeth were then transferred into sterile vials.

Bacteriological Analysis

After the procedure, teeth were placed in vials containing 2 mL of nutrient broth and incubated at 37°C for 24 hours. The vials were then evaluated for turbidity after 24 hours of incubation. After that, 2 mL of broth from all the vials containing treated samples was collected and seeded on Petri dishes containing UTI Hichrome Media. Total 120 Petri dishes were used for each treated sample to count the CFUs using the pour plate method in which the sterile Petri dish is inoculated with 2 mL of nutrient broth containing treated samples followed by pouring of melted 20 mL of UTI Hichrome Agar into the Petri dish. Then, it was mixed thoroughly by tilting and swirling the dish after which the agar was allowed to completely gel without disturbing it for 10 minutes. The Petri dishes were then kept in an inverted position in the incubator; incubation lasted for 48 hours at 37°C. The grown colonies were observed and CFUs were counted using open CFU (3.9.0) software (Fig. 3).

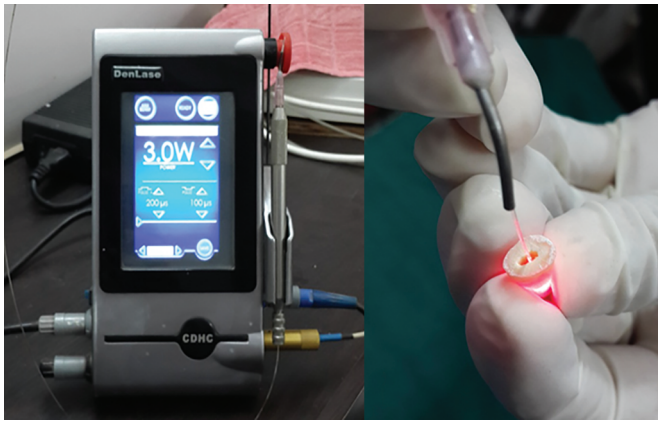


Fig. 2: Teeth samples treated with 980 nm diode laser

Statistical Analysis

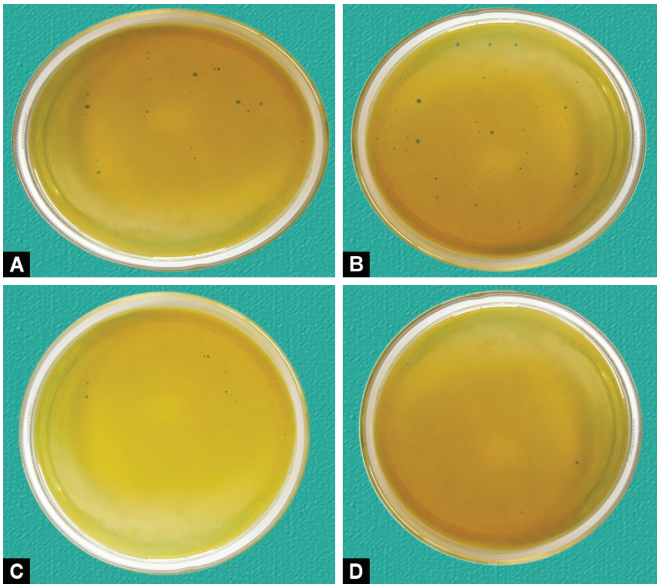
The data thus obtained were subjected to statistical analysis which was performed using SPSS (Statistical Package for Social Science) version 14 for Windows 7. Kruskal–Wallis test, Mann–Whitney test, Chi-square test, and Wilcoxon signed-rank test were used to compare the CFUs obtained at the end of the incubation period among the various groups for the evaluation of antibacterial efficacy of various disinfection protocols. The significance level for all the statistical tests utilized in this study was set at $p < 0.05$ (5%).

Results

A Kruskal–Wallis test was conducted to assess differentiation among the four test groups (conventional sodium hypochlorite, ozonated water, high-power diode laser, and diode laser along with ozonated water) on median change in a total amount of CFUs after treatment with respective test agents. The test, which was rectified for tied ranks, was very highly significant X^2 (4, $N = 120$) = 105.186, $p = 0.001$. Maximum efficacy was shown by group IV followed by groups III, I, and II, respectively (Table 1 and Fig. 4). Group IV showed the least mean rank values (20.75) for average CFU's count than the other three groups with the highest mean rank values (105.37) for ozonated water group (Table 2). A follow-up test, i.e., the Mann–Whitney test for intergroup comparison, was conducted to evaluate pairwise differences among the four groups. The outcome of this test indicated a significant difference among group I and group II, group I and group III, group I and group IV, group II and group III, group II and group IV, and group III and group IV with the *p* value <0.001 (Table 3).

Discussion

Diseases of the root canal system consist of multi microbial flora with a relatively equivalent amount of gram-positive and gram-



Figs 3A to D: CFUs (colony-forming units) after treatment: (A) Sodium hypochlorite group (group I); (B) Ozonated water group (group II); (C) Laser and saline group (group III); (D) Laser and ozonated water group (group IV)

Table 1: Nonparametric tests for comparison of averages between groups

CFU/mL	N (total number of samples)	Percentiles			Interquartile range (IQR)
		25	50 (Median)	75	
Laser and normal saline (group III)	30	5.00	8.00	9.00	4.00
Laser and ozonated water (group IV)	30	2.75	4.00	6.00	3.25
NaOCl (group I)	30	18.00	21.00	22.00	4.00
Ozonated water (group II)	30	27.75	31.00	32.00	4.25

The median values of the experimental and control group comprising 30 samples each using non-parametric test and it was observed that group II (ozonated water) showed the highest median value (31) and the lowest median value (4) was observed for group IV (laser and ozonated water)

Table 2: Kruskal–Wallis test for comparing average CFUs between four groups simultaneously

Group	N	Mean rank
NaOCl (control) (group I)	30	75.63
Ozonated water (group II)	30	105.37
Laser and normal saline (group III)	30	40.25
Laser and ozonated water (group IV)	30	20.75
Total	120	

	CFU/mL
Chi-square	105.186
Df	3
p value	<0.001

The Kruskal–Wallis *H* test which showed that there was a highly statistically significant difference in colony-forming units between the different test groups, χ^2 (4) = 105.186, $p = 0.001$, with a mean rank score of 75.63 for group I, 105.37 for group II, 40.25 for group III, and 20.75 for group IV

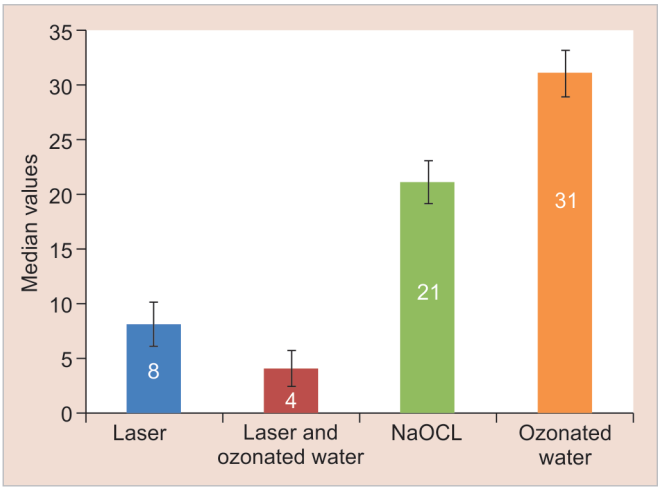


Fig. 4: Median values for all the groups

Table 3: Comparison table of all the groups with *U* (Mann–Whitney test), *W* (Wilcoxon signed rank test), *Z*-test, and *p* values

Comparison	N (total no. of samples)	U (Mann-Whitney test)	W (Wilcoxon test)	Z-test	p value
Group I and group II	60	4.00	469.00	−6.618	<0.001
Group I and group III	60	0.00	465.00	−6.675	<0.001
Group I and group IV	60	0.00	465.00	−6.675	<0.001
Group II and group III	60	0.00	465.00	−6.670	<0.001
Group II and group IV	60	0.00	465.00	−6.669	<0.001
Group III and group IV	60	157.50	622.50	−4.351	<0.001

The statistical result of the intergroup comparison. From the Mann–Whitney test (*U*), Wilcoxon signed-rank test (*W*), and *z*-test the probability value (*p* value) for all the groups is <0.001, which is <0.05 and is very highly significant

negative bacteria, the existence of which is responsible for the failure of root canal treatment. Thus, the fundamental aim of standard root canal treatment is to attain a sterile, prepared root surface free of endodontic pathogens and there toxins which aid in the placement of an adequate root filling material.[11] Studies demonstrated that microorganisms are capable of infiltrating the dentinal lumen approximately to a depth of 1,000 to 1,100 µm, on the other hand, the irrigation done with caustic irrigants penetrate up to 130 µm in the dentinal lumen.[12] This lab research assessed the antimicrobial effects of four sterilization methods, which could be used in addition to chemomechanical canal preparation. The microbial marker used was *E. faecalis*, as it often survives chemomechanical preparation because of its defiance to antimicrobial agents and its potential to cause a monoinfection in the root canals.[13]

The most commonly and regularly used endodontic irrigant is sodium hypochlorite. It possesses the dual benefit of pulpal cessation and antimicrobial potential. Sodium hypochlorite has strong basic (pH > 11) properties due to its chloramine molecules which acts as an organic solvent, causing amino acid degradation and hydrolysis. Sodium hypochlorite proved to be an efficient irrigant for all strains of *E. faecalis* including its existence as a biofilm. It breaks down bacterial proteins into amino acids via the action of free chlorine in it which dissolves vital and necrotic tissue faster with higher concentrations[14] but are more toxic if they come in contact with vital tissue. A 5.25% sodium hypochlorite solution is more effective in a shorter time because of its high concentration of hypochlorous acid when in contact with organic tissues.[15] In cases of extravasation, the 5.25% sodium hypochlorite can, due to its high cytotoxicity,[16,17] cause sequelae such as pain, swelling, bruising, and stupor. Eldeniz et al.[17] observed that 3% sodium hypochlorite inhibited the growth of *E. faecalis* and provided complete elimination of the bacteria in all canals. According to Mithra et al.,[18] 3% sodium hypochlorite was the most effective

irrigant comparatively and in a combination of 2% CHX and laser was as effective as 3% sodium hypochlorite.[19] Thus in our study, we have used 3% sodium hypochlorite as a positive control to minimize cytotoxicity yet retain antimicrobial efficacy.

The powerful bactericidal property of ozone can kill microorganism efficiently; when oxygen is passed through high-voltage strongly reactive oxygen molecule, i.e., ozone is generated. Aqueous ozone can be used as a conventional irrigant instead of using irrigation chemicals for endodontic treatment. Ozone was found to be successful in eliminating a wide array of pathogenic microflora like *E. faecalis*, *Candida albicans*, *Peptostreptococcus micros*, and *Pseudomonas aeruginosa* from root canals and dentinal lumen. In this study, the ozone (O_3) was generated by discharging electrical current on oxygen molecules having the highest purity using a generator manufactured by Kent healthcare products. But gaseous ozone when inhaled has pernicious effects on the respiratory system, hence, aqueous ozone might be favorable in managing oral infections.[20] Aqueous ozone was made by bubbling ozone gas through sterile distilled water (O_3 concentration 24 mg/L). The selection of the ozonated water concentration (24 mg/L) was according to the higher concentration, which the generators can produce.[21] Ozone output of the Kent Ozone generator is 200 mg/hour and so to obtain ozone concentration of 24 mg/L, bubbling of ozone through sterile distilled water was carried out for 7 minutes. According to Nagayoshi et al.,[21] aqueous ozone had nearly equivalent antimicrobial efficacy like 2.5% sodium hypochlorite when used as an irrigant for endodontic treatment. On the contrary, Hems et al.[9] evaluated the potency of ozone to cease an *E. faecalis* strain and also confirmed that its antibacterial efficiency was not comparable to sodium hypochlorite.[22] Similarly, the findings of this study clearly showed the superiority of the control, i.e., 3% sodium hypochlorite over ozonated water irrigation with the results showing higher CFUs/mL in ozonated water group than the control group. The antimicrobial efficacy of various lasers against *E. faecalis* has been evaluated and documented in the dental literature.[23] It was proposed that, along with the refined elimination of debris and smear layer, dental lasers can provide significant accessibility to previously impermeable parts of the dentinal tubules because of their increased penetration properties; consequently, they may have additional antimicrobial effects to help in the reduction of bacteria in the root canal. Studies performed in relation to the antimicrobial effect of diode laser with different parameters showed that this laser can be efficacious in the reduction of intracanal bacteria and penetrates to a depth of 500 µm in dentin. Research studies in which diode laser radiation when used in combination with sodium hypochlorite and oxygenated water for disinfection of root canal, superior results were obtained.[24]

In recent years, different laser systems are used in the endodontic field, which is effective for root canal disinfection. Initially, Nd:YAG laser was first used for root canal disinfection, introduced by Hardee and Myers and McDaniel.[24,25] The diode laser is a compact device and is now used in varied fields of dentistry. Moritz et al.[26] introduced a diode laser for root canal disinfection. *Enterococcus faecalis* is a non-sporiferous vegetative microorganism and is resistant to high temperatures, commonly seen in cases of treatment defiant infection. Due to its heat resistance, *E. faecalis* was preferred for this study to examine the outcome of the laser irradiation. Premolar teeth with single canals were prepared up to #40 K-file to achieve adequate size and easy access for the fiber tip.

The methodology used for counting the CFUs in the present study revealed that diode laser irradiation produced an elevated level of sterilization in contrast to other groups without laser irradiation with a statistically significant difference. Unlike neodymium laser which provokes dentine melting, the diode laser diminishes dentine permeability.[27] Light emitted by diode laser acquiesce for higher absorption by water than dental tissues, this property leads to a higher amount of laser light permeability across the dentinal lumen with less reciprocal action on the dentinal tissues, which makes it desirable to work on the pathogenic microflora present in the dentinal tubules. Optic fibers of diode laser have very fine diameters (200–320 µm) which allow sufficient transmission of laser light through the root canal region, the antibacterial effect detected reaches up to the depth of 1 mm and above into the dentin, surmounting the effective range of chemical disinfectants, such as sodium hypochlorite and exhibit marked effectiveness against *E. faecalis* even in the abysmal layers of dentin.[27] Moritz et al.[26] determined that irradiation with a diode laser in two consequent sessions resulted in substantial eradication of bacteria and implied that the diode laser is considered equivalent to the Nd:YAG laser in endodontic treatment. Schoop et al.[27] stated that Nd:YAG and diode lasers at 1 W are potent against *E. faecalis*; while the power elevated to 1.5 W; only the diode laser was effective against the microorganisms. In the present study, the diode laser was used at a power of 3 W, although complete sterilization cannot be achieved, a significant bacterial reduction was seen. The parameters used in this study were considered safe in accordance with Radaelli et al.[28] The diode laser application in endodontic treatment as a support results in an increased success rate, which should be accustomed by performing *in vivo* studies. Furthermore, the findings revealed significant differences between conventional sodium hypochlorite irrigation, ozonated water, high-power diode laser, and diode laser along with ozonated water, and the highest reduction in CFUs was seen in the diode laser along with ozonated water group which can be partially explained by the combined antibacterial effect of both the test agents together. In this study, the 980 nm diode laser along with ozonated water and the 980 nm diode laser along with saline irrigation were superior to 3% sodium hypochlorite and ozonated water when used as an irrigant for the eradication of *E. faecalis* from the root canal region.[29] Nevertheless, to determine the most efficient root canal sterilization protocol, the effectiveness of the procedure has to be assessed on multiple species biofilm. Hence, it is imperative to assess their existent beneficence to traditional chemomechanical preparation *in vivo* studies.

Conclusion

Depending on the outcome of the current study, the subsequent inferences could be summed up:

- Significant reduction is seen in CFUs in all the test groups.
- There has been a very highly statistically significant difference among all four groups.
- Highest number of reduction in CFUs was seen in group IV (laser and ozonated water) and hence it could be concluded that laser and ozonated water when used together were most efficacious in eradication and sterilization of the root canals contaminated with *E. faecalis*.

Clinical Significance

Thus, utilizing the combination of laser radiation and aqueous ozone can be used as a possible alternative against various caustic endodontic irrigants and can replace them with relatively mild biocompatible irrigants that help in the eradication of *E. faecalis* within the root canals.

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