

Comparative Evaluation of Antibacterial Efficacy of 5% Sodium Hypochlorite, 940 nm Diode Laser, and Gaseous Ozone against *Enterococcus faecalis* Biofilm formed on Tooth Substrate: A Scanning Electron Microscope Study

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ABSTRACT

Aim: To comparatively assess the antibacterial effectiveness of 5% sodium hypochlorite, 940 nm diode laser, and gaseous ozone against *Enterococcus faecalis* biofilm produced on the substrate.

Materials and methods: Freshly extracted maxillary central incisor teeth of human source were decoronated and vertically sectioned after biomechanical preparation.

The samples were then, after sterilization, placed in Eppendorf tubes filled with 1 mL of bacterial solution containing 1.5×10^5 colony-forming units (CFUs)/mL of *E. faecalis*. Then, these adulterated samples were divided into four groups ($n = 8$) depending upon the method of disinfection used: group I, 5% sodium hypochlorite (positive control); group II, normal saline (negative control); group III, gaseous ozone; and group IV, 940 nm diode laser.

At the conclusion of 3 weeks, all the samples were disinfected according to their groups and were analyzed qualitatively and quantitatively.

Results: The positive control group (5% sodium hypochlorite) showed statistically significant results in comparison with the other three groups ($p < 0.05$). Statistically, there was no significant difference found between the experimental groups, i.e., ozone group and diode laser group ($p > 0.05$).

Conclusion: A 5% sodium hypochlorite showed the highest antibacterial effect against *E. faecalis* biofilm formed on substrate, i.e., tooth. Both diode laser and gaseous ozone groups have a statistically significant antibacterial action on the infected root canals.

Keywords: Biofilm, Diode laser, *Enterococcus faecalis*, Ozone, Root canal disinfection.

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INTRODUCTION

In nature, microorganisms play a pivotal role in pulpal death and periapical infections.¹

The ability of the microorganisms to colonize in the root canal system plays the primary etiologic factor in pulpal and periradicular diseases.² The primary aim of an endodontic treatment is to eliminate or substantially decrease the microbial load in the root canal system, which is conventionally achieved by chemomechanical instrumentation, using various mixtures of canal irrigants and files, followed by canal obturation, thereby preventing recolonization of bacteria.

The anatomical complexity of the root canal system acts as a reservoir for numerous bacterial species, which might result in biofilms. Biofilm is defined as a sessile and multicellular microbial community characterized by cells that are firmly attached to a surface and enmeshed in a self-produced matrix of extracellular polymeric substance.³ Oral bacteria grow exclusively (or almost) in biofilms.

Thus, the main goal of oral disinfection is mainly biofilm eradication, along with the destruction of the remaining active bacteria.⁴ The *E. faecalis* is a Gram-positive facultative anaerobe that is the most commonly isolated and persistent bacteria in root canals. It survives endodontic treatment procedures, shows strong adhesion to collagen, attaches to the dentin, and infests the dentinal tubules.⁵

It also resists high concentrations of drugs and wide variations in pH and shows high bacterial resistance to several irrigation solutions and medications used in endodontic therapy. And more importantly, these bacteria have the ability to produce biofilms. Therefore, *E. faecalis* can survive for a long time in the root canals.^{6,7} The studies related to the eradication of root canal infections

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use *E. faecalis* biofilm models since it is the representative bacteria of secondary infections in both its planktonic and biofilm forms.^{8,9} The commonly utilized irrigants, for their antibacterial efficacy, are sodium hypochlorite and chlorhexidine. However, the effectiveness of these commonly used strategies (sodium hypochlorite, chlorhexidine, alone or combined) is limited by the emergence of resistant strains of microorganisms and biofilm formation. Persistence of even a small proportion of viable bacteria can largely, if not completely, regenerate the community.³ Also, though sodium hypochlorite is proven to be the most efficacious because of its ability to fix necrotic tissues and its antibacterial action,¹⁰ it presents with some disadvantages like its unpleasant taste, cytotoxic effect on periradicular tissues, caustic nature, irritation to the tissues, inflammation to the gingiva, bad odor, incomplete elimination of the smear layer, and incomplete eradication of all bacteria from infected root canals.¹¹

Many researchers are still looking for better disinfection procedures that will be effective against all bacteria, and simultaneously be biocompatible with periradicular tissues so as to eradicate contamination from root canals. Ozone (O₃), a natural, pale blue gas that is found in our environment, is a strong oxidizing agent and can be produced by generators.¹² Ozone causes cellular lysis as an antimicrobial agent. In the presence of liquid, ozone generates oxides which act as free radicals and these radicals disrupt the osmotic balance of the cell membrane causing its lysis. This process depends upon the rate of reaction time.¹³ In dentistry, ozone is used to enhance healing of soft tissues postsurgically. It is also used for removal of root caries and in endodontic procedures.¹⁴ The research on ozone systems still continues to progress, so that they can be used more effectively in root canals.¹⁵ Among the new advancements in the field of lasers, a diode device is interesting for researchers and clinicians alike due to its small dimension, cost effectiveness, power outputs, and different operating modes.¹⁶ Diode lasers can be used as an accessory to root canal disinfection.

There have been different wavelengths for diodes described in endodontics: 810,¹⁷ 830,¹⁸ 940,¹⁹ and 980 nm.¹⁶ Recently, the 940 nm wavelength has gained popularity because it was found to generate a limited form of cavitation in aqueous fluids around the top of the fiber tip.¹⁹

Just as with the neodymium-doped yttrium aluminum garnet (1,064 nm) laser, this wavelength of the diode laser has a deep penetration into the dentinal tubules.¹⁶ Taking into account the role of *E. faecalis* and its by-products in the causation of persistent pulpal and periapical diseases, the aim of this study was to assess the efficacy of ozone and diode laser as newer antimicrobial strategies against the conventional sodium hypochlorite in the eradication of *E. faecalis* biofilm in root canals.

MATERIALS AND METHODS

Preparation of Teeth Specimens

This study used 48 freshly extracted human single-rooted maxillary central incisors with matured closed apices that had not undergone any endodontic treatment. Superficial debris, remnants of periodontal ligament fibers, and calculus were removed from the root surfaces with the help of a periodontal scaler, mechanically.

In order to confirm the canal anatomy, each tooth was radiographed buccolingually and mesiodistally. The teeth were then stored in normal saline at room temperature for 24 hours to prevent dehydration. Then, they were sectioned horizontally below the cemento-enamel junction with a diamond disk to produce a standardized root length of 12 mm in size.

Canal patency was established with a size 15 K-file (Mani Co., Tokyo, Japan). The canals were then instrumented using the crown-down technique and rotary instruments (ProTaper, Dentsply Maillefer Ballaigues, Switzerland), and the canals were enlarged to a standardized apical size F3 for each tooth sample. During the cleaning and shaping procedure, there was sequential use of 2 mL of 2.5% sodium hypochlorite, and 17% ethylenediaminetetraacetic acid was used alternatively between each instrument.

For the final irrigation wash, 2% chlorhexidine was used. Then, the teeth were vertically sectioned in a mesio-distal direction into two halves, along their midsagittal plane, to achieve a flat surface so that they can be placed in the Eppendorf tubes, thus exposing maximum root canal surface to *E. faecalis*, so that it can form a biofilm. The sectioned samples were then divided into four experimental groups.

Each group consisted of 12 samples (n = 12). They were assigned as group I, 5% sodium hypochlorite (positive control); group II, normal saline (negative control); group III, gaseous ozone; and group IV, 940 nm diode laser. Then, each specimen was immersed in 1 mL normal saline within a 2 mL Eppendorf tube for sterilization. This was then autoclaved at 121°C for 20 minutes.

Preparation and Inoculation of *E. faecalis*

A pure culture of *E. faecalis* (American Type Culture Collection 29212) (Sola Civil Medical College and Hospital, Ahmedabad, India) was developed on Mueller-Hinton agar (Himedia, Mumbai, India), inoculated in Mueller-Hinton broth (Himedia, Mumbai, India), incubated at 37°C overnight, and adjusted to an optical density (OD₆₀₀) of 1 and McFarland standard to provide a suspension of approximately 1.5 × 10⁵ CFU/mL.

Root Canal Infection

The bacterium was cultured as described previously, and the tubes containing tooth samples were inoculated with 1 mL of bacterial solution and incubated at 37°C. The culture medium was replenished every alternate day for 3 weeks to avoid nutrient depletion and accumulation of toxic end products. The samples were taken from each well with a sterile paper point, inoculated onto Mueller-Hinton agar plates, and incubated at 37°C for 24 hours to check for cell viability and purity of culture.

At the end of 3 weeks, all the samples were disinfected according to their assigned groups as group I, 5% sodium hypochlorite (positive control); group II, normal saline (negative control); group III, gaseous ozone; and group IV, 940 nm diode laser.

Experimental Groups

Group I: 5% sodium hypochlorite (n = 12): The specimens were immersed in 1 mL of 5% sodium hypochlorite for 5 minutes.

After this procedure, the specimens were stored in a sterile saline solution. Group II: normal saline (n = 12)⁶: the specimens were immersed in 1 mL of normal saline for 2 minutes. Group III: gaseous ozone (n = 12): an ozone generator device, (Sonimix 3001 Ozone Generator, LN Industries SA, Geneva) was used by attaching a sterile cannula on the exposed surfaces of the root canal. The ozone analyzer was used to check the values and calibrated to 1.4 ppm.

The generator was stabilized for 1 hour before exposing the specimen. The specimens were exposed to ozone gas through the sterile cannula for 240 seconds. Group IV: diode laser (n = 12): Laser treatment was performed with a diode laser (Ezlase 940, Biolase, San Clemente, California, USA) and the endodontic tip (ezTip Endo, 14 mm/200 mm), at a wavelength of 940 nm and output power of 3.5 W with a repeated pulse mode using a pulse duration of 0.05 ms

and a pulse interval of 0.2 ms according to the instructions of the manufacturer. The exposed tooth surface was irradiated for 30 seconds on. After the treatment, the biofilm on the root canal portion was scraped off and inoculated on Mueller-Hinton agar plates and incubated for 24 hours at 37°C for qualitative analysis.

The quantitative analysis was performed by vortexing the tooth samples with sterile saline for a few minutes followed by serial dilution method for all the groups. All the procedures were carried out in a laminar flow chamber.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance and compared by *post hoc* test using Statistical Package for the Social Sciences software (student version 7.01; SPSS Inc, Chicago, IL). The criterion for statistical significance was defined as $p < 0.05$.⁷

RESULTS

After 21 days of incubation period, the initial total CFU count, which was 10^5 , had increased to 10^8 in each specimen. The *E. faecalis* biofilms could not be eliminated 100% from the root canals of the positive control group. Many strata of bacteria were still agglomerated around the opening of the dentinal tubules.

But, after the use of sodium hypochlorite, ozone, and diode laser, it was noted that structurally the biofilm was destroyed and replaced with ruptured bacterial colonies. All the treated groups showed reduced CFU of *E. faecalis* as compared with the negative control group ($p < 0.05$). The positive control group (5% sodium hypochlorite) showed statistically significant results when compared with the other three groups ($p < 0.05$) (Figs 1 to 8).

Statistically, there was no significant difference found between the experimental groups, i.e., ozone and diode laser groups ($p > 0.05$) as shown in Tables 1 and 2.

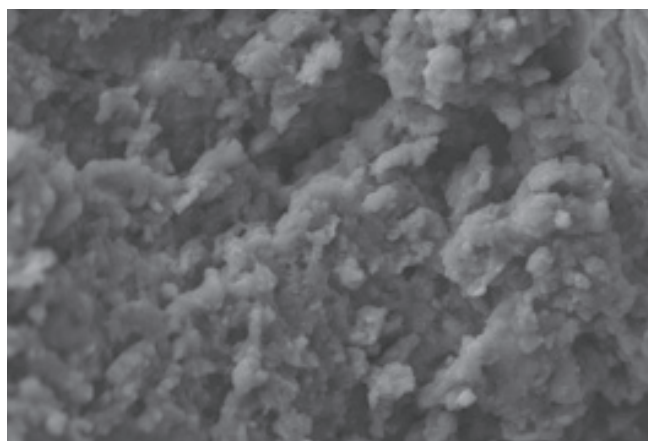


Fig. 1: Twenty-one-day *E. faecalis* biofilm

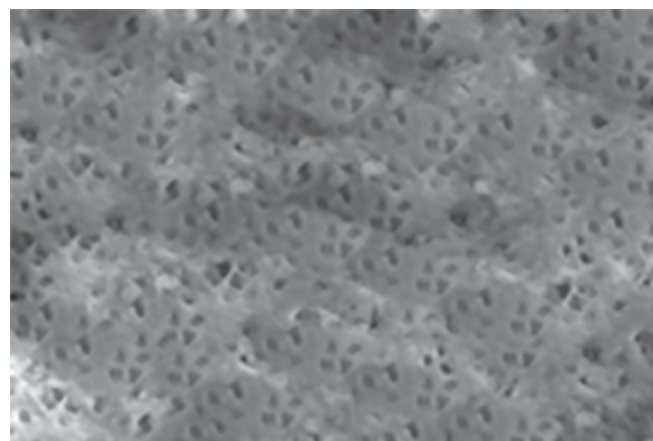


Fig. 2: Group I: treated with 5% sodium hypochlorite for 5 minutes (1,000×)

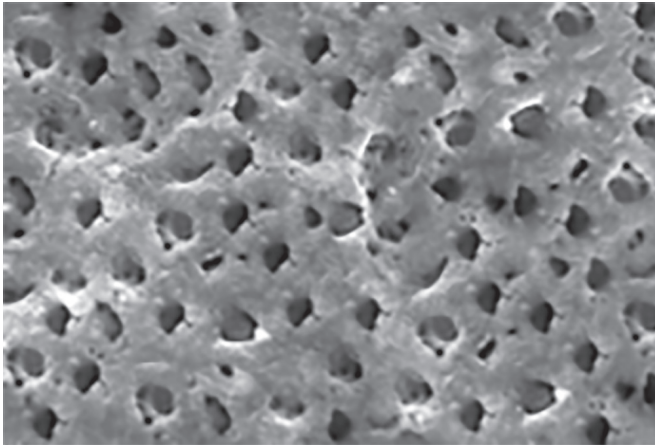


Fig. 3: Group I: treated with 5% sodium hypochlorite for 5 minutes (1,500×)

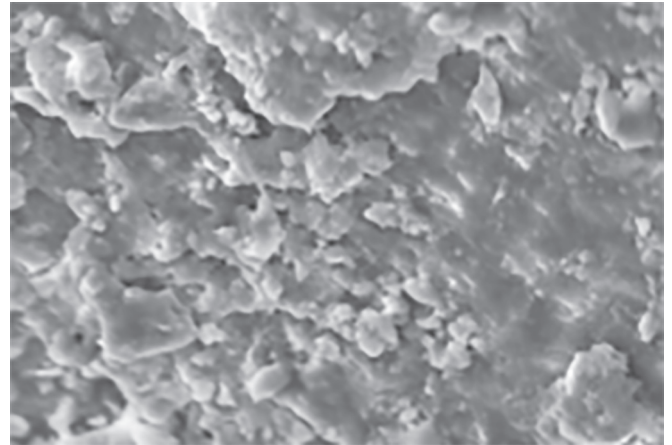


Fig. 4: Group II: treated with normal saline for 2 minutes

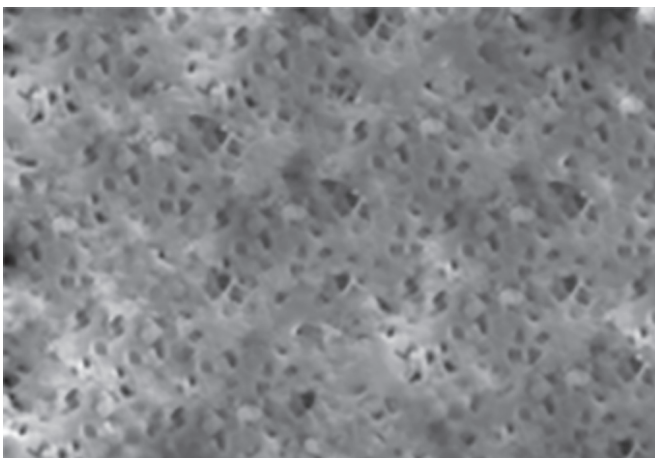


Fig. 5: Group III: treated with gaseous ozone for 240 seconds (1,000×)

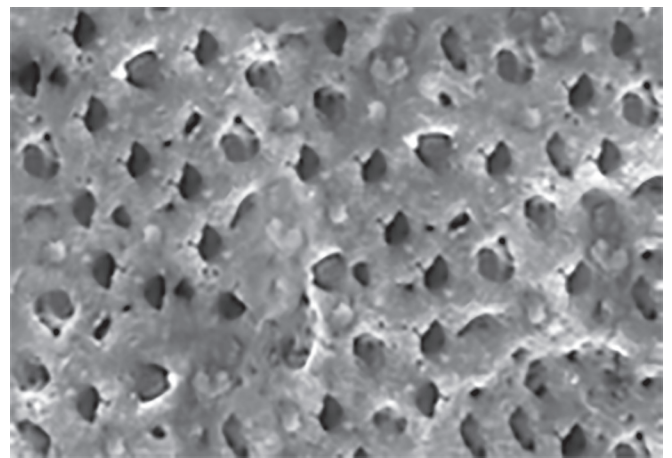


Fig. 6: Group III: treated with gaseous ozone for 240 seconds (1,500×)

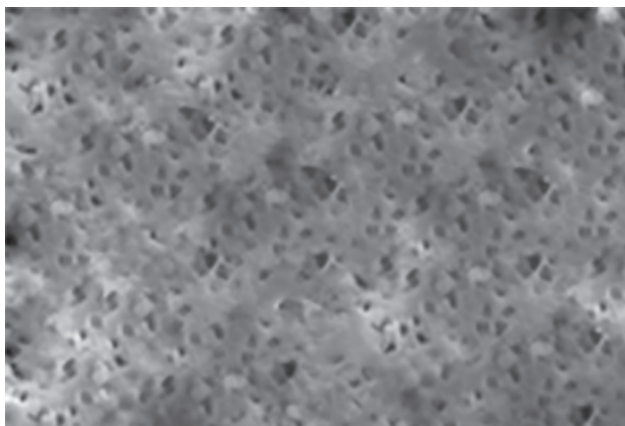


Fig. 7: Group IV: treated with 940 nm diode laser for 30 seconds (1,000×)

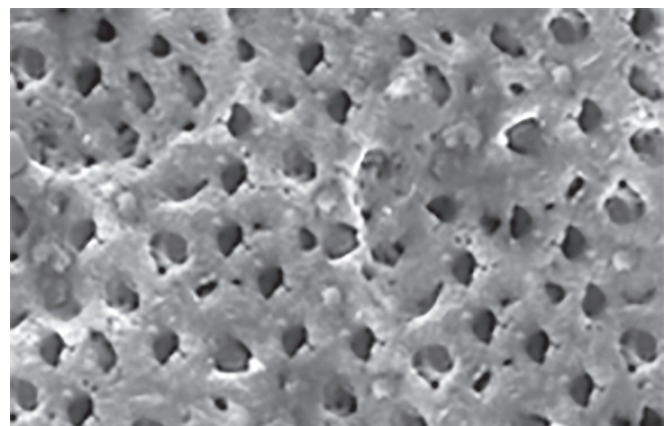


Fig. 8: Group IV: treated with 940 nm diode laser for 30 seconds (1,500×)

Table 1: One-way ANOVA ($\times 10^5$ CFU/mL)

	n	Mean	Std. deviation	Std. error	95% confidence interval for mean		Minimum	Maximum	ANOVA p-value
					Lower bound	Upper bound			
Hypochlorite	8	700.5	96.39	34.08	619.92	781.08	591	861	<0.001
Normal saline	8	64,251.25	14,877.66	5,260.05	51,813.22	76,689.28	41,300.00	81,300.00	
Laser	8	5,533.75	1,684.47	595.55	4,125.50	6,942.00	3,730.00	7,970.00	
Ozone	8	6,173.75	1,971.00	696.85	4,525.96	7,821.54	3,290.00	8,980.00	

ANOVA: Analysis of variance

Table 2: Post hoc test (multiple comparisons Tukey HSD)

(I) Group	(J) Group	Mean difference (I-J)	Std. error	p-value	95% confidence interval	
					Lower bound	Upper bound
Hypochlorite	Normal saline	-63,550.75	3,775.55	<0.001	-73,859.17	-53,242.33
	Laser	-4,833.25	3,775.55	0.583	-15,141.67	5,475.17
	Ozone	-5,473.25	3,775.55	0.48	-15,781.67	4,835.17
Normal saline	Laser	58,717.5	3,775.55	<0.001	48,409.08	69,025.92
	Ozone	58,077.5	3,775.55	<0.001	47,769.08	68,385.92
Ozone	Laser	-640	3,775.55	0.998	-10,948.42	9,668.42

DISCUSSION

The *E. faecalis* is the most important *Enterococcus* species persisting in treated root canals and are resistant to traditional antibiotics.²⁰ When *E. faecalis* develops into a biofilm, the structure of the biofilm becomes complex and its genetic and metabolic processes are altered; this prevents the entry as well as the action of several antimicrobial agents.²¹ The antibiotic resistance increases up to 1,500 times when compared with its planktonic form.²² The dissolution of the dentin surface by the *E. faecalis* and its ability to form calcified biofilms on root dentin may lead to its persistence after endodontic treatment.²³

It has been proven that the chemical nature of the substrate influences the biofilm-forming capacity and its structural organization. So, the experiments conducted on polycarbonate or glass substrate are not true measures of the bacteria-substrate interactions.²⁴ Hence, in accordance with the methodology done by Kishen et al²³ a tooth substrate was used to form the *E. faecalis* biofilm in this study.

All the groups were tested while being in direct contact with the biofilm formed on tooth substrate at 21 days. Sodium hypochlorite is widely used as an irrigant during endodontic treatment. It can create large zones of inhibition against *E. faecalis*.²⁵ However, the data regarding the inhibitory concentration and application time of sodium hypochlorite against *E. faecalis* are not very clear enough in the literature yet.²⁶ In our study, a significant effect was achieved with 5% sodium hypochlorite irrigation for 5 minutes, in accordance with the previous studies. The bactericidal effectiveness of ozone is based on forming oxygen radicals in aqueous solutions, as a result of which the cell membranes become damaged due to altered osmotic stability and permeability.²⁷ However, there is no unanimous agreement when it comes to the application manner, time, and dosages of ozone needed to achieve significant results.

Hems et al²⁸ reported that when *E. faecalis* cells were treated with ozone, over time periods of 30 to 240 seconds, using nutrient broth as the medium, there was a decrease in the bacterial counts up to 1- to 2-log.¹⁰ Also, a significant decrease could be achieved only after a 240 seconds application.

In the present study, ozone had an important antibacterial effect eliminating a significant amount of *E. faecalis* biofilms in 240 seconds.²⁹⁻³⁵ There were significant reductions of *E. faecalis* biofilms in the ozone group ($p < 0.05$); however, it was not superior to 5% sodium hypochlorite. A number of studies have put forth that the use of diode laser as an accessory to conventional root canal disinfection can have an added value.¹⁸

It became clear from these studies that it was difficult to compare the results due to differences in wavelength and settings. Nevertheless, the use of diode lasers remains interesting because of its small dimensions, cost-effectiveness, power outputs, and operating modes.¹⁶ For the present study, the manufacturer’s recommendations were followed, using a 940 nm diode laser at 3.5 J powers, a length of pulse of 0.05 ms, and amplitude of 0.2 ms. A special tip for the laser made by the same manufacturer (ezTip Endo, 14 mm/200 um) was used for ease of use.

This power of diode laser has to be taken into account because the thermal effects of the diode laser can cause many dentinal cracks, destroy the periodontal ligament, and cause root resorption. Also, the temperature changes should be examined in future investigations at this power with this length of pulse and amplitude.

CONCLUSION

Under the limitations of this study, it can be concluded that sodium hypochlorite is still the most effective irrigant when it comes to killing endodontic pathogens. Further investigations are still needed to comprehend better the proper application and maintaining the required protocols of ozone and diode laser.

Ozone and diode laser, for the current scenario, could be considered ideal devices, if used toward the end of canal treatment, as an adjunct to improve the effect of the conventional irrigating solutions.

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