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Original Research Paper

Dental Science

IN VITRO EVALUATION OF ANTIMICROBIAL EFFICACY OF DIODE LASER WHEN USED ADJUNCTIVELY WITH SODIUM HYPOCHLORITE, METHYLENE BLUE OR TOLUIDINE BLUE IN ENTEROCOCCUS FAECALIS CONTAMINATED ROOT CANALS

Dr. N. Shubhashini MDS	Professor, Department of Conservative Dentistry & Endodontics, RajaRajeswari Dental College & Hospital, Bangalore.
Dr. Vinaya Kumar R MDS*	Professor, Department of Periodontology, RajaRajeswari Dental College & Hospital, Bangalore. *Corresponding Author
Dr. Annapoorna Kini MDS	Professor, Department of Conservative Dentistry & Endodontics, RajaRajeswari Dental College & Hospital, Bangalore.
Dr. Swetha H.B. MDS	Professor, Department of Conservative Dentistry & Endodontics, RajaRajeswari Dental College & Hospital, Bangalore.
Dr. Srikanth Choudhary B.S. MDS	Professor, Department of Conservative Dentistry & Endodontics, HKDET's Dental College Hospital & Research Institute, Humnabad.
Dr. Nithin Kumar Shetty MDS	Assistant Professor, Department of Conservative Dentistry & Endodontics, Faculty of Dental Sciences, Ramaiah University of Applied Sciences, Bangalore.

ABSTRACT

Aim: To evaluate antimicrobial effect of diode laser when used adjunctively with sodium hypochlorite, methylene blue or toluidine blue in Enterococcus faecalis inoculated root canals.

Methodology: 70 extracted uniradicular premolars were prepared with ProTaper files and sterilized. 5 were kept aside as negative control (G1) and the remaining 65 were inoculated with Enterococcus faecalis and incubated for 7 days. Following this, 5 were kept as positive control (G2) and the other 60 were randomly distributed into six groups: G3, diode laser irradiation (1.5W) with sodium hypochlorite; G4, diode laser (3W) with sodium hypochlorite; G5, diode laser (1.5W) with methylene blue; G6, diode laser (3W) with methylene blue; G7, diode laser (1.5W) with toluidine blue; G8, diode laser (3W) with toluidine blue. Subsequently, turbidity was assessed and CFU count determined following intracanal sampling and plating.

Results: G4 exhibited significantly lower mean CFUs/mL ($P<0.001$) among the experimental groups. G4 demonstrated the most (98.8%) while G7 exhibited the least antibacterial effect (28.66%). Highest turbidity was observed in G5 (0.1310) while the lowest was noted in G4 (0.0287).

Conclusion: Diode laser used adjunctively with sodium hypochlorite exhibited superior antibacterial efficacy when compared to its use with photosensitizer dyes like methylene blue and toluidine blue.

KEYWORDS : Enterococcus faecalis, lasers, methylene blue, sodium hypochlorite, toluidine blue.

INTRODUCTION

The effectiveness or the success of root canal treatment depends on mechanical instrumentation along with the use of appropriate irrigants and intra canal medicaments placed between appointments. Despite mechanical instrumentation (rotary/hand), large areas in the root canal system remain untouched. Hence, irrigants have been used as an adjunct to instrumentation in order to reach these inaccessible areas.[1] Sodium hypochlorite (NaOCl) is the most commonly used endodontic irrigant as its efficacy and advantages has been demonstrated by numerous studies. However, limitations such as its high tissue toxicity and low tubule penetration depth have reduced its use.[2]

E. faecalis is a gram positive facultative anaerobic bacterium that has been implicated in resistance/reinfection leading to failure of root canal treatment. It persists and remains viable within the dentinal tubules despite chemo-mechanical instrumentation and use of intracanal medicaments as it is found at depths greater than 1mm in the root dentine, while irrigants reach a maximum depth of only 100μm.[3,4,5]

Novel approaches in disinfecting the root canal such as lasers and photoactivated disinfection have been recently advocated.[1] Laser has a better penetrability in areas inaccessible to irrigants and/or intracanal medicaments.[6] Lasers with wide range of wavelengths have been used in endodontics, namely Nd:YAG laser, diode laser, Er:YAG laser and Er,Cr:YSGG laser. The diode laser is comparable to the Nd:YAG laser in terms of its effectiveness in root canal disinfection.[5] The antibacterial effect is dose dependent and adjunctive use of photosensitizer dyes has also been evaluated.[1]

Limited data is available in literature comparing the efficacy of the 'gold standard' irrigant – NaOCl and the more recently introduced photosensitizer dyes in disinfecting *E. faecalis* contaminated root canals when used in conjunction with the diode laser. Hence this in-vitro study was undertaken to evaluate and compare the antibacterial efficacy of diode laser irradiation at 1.5W & 3W in the disinfection of root canals when used in combination with sodium hypochlorite, toluidene blue and methylene blue dyes.

MATERIALS AND METHODS

Inoculum Preparation

A pure strain of *E. faecalis* (ATCC 29212) was procured from the Department of Microbiology, RajaRajeswari Medical College & Hospital, Bangalore. The bacterium was sub-cultured in nutrient broth at 37°C for 48 hours before inoculating in the root canals.

Preparation Of Samples

70 extracted single rooted premolar teeth were stored in 5.2%

NaOCl for 30 minutes to eliminate organic residues and left in saline solution until the study commenced. The crowns of the extracted teeth were sectioned at the CEJ using high speed diamond disc to obtain root length of 15mm. The working length was determined by inserting #15 K file into the canal until it was visible at the apex following which 1mm was deducted from the total length. All root canals were instrumented with hand Protaper system according to the manufacturer's instructions up to F2 (Size 20; 6%). The canals were irrigated with 2.5% NaOCl between each instrument with a 2mL disposable syringe and a 30-gauge needle. After instrumentation, canals were irrigated with 15% EDTA for 2 minutes followed by 1mL saline solution. Finally, the canals were dried with sterile F2 paper points. The apical foramen was sealed with composite resin and the root surface was painted with a double transparent nail polish to prevent leakage. Samples were then autoclaved at 134°C for 15 minutes. At this stage, five teeth were randomly kept aside as negative control (Group 1).

Study Model

The rest of the samples (65 teeth) were filled with 10μL of the bacterial suspension using sterile 1mL insulin syringes without overflowing and placed in a upright position in a tray. The suspension was carried to the entire length of the root canal with a #15 size K file. The samples were incubated at 37°C for 7 days in 100% relative humidity. Regular re-inoculation was performed on 1st, 4th and 6th day after initial inoculation. At the end of 7th day, 5 teeth were randomly kept as positive control (Group 2). In the other 60 samples the residual medium in the canal was removed with sterile paper points. The samples were then randomly divided into 6 experimental groups of 10 teeth each.

Experimental Protocol

Group 3

The samples were irrigated with 5mL of 2.5% NaOCl for 60 seconds using a 5mL syringe and 30 gauge needle that was placed 2 mm short of working length followed by diode laser irradiation (Oscillatory technique developed by Gutknecht et al.) at 1.5W. The optical fiber was introduced 1 mm short of the apex and recessed in helicoidal movements at a speed of approximately 2mm/s for 5 seconds. This was repeated 4 times with a 10 second rest period between irradiation to avoid heat build-up.

Group 4

Group 4 samples followed the same protocol as Group 3 but with laser irradiation done at 3W.

Group 5

In Group 5 samples, the canals were filled with methylene blue (15μg/mL) to the level of the access cavity using a 30 gauge needle adapted to a disposable plastic syringe. The solution was agitated with a #15 K file and left undisturbed in the canal for 2 minutes as a pre-irradiation time. This was followed by irradiation with 1.5W diode laser as per the Gutknecht's technique described elaborately in Group 3.

Group 6

In Group 6 samples, the experimental protocol followed was same as that of Group 5 but laser irradiation was performed at 3W.

Group 7

In Group 7, the canals were filled with toluidine blue (15μg/mL). The solution was agitated with a size 15 K file and left undisturbed in the canal for 2 minutes prior to irradiation. Diode laser irradiation followed with energy set at 1.5W.

Group 8

In Group 8, the same experimental procedure as in Group 7 was followed and the samples irradiated with diode laser at 3W.

Post Bacteriological Evaluation

Immediately following the laser irradiation, the root canals were rinsed with 100μL of physiological saline solution and inoculated into autoclaved sterile BHB (Brain heart infusion broth) media and incubated for 24 hours at 37°C. Following incubation, OD was taken at 600nm using UV visible spectrophotometer for turbidity and growth analysis. Furthermore, the sample was serially diluted to 10^{-6} to obtain isolated colonies. 100μL diluted sample was inoculated into BH infusion agar and incubated for 24 hours at 37°C. After incubation, the number of colonies was counted using colony counter with same *E. faecalis* as positive control and Agar plate without any inoculation as negative control (Figure 1&2).

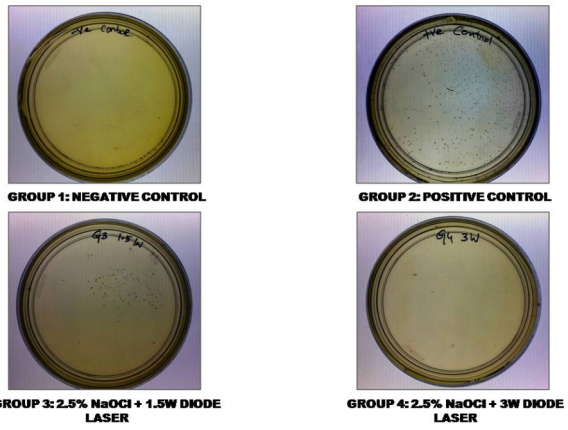

Figure 1. CFUs In Negative Control, Positive Control, Group 3 & 4

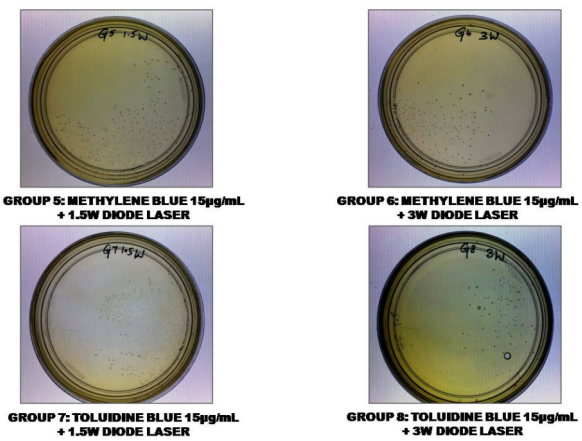

Figure 2. CFUs Observed In Group 5, 6, 7 & 8

Statistical Analyses

Statistical Package for Social Sciences (SPSS) for Windows, Version 22.0 was used to perform statistical analyses. Descriptive analysis of all study parameters was done using mean, standard deviation for continuous data & frequency and percentage for categorical data. Kruskal Wallis test followed by Bonferroni Post hoc Analysis was used to compare the mean CFUs and turbidity scores between different study groups. The level of significance (P-value) was set at $P<0.05$.

RESULTS

Colony counts were done using a magnifying lens. The mean CFUs/mL in the negative control group (Group 1) was 0 while it was $2.76x10^{8}$ for the positive control samples (Group 2). Among the experimental groups, lowest mean CFUs/mL was observed in Group 4 ($0.033x10^{8}$) while the highest was noted in Group 7 ($1.969x10^{8}$). Further, Group 4 (NaOCL+3W laser)

exhibited a statistically significant difference in mean CFUs/mL (P<0.001) when compared with all the other experimental groups. When compared to the photosensitizers (Group 5 to 8), the NaOCl groups (Group 3 & 4) exhibited lower mean CFUs/mL. Among the photosensitizer dyes, methylene blue (Group 5) showed lower mean CFUs/mL when used adjunctively with diode laser at 1.5W while toluidine blue (Group 8) demonstrated lower mean CFUs/mL at 3W (Figure 3, Table 1).

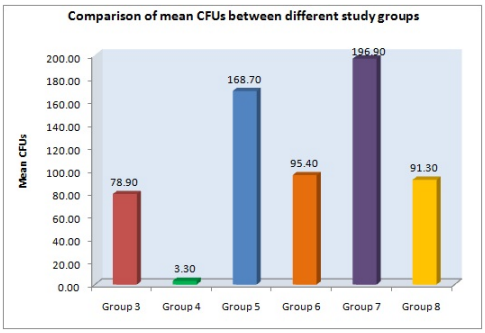

Figure 3. Comparison Of Mean CFUs (x10^6/ml) Between Different Study Groups.

Consistent with the above findings, the bacterial death percentage was highest in Group 4 (98.8%) followed by Group 3 (71.41%), Group 8 (66.92%), Group 6 (65.43%), Group 5 (38.87%) with lowest bacterial death observed in Group 7 (28.66%) (Figure 4).

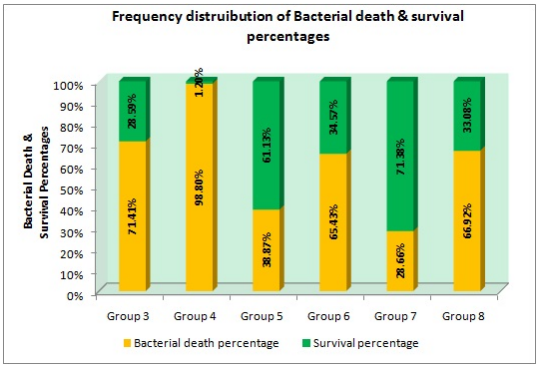

Figure 4. Frequency Distribution Of Bacterial Death & Survival Percentages Among The Study Groups

The mean turbidity was observed to be highest in Group 5 (0.1310) while the lowest turbidity was noted in Group 4 (0.0287). The Group 4 mean turbidity score was statistically significantly lower to other study groups with the exception of Group 8 (P=0.06) (Figure 5, Table 1).

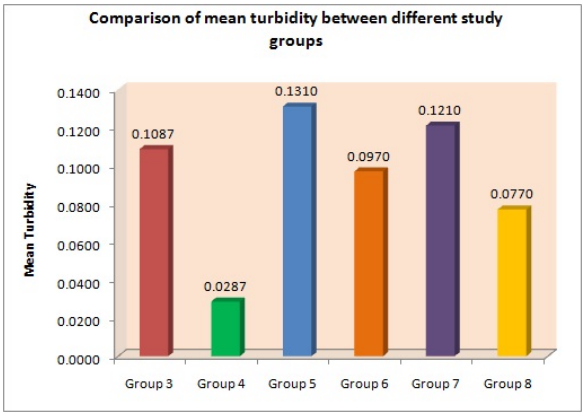

Figure 5. Comparison Of Mean Turbidity Between Different Study Groups

Table 1. Multiple Comparison Using Bonferroni's Post Hoc Analysis For Cfus & Turbidity

GROUPS	CFUs	TURBIDITY			
(I) Group	(J) Group	Mean Diff.(I-J)	P-Value	Mean Diff.(I-J)	P-Value
Group 3	Group 4	75.60	<0.001*	0.0800	0.001*
	Group 5	-89.80	<0.001*	-0.0223	1.00
	Group 6	-16.50	0.22	0.0117	1.00
	Group 7	-118.00	<0.001*	-0.0123	1.00
	Group 8	-12.40	0.96	0.0317	0.58
Group 4	Group 5	-165.40	<0.001*	-0.1023	<0.001*
	Group 6	-92.10	<0.001*	-0.0683	0.004*
	Group 7	-193.60	<0.001*	-0.0923	<0.001*
	Group 8	-88.00	<0.001*	-0.0483	0.06
Group 5	Group 6	73.30	<0.001*	0.0340	0.42
	Group 7	-28.20	0.001*	0.0100	1.00
	Group 8	77.40	<0.001*	0.0540	0.03*
Group 6	Group 7	-101.50	<0.001*	-0.0240	1.00
	Group 8	4.10	1.00	0.0200	1.00
Group 7	Group 8	105.60	<0.001*	0.0440	0.11

Note: * - Statistically Significant

To summarize the results, Group 4 demonstrated better results than Group 3 probably owing to the greater intensity of laser energy employed. Among the photosensitizer dye groups, the methylene blue dye showed greater bactericidal effect when used along with the diode laser at 1.5W while the toluidine blue dye was superior when used as an adjunct to laser at 3W.

DISCUSSION

Microorganisms in the root canals have long been considered as the key etiologic factors in the development of pulp and periapical lesions.[7] Therefore, effective decontamination of the root canal system is critical in achieving endodontic success. Chemical agents and dental lasers are presently being used to perform this role concurrently with the mechanical action of endodontic instruments.[8]

The present study compared the efficacy of a 810nm diode laser with NaOCl, methylene blue or toluidine blue in eradicating *E. faecalis* from the root canal system.

E. faecalis was selected as the test organism because of its ability to colonize the root canal in biofilms, representing the in vivo growth condition. In addition, it often survives chemomechanical preparation due to its resistance to antimicrobial agents and its ability to cause a monoinfection in the root canals.[1] It can proficiently invade the dentinal tubules, survive intracanal medication (e.g., calcium hydroxide), adapt to altered nutrient supply and continue to remain viable in the root canal.[4] Moreover, *E. faecalis* is commonly associated with root canal failure cases and persistent apical periodontitis.[9] All the above reasons, justify the selection of E. faecalis as the test organism in the present study.

Conventionally, irrigants have been delivered using a syringe and needle. A drawback with this irrigation method is that as the peak streaming velocity is present only in the lumen and around the needle tip, the irrigant is not adequately replaced throughout the canal system. Further, the commonly used irrigant – NaOCl does not directly contact the anatomically complex dentinal walls owing to its high surface tension. In fact, a study has demonstrated that 40-60% of the canals still contained cultivable bacteria following NaOCl syringe/needle irrigation and instrumentation.[1] Further, previous studies have shown that the depth of penetration of irrigants is limited to 100 μm while *E. faecalis* is known to penetrate upto a depth of 600-1000 μm. This demonstrates that disinfection of root dentin by chemo-mechanical means alone is unachievable. Adjunctive use of lasers ensure superior

bactericidal effect due to greater depth of penetration (upto 1000 μm) owing to their inherent properties of light scattering, local intensity enhancement and attenuation.[4]

Relatively new approaches to root canal disinfection include the use of diode lasers as well as photodynamic therapy. The laser light is thought to be able to reach areas that are impossible with the traditional techniques. The bactericidal effect of lasers is based on dose-dependent heat generation. Its antimicrobial effectiveness against diverse organisms has already been demonstrated in previous studies. Nevertheless, according to some, it was not more effective than NaOCl irrigation. Lasers can also induce dentine charring, ankylosis, root resorption and periradicular necrosis.[1]

Sohrabi et al. found 5.25% NaOCl reduced *E. faecalis* more effectively than the diode laser when used individually and recommended combining laser irradiation with chemical irrigation to evaluate the antibacterial effect of the diode laser.[2] dos Santos et al. in their in vitro study opined that use of a high-power diode laser in association with 2.5% NaOCl improved the antimicrobial effect of the chemical irrigant in *E. faecalis* infected root canals.[8] Another subsequent in vitro study too concluded that both diode laser alone and diode laser used in conjunction with NaOCl completely eradicated *E. faecalis* from the root canal.[4]

Photodynamic therapy (PDT) is an antimicrobial strategy defined as "light induced inactivation of cells, microorg anisms and molecules". In principle, it uses a nontoxic photosensitizer that is selectively absorbed in a target tissue and a low-intensity light source. Upon photo-induced activation of the photosensitizer, in the presence of oxygen, a series of reactions produce free radicals and singlet oxygen molecules leading to bacterial eradication.[10]

In a study, root canals were contaminated with *A. israelii*, *F. nucelatum*, *P. gingivalis* and *P. intermedia*. Photodynamic therapy done by incubating the canal with methylene blue followed by irradiation with diode laser led to a 80% reduction in colonies.[11] Similarly, in another study where toluidine blue was used followed by diode laser application, 99.9% reduction in bacterial count was observed. These results demonstrate that PDT is effective in disinfection of *E. faecalis* contaminated canals.[12]

CONCLUSION

From the results of our study, it can be concluded that 2.5% NaOCl when used in association with diode laser exhibited superior disinfection of the *E. faecalis* contaminated root canals when compared to diode laser used with the dyes methylene blue or toluidine blue. In the groups where NaOCl was used as irrigant, diode laser when used at a higher power of 3W demonstrated superior elimination of *E. faecalis* than at 1.5W power. Both the photosensitizer dyes when used with the diode laser at 1.5W & 3W exhibited similar disinfection ability. However, it was significantly lower when compared to NaOCl groups. Further studies need to be undertaken using different photosensitizers to determine and develop suitable disinfection protocols to ensure they can match up to the decontamination efficacy demonstrated by NaOCl.

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