Original Article

Combined Sodium Hypochlorite and 940 nm Diode Laser Treatment Against Mature E. Faecalis Biofilms in-vitro

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Abstract:

Background: Laser irradiation has been introduced in endodontic treatment due to its bactericidal effect. The aim of this study is to evaluate the bactericidal efficacy of a 940 nm diode laser alone or in combination with 5% sodium hypochlorite (NaOCl) against mature biofilms of *E. Faecalis*.

Methods: Sixty-eight (60 for the three groups, 4 for SEM and 4 as negative controls) single-rooted human central incisors were prepared and contaminated with *E. Faecalis*. After two weeks of incubation, specimens were randomly divided in three groups; group 1 (n =20), the teeth were irradiated with a 940 nm diode laser; group 2 (n=20), specimens were rinsed with 5% NaOCl; group 3 (n=20), the teeth were rinsed with 5% NaOCl and then were irradiated with 940 nm diode laser. Four teeth were used to observe the biofilms by SEM. Intracanal bacteria sampling was done, and the samples were plated to determinate the CFU count.

Results: At 24 hours and 7 days, group 3 showed a significant difference (P=0,02; P=0,00) in disinfection if compared to group 1 but did not show this difference if compared to group 2 (P=1, P=0,66), although group 3 obtaining a more extensive disinfection. Groups 1 and 2 did not show difference after 24 hours (P=0,09) but showed a significant difference 7 days afterwards (P=0,04).

Conclusion: The combination of sodium hypochlorite and diode laser light (940 nm) has a synergistic effect, intensifying the bactericidal action.

Keywords: *E. Faecalis*; diode laser; biofilm;disinfection.

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Introduction

Microorganisms play a crucial role in pulp death and periapical infections (1). Although initial root canal therapy has been shown to be a predictable procedure with a high degree of success, failures can occur after treatment. Recent publications reported failure rates of

14-16% for initial root canal treatment (2, 3). Hence, the presence of bacteria persisting in the root canal system after the phase of cleaning and disinfection in (re)treatment has a significant impact on this success rate (4). The elimination of all bacteria from the root canal system before obturation is very complicated (5), and therefore additional methods to the traditional treatments have been proposed in order to overcome the limitations of incomplete elimination of biofilms from endodontic sites (6).

Endodontic failures result from the passage of remnant microorganisms that have not been eliminated during therapy and that have not been sufficiently entombed during subsequent obturation of the root canal system. Most common Genera of endodontic failure include Enterococcus (notably *E. Faecalis*), Prevotella, Propionibacterium and Actinomyces (7). *E. Faecalis* is part of the normal human flora and is usually found in relatively small concentrations in the mouth. It is the species that is more frequently isolated in teeth with persistent infection after endodontic treatment (8). Different bacteriological examinations have shown that the *E. Faecalis* is present in 30-48% of teeth with post-treatment infection (9). These findings highlight the ability of *E. Faecalis* to survive in tough environmental conditions, such as root canals after endodontic treatment (10). It has been reported that *E. Faecalis* survives endodontic treatment procedures, resisting high concentrations of drugs and wide variations in pH (11, 12). The ability of these bacteria to form biofilms is widely known. A biofilm can be defined as a community of bacterial cells absorbed into a solid surface wrapped in an extra-cellular matrix. Biofilm allows microorganisms to survive under extreme environmental conditions and gives them greater resistance to antibacterial agents (13).

Diode lasers can be used as an adjunct for root canal disinfection (14, 15). Among the new tendencies in the field of lasers, a diode device is interesting for researchers and clinicians because of its small dimensions, low cost, power outputs and operating modes (14). Different wavelengths for diode in endodontics have been described: 810 nm (16), 830 nm (17), 940 nm (15), 980 nm (14). Recently the 940 nm wavelength came to the attention, as it was demonstrated that a limited form of cavitation could be generated in aqueous fluids around the top of the fibre tip (15). It is also known that this wavelength as is the case for the Neodymium-Doped Yttrium Aluminium Garnet(Nd:YAG) (1064 nm) has a deep penetration into the dentinal tubules (14).

The aim of this study is to evaluate the bactericidal efficacy of a 940 nm diode laser alone or in combination with 5% sodium hypochlorite (NaOCl) against mature biofilms of *E. Faecalis*.

Methods

Preparation of Tooth Specimens

This study used 68 extracted human single-rooted teeth with fully formed apices (maxillary central incisors with substantially equal canal curvature and morphology) that had not undergone prior endodontic treatment. After root surface debridement, specimens were immersed in a 5% NaOCl solution (Niclor 5; OGNA, Muggiò, Italy) for 1 hour and then stored in saline solution one day at 37°C until preparation. The presence of a single canal was verified radiographically and by direct exploration. The same operator performed all experimental procedures. Each specimen was sectioned to obtain a working length of 16 mm. The working length (taken 1 mm short from the apical foramen) was established under a microscope (M525 F40; Leica, Heerbrugg, Switzerland) at 10× magnifications with the tip of the instrument visible at the apical foramen. Patency of each root canal was assessed using K-Flexofiles (Dentsply Maillefer, Ballaigues, Switzerland) up to #20. All canals were prepared using ProTaper S1-S2-F1-F2-F3-F4 (Dentsply Maillefer). Irrigation was performed with a 30-gauge needle (ProRinse; Dentsply Tulsa Dental Specialties, Tulsa, OK, USA) using 3 ml of 5.25% NaOCl after each file. The irrigation needles were introduced passively up to 2 mm from the working length, and the rate of delivery was fixed at 3 ml/min. The total irrigation time was 10 min/specimen. After instrumentation, all teeth were irrigated for 3 min with 3 ml of 10% EDTA (Tubuliclean; OGNA, Milan, Italy) followed by a 3-min final rinse with 5% NaOCl, activating both solutions with an ultrasonic tip for 30 seconds (Irri-Safe; Satelec, Acteón Group, Merignac Cedex, France). A final wash was made with distilled water. After drying with paper points, the roots were inspected under the microscope at 10× magnifications to verify the absence of cracks and canal cleanliness. After the completion of the shaping procedures, the apical foramen was sealed using light-cured restorative composite (CeramX Duo, Dentsply-Friadent, Roskilde, Denmark). Each specimen was transferred to a plastic cryo-tube (Cryo. S, PP Greiner Bio-One Gmbh., Frickenhausen, Germany) containing

sterile brain heart infusion (BHI) broth (Merck KGaA, Darmstadt, Germany) and autoclaved at 121ºC for 20 minutes. Samples were incubated in their sealed tubes for 48 h at 37ºC. Daily inspection revealed no signs of turbidity. Four teeth were selected randomly to serve as negative controls.

Experiment Root Canal Infection

An overnight pure culture of E. Faecalis (ATCC 29212) in BHI was used for inoculation. The bacterial suspension was adjusted spectrophotometrically to match the turbidity of a McFarland 0.5 scale. Aliquots of 20 µl of the suspension were inoculated into each canal, then specimens were coronary sealed with Cavit (3M ESPE, Seefeld, Germany) and incubated for two weeks under aerobic conditions at 37ºC into Falcon tubes (Eurolab, Barcelona, Spain) with 5ml of BHI, changing it every 3 days.

Scanning Electron Microscopy

Four root specimens were selected for scanning electron microscopy (SEM) to confirm the established biofilm infection. Each specimen was split into two halves, with a pointed stainless steel chisel inserted in pre-fracture grooves, and fixed in 5 ml of 3.7% glutaraldehyde in 0.2 M sodium cacodylate buffered solution at 4ºC for 24 hours. Following dehydration in graded concentrations of ethanol, specimens were air dried and mounted on SEM stubs for gold sputtering and observation with a JEOLJSM 6400 scanning electron microscope (JEOL Corporation, Tokyo, Japan). SEM microphotographs were obtained at different magnifications in representative areas of the root canal system.

Disinfection of the Root Canals and Assessment

Before handling the specimens, the outer surface was disinfected with a cloth of chlorhexidine for 3 minutes, plunged in sterile distilled water for another 2 minutes and dried with sterile cotton gauze for 30 seconds. The provisional seal (Cavit, 3M ESPE, Seefeld, Germany) was eliminated. The extent of infection in each specimen was checked before the introduction of the respective experimental treatments with laser, NaOCl or combined: two sterile tips were introduced in the specimens during 30 seconds and then incubated in 5 ml of BHI at 37ºC. After 24 hours, infection was confirmed by turbidimetry (Dinko D-101, Barcelona, Spain; at 590 ηm of wavelength) and CFU count. Briefly, after tenfold serial dilution in saline, aliquots of 0.1 ml were plated onto BHI agar plates and incubated at 37ºC for 48 hours. The colony forming units (CFUs) grown were counted and then transformed into actual counts based on the known dilution factors. All samples showed bacterial growth at 48 hours.

Experimental Groups

Group A (n = 20): Diode Laser

Laser treatment was performed with a diode laser (Ezlase 940, Biolase, San Clemente, California, USA) and the endodontic tip (ezTip Endo, 14 mm / 200 µm), 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 laser irradiation was delivered for 1 minute into the canal up to 1 mm short of the working length, with circling movements from the apical part towards the coronal part (step-back technique) i.e. 4 times an exposure of 15 seconds with a dwell time of 20 seconds. The laser was inserted directly in the root canal without any root canal treatment. After this procedure, 20 µl of sterile saline solution was introduced in the samples, and then they were coronary sealed with Cavit (3M ESPE, Seefeld, Germany). Fibers were sterilized between irradiations for each tooth. Then, specimens were incubated under aerobic conditions at 37ºC into Falcon tubes (Eurolab, Barcelona, Spain) with 5ml of BHI up to 7 days.

Group B (n = 20): Sodium Hypochlorite

Specimens were irrigated with 5 ml of 5% NaOCl for 1 minute. Irrigation was performed with a 30-gauge needle (ProRinse; Dentsply Tulsa Dental Specialties, Tulsa, OK, USA). The irrigation needles were introduced passively up to 2 mm from the working length. A paper point was introduced in the specimens to dry the root canals during 30 seconds. After this procedure, a sterile saline solution was introduced in the specimens. Then, samples were coronary sealed and incubated under the same conditions as group A

Group C (n = 20): Sodium Hypochlorite and Laser

The irrigation procedure was performed similar to

that in-group B. After irrigation with 5% NaOCl laser irradiation was performed as in group A directly in the remaining NaOCl solution. Then, a sterile saline solution was introduced in the specimens and they were coronary sealed and incubated in the same conditions as for groups A and B.

Determination of the Number of Bacteria

The specimens were processed at two intervals of time, 24 hours and one week after the experimental disinfection procedures. After opening the teeth, the canals were refilled with normal saline as a transfer fluid. Sampling from inside the canals consisted of using a sterile #25 K-File (Dentsply, Maillefer) with circumferential filing during 20 seconds in order to disrupt the biofilm and to collect dentin chips. Sterile paper points were used to collect the transfer fluid and dentin chips. Both sterile paper points and sampling K-files were placed into a test tube containing 4ml of sterile BHI and vortexed for 1 minute.

After 24 hours, the bacterial growth was confirmed by turbidimetry and posterior CFU´s counting. After sampling, 20 µl of sterile saline solution was introduced in samples, and then they were coronary sealed with Cavit. Specimens were then sealed again with Cavit and incubated in BHI up to 7 days. The previously described procedure was performed once more. The colony-forming units (CFUs) were calculated as described in the processing of the infected teeth. The colonies were confirmed to be E. Faecalis by colony morphology, Gram staining and PCR.

Statistical Analysis

Quantitative data are expressed as medians [Percentil25, Percentil75] and range. Comparisons of CFU values between groups were analyzed by Kruskal-Wallis and Chi-square tests with Bonferroni correction. P values <0.05 were considered statistically significant. Analyses were performed using the SPSS program (SPSS INC, Chicago, Il, USA) version 17.0.

Results

Effectiveness of Disinfection Procedures

Absence of growth was seen in the negative controls. All specimens of the three groups showed bacterial growth prior to the experiment disinfection procedures. The number of CFUs is shown in Table 1.

There were no statistically significant differences between the 3 groups (P=0.210).

After 24 hours, bacterial growth was observed in 30% of the teeth with a range between 11 x 10^7 to 167 x 10^7 in group 1. In group 2, only one sample showed bacterial growth, with a value of 264 x 10^7. In group 3 no bacterial growth was observed after 24 hours. A statistically significant difference was seen between groups 1 and 3 (P=0.002); however, no statistically significant differences between groups 2 and 3 were observed (P=1) nor between 1 and 2 (P=0.091) (Table 2)

At day 7, the laser group showed bacterial growth in 70% of the samples with a range of 12 x 10^7 to 230 x 10^7. Group 2 showed bacterial growth in 20% of the samples, with a range 120 x 10^7 to 232 x 10^7. In group 3, we only observed bacterial growth in 10% of the samples, with values of 120 x 10^7 and 136 x 10^7. In this way, after 7 days, we observed a statistically significant difference between group 1 and 2 (P=0,04) and group 1 and 3 (P=0,000). We did not observe statistically significant differences between groups 2 and 3. (Table 2)

E. Faecalis biofilm successfully formed in the root canals (as shown in Figure 1 by SEM). Many layers of bacteria accumulated around the opening of the dentinal tubules and on the root walls. Bacterial

Table 1: Specimens CFU prior to the treatment

Groups	CFU/ml			
	Median (x 10^7)	P25 (x 10^7)	P75 (x 10^7)	Range (x 10^7)
Group 1 (N=20)	128,50	61,50	198,00	4-280
Group 2 (N=20)	119,00	59,25	196,75	45-305
Group 3 (N=20)	148,00	101,25	210,75	45-317

Group 1: Diode laser irradiation, Group 2: Irrigation with 5% NaOCl, Group 3: Combined laser and NaOCl treatment.

Table 2: Number of infected teeth after disinfection procedures at day 1 and 7

Groups	n	Number of infected teeth	
		At day 1	At day 7
Group 1	20	6 a	14 a
Group 2	20	1 ab	4 b
Group 3	20	0 b	2 b

For each time period groups with the same letter (a, b) are not significantly different at P < 0.05
Group 1: Diode laser irradiation, Group 2: Irrigation with 5% NaOCl, Group 3: Combined laser and NaOCl treatment

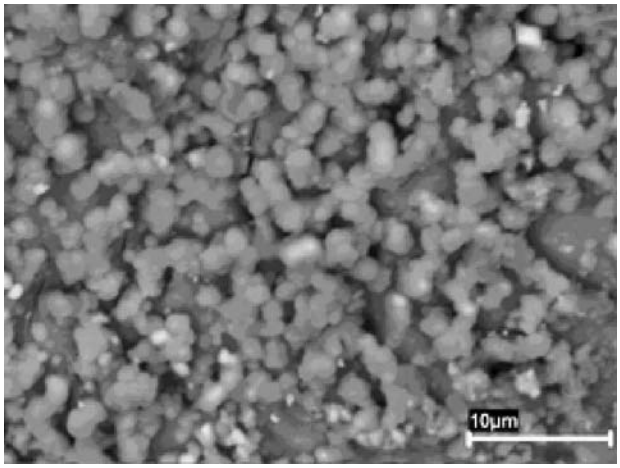

Figure 1. Representative SEM image of specimens inoculated with E. Faecalis after 14 days (5000 ×).

structures connecting to each other and dividing can be seen.

Discussion

E. Faecalis results to be the species most commonly associated with post-treatment apical periodontal disease (18). It has been widely used as a microbiological marker for studies *in vitro* as there is plenty of evidence that it colonizes the root canal and dentinal tubules successfully, forms biofilms and survives endodontic treatments (19). The strain used in this study suitably colonized the walls of the root canal after 14 days of incubation, forming a mature biofilm in different areas of the root canal, as proven by SEM (Figure 1).

A number of studies have shown that the use of diode laser as an adjunct to conventional root canal disinfection can have an added value (15, 17). From these studies it became clear 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, low cost, power outputs and operating modes (14). For our study we followed the manufacturer´s recommendations, 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. We also used a special tip for the laser made by the same manufacturer (ezTip Endo, 14mm / 200um). We have to take into account this power because thermal effects can destroy the periodontal ligament and cause root resorption, Temperature changes should be examined in future investigations at this power with this length of pulse and amplitude. We have also decided to use the sodium hypochlorite as a contributor for the laser. The set-up from this study differed from others where short incubation times did not enable bacteria to sufficiently colonize the root canal walls. In this study the evaluation of the bactericidal effect of the diode laser was evaluated as a function of time taking into account the eventual recovery of remaining bacteria.

After 24 hours we observed that the three disinfection protocols reduced the bacterial load, with group 3 obtaining a more extensive disinfection. This may be due not only to the combined action of hypochlorite of sodium and laser, but also to the capacity of the laser to heat sodium hypochlorite (20).

After 7 days, however, we observed a high microbial growing in the laser group (70% of the samples showed infection). This is not due to the complexity of the root canal system as single rooted maxillary central incisors were used. An explanation can be provided by the fact that laser light cannot reach all areas with the same effectiveness as is the case with sodium hypochlorite rinsing. A shortcoming of the use of laser fibres is the need for a spiral motion to expose the root canal wall to the laser light ending up in areas that might not have been exposed sufficiently or not to the laser beam. On the other hand, groups 2 and 3 did show a statistically significant difference with the laser group, confirming a good initial bactericidal effect. The present results are in agreement with Lee et al. demonstrating bacterial reduction with 90% for conventional disinfection techniques. The combined disinfection procedure may end up in a 98% bacterial reduction (21). This has also been shown by Benedicenti et al. demonstrating a synergistic effect of the use of NaOCl, citric acid and diode laser energy (810 nm) resulting in increased treatment efficacy and leading to significantly better decontamination of the root canal (22). In a recent study, Preethee demonstrated superior bactericidal efficacy of 908 nm diode laser combined with irrigants than canals disinfected with this alone (23). The present findings also demonstrate the need for a tight root canal filling immediately after the cleaning and shaping procedure resulting in entombment of remaining bacteria and hence avoiding the recolonization of the root canal system. This is more specific for *E. Faecalis* where it has been demonstrated that they can maintain viability in obturated root canals (24, 25).

Within the limitations of this study, we can conclude that the combination of sodium hypochlorite and

diode laser light (940 nm) has a synergistic effect, intensifying the bactericidal action.

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