Lasers in Medical Science (2019) 34:1717–1721
https://doi.org/10.1007/s10103-019-02763-y

BRIEF REPORT

Enhancing Er:YAG bactericidal effect against *Enterococcus faecalis* biofilm in vitro

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Received: 30 July 2018 / Accepted: 27 February 2019 / Published online: 8 March 2019

Introduction

Microorganisms and their products are etiologic factors in pulp and periapical pathologies. The success of endodontic therapy relies on efficient disinfection of the root canal system and prevention of recontamination [1]. Still, studies show that even meticulous instrumentation and irrigation with NaOCl cannot render the canal bacteria free, mainly due to the presence of lateral canals, ramifications, and apical deltas [2, 3]. Furthermore, bacteria penetrate into the deeper layers of root dentin [3], which are not accessible to irrigating agents [4].

Different techniques and devices have been introduced in order to improve the disinfection of the root canal system, such as fine diameter irrigation needles and sonic and ultrasonic activation which further amplify the removal of organic tissue and debris from hard-to-reach areas in the root canal system. While these methods may improve root canal disinfection, the complete eradication of bacteria and their toxic byproducts from distant areas of the tubular system remains a major challenge [5]. In addition, certain microorganisms like *Enterococcus faecalis*, a keystone endodontic pathogen, is known to form intra- and extra-radicular biofilm, which further weakens the ability to control the infection. *E. faecalis* can survive endodontic treatment procedures by resisting high concentrations of intracanal medicaments and wide variations in pH and are also able to penetrate the dentinal tubules up to a depth of 800 μm [6]. These altered microbial genetic and metabolic processes are thought to be some of the contributing factors that allow their persistence in failed treatments [7].

Laser application was found to be safe and efficient in the eradication of bacteria and the removal of the smear layer from root canals [8, 9]. The Er:YAG (erbium:yttrium aluminum garnet) laser yields a bactericidal effect on root canal surfaces and in the deeper dentin layers, and has been shown by several authors to be highly effective against several bacterial species [10, 11]. Mehl et al. investigated the antimicrobial properties of Er:YAG-laser radiation in root canals and found reduced bacterial counts of *Escherichia coli* and *Staphylococcus aureus* following Er:YAG irradiation [12]. Similar results were found by Schoop et al., (2002) who demonstrated the bactericidal effect of Er:YAG laser on extracted premolars contaminated with six different species [13].

The aim of the present study was to evaluate the antimicrobial activity of Er:YAG laser on *E. faecalis* biofilm in vitro and the impact of different parameters of the laser on its bactericidal effect.

Materials and methods

Bacterial preparation

The standard strain of *E. faecalis* (V583) was grown in brain heart infusion (BHI) broth overnight at 37 °C. Then, the bacterial culture was adjusted to OD_{600}, corresponding to a cell density of 1.5×10^8 colony forming units per milliliter. After 24 h, the inoculation was transferred into high glucose (2%) BHI broth. Aliquots of 2 ml were seeded in 24-well plates (Nunc, Roskilde, Denmark) and incubated overnight at 37 °C. The newly formed biofilms were washed twice in sterile phosphate-buffered saline (PBS) and each well was filled with 400 μl of PBS.

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Experimental design

Biofilms were exposed to Er: YAG (Light Instruments, Yocneam, Israel) at three areas in each well. Water spray was turned off to avoid dilution of samples, but as stated above, the biofilms were completely saturated in PBS. Different irradiation settings were tested:

Group A: The handpiece emitting the beam was fixed at different distances from the biofilm: 10 mm versus 1 mm. Other settings used: 0.5 W, 10 Hz, duration 10 s, and tip diameter 0.4 mm.
Group B: Different output intensities were examined: 0.5 W versus 1 W. Other settings used: 1-mm distance, 10 Hz, duration 10 s, and tip diameter 0.4 mm.
Group C: Different tip diameters were examined: 1 mm versus 0.4 mm. Other settings used: 1-mm distance, 10 Hz, 0.5 W, duration 10 s.
Group D: Different durations of irradiation were examined: 10 s versus 5 s. Other settings used: 1-mm distance, 10 Hz, 0.5 W, tip diameter 0.4 mm.

Non-treated biofilm served as controls. One parameter only was modified in each experiment.

Biofilm staining and fluorescent microscopy

Following Er:YAG treatments, biofilms were stained with a live/dead bacterial viability stain (BacLight Bacterial Viability Kit; Molecular Probes, Waltham, MA USA) according to the manufacturer's instructions. Each sample was processed and analyzed individually under an inverted fluorescent microscope (Nikon TL, Nikon Instruments Inc., NY, USA) using a × 10 magnification. Images were analyzed with specific software (NIS Elements, Nikon Instruments Inc., NY, USA). Diameters of the irradiated areas (rounded biofilm free areas) were measured by a calibrated examiner. Images were also analyzed by ImageJ (Wayne Rasband, National Institute of Health, USA) software in order to quantify live versus dead biofilm ratios, differentially stained as described above. Percentages of live and dead bacteria in every experiment were determined.

Tetrazolium reduction assay

Tetrazolium salt assay (MTT, Calbiochem, Darmstadt, Germany) was used to measure the metabolic activity of the biofilm. The experiments were performed in 96-well plates with a single Er:YAG exposure site/well (instead of three sites/well). The assay was performed according to Kairo et al. [14], with minor modifications. Briefly, after irradiation of the biofilm with Er:YAG, the wells were washed twice with sterile PBS after which 50 μl of 0.1% MTT solution was added to each well. Plates were incubated for 2 h at 37 °C. At the end of incubation period, wells were washed again with sterile PBS and filled with dimethyl sulfoxide for 15 min in order to dissolve the reduced tetrazolium salts in the biofilm. Finally, 100 μl of the solubilized MTT solutions were transferred to new wells of a 96-well plate and analyzed with a plate reader (Infinite 200 PRO, Tecan Trading AG, Switzerland) at 570 nm with a reference wavelength of 620 nm.

Data analysis

All experiments were done in duplicates and 3 exposures sites/well. Each experiment was repeated at least 3 times. One-way repeated measure analysis of variance (RM ANOVA) was used to test the significance of the differences between the experimental groups. The inter-group differences were tested for significance using the Student's *t* test with Bonferroni correction for multiple testing.

Results

Analysis of the substrates revealed that bacterial adhesion with subsequent biofilm formation occurred in all groups. Biofilm-irradiated areas (IA) were observed in all irradiated groups and were completely free of bacteria. Figure 1 represents images obtained from the biofilms following irradiation with different laser parameters (Fig. 1a–e), as well as control groups that were not irradiated (Fig. 1f). The images show the presence of microorganisms throughout the entire extension of the substrate, with a marked predominance of live cells, with the exception of areas completely free of bacteria corresponding to the laser-irradiated areas.

The diameter of the IA increased significantly following long exposure time duration ($P < 0.05$), large tip size ($P < 0.005$), and the close proximity of the tip to the biofilm ($P < 0.005$), as shown in Table 1. Laser intensity was not found to have a significant effect on the diameter of the IA, as shown in Table 1.

Quantification of the ratio between live and dead biofilm did not show a significant difference between groups (data not shown).

E. faecalis ability to form viable biofilm was tested through tetrazolium salt reduction assay. Absorption was measured at 570 nm with a reference length of 620 nm. Higher OD values correspond to higher metabolic activity in *E. faecalis* biofilm. Bacterial cultures irradiated with Er:YAG laser showed a significant reduction in viable biomass compared to the control group, as shown in Fig. 2. Laser emitted at a higher intensity provided the highest reduction in bacterial viability, among all tested parameters ($P < 0.005$). Using a larger tip or irradiating at closer proximity to the biofilm was also found to

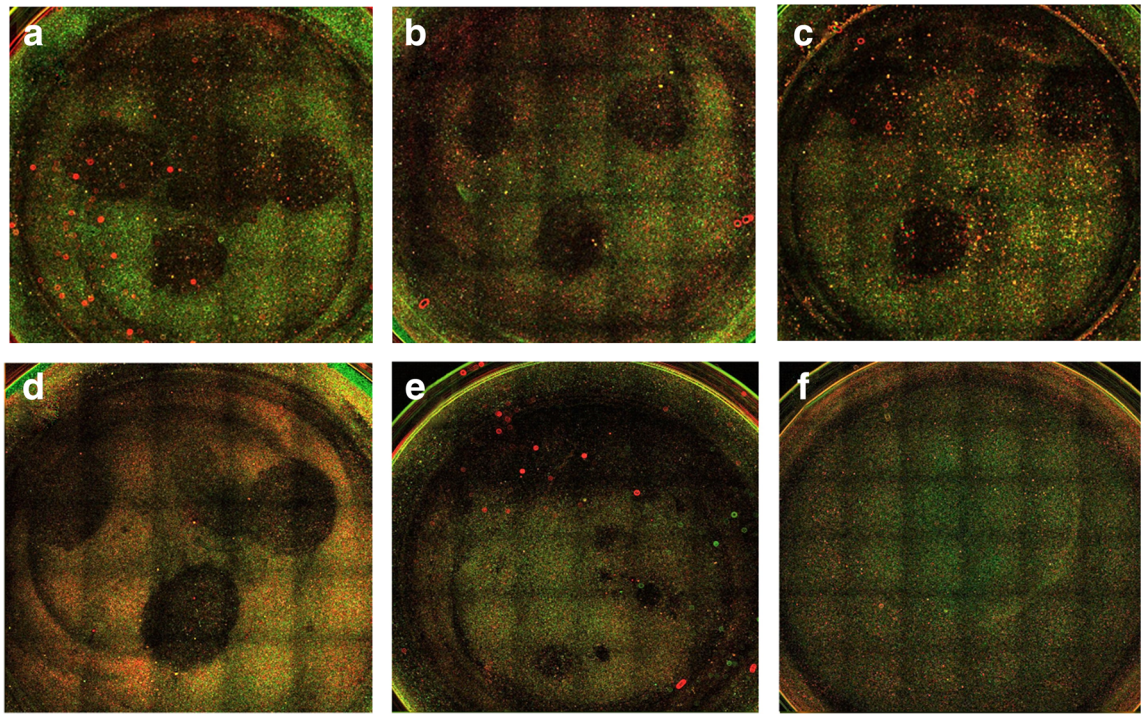

Fig. 1 Microscopic images of live/dead staining of biofilm-irradiated areas in relation to different laser parameters as detailed as follows (underline highlights the tested parameter): **a** intensity, 0.5 W; tip size, 0.4mm; distance from the biofilm, 1 mm; duration, 10 s. **b** Intensity, 0.5 W; tip size, 0.4 mm; distance from the biofilm, 1 mm; duration: 5 s. **c** Intensity, 1 W; tip size, 0.4 mm; distance from the biofilm, 1 mm; duration, 10 s. **d** Intensity, 0.5 W; tip size, 1 mm; distance from the biofilm, 1 mm; duration, 10 s. **e** Intensity, 0.5 W; tip size, 0.4 mm; distance from the biofilm, 10 mm; duration, 10 s. **f** Negative control

significantly reduce bacterial viability within the biofilm ($P < 0.05$), but to a lesser degree, as shown in Table 2.

Discussion

E. faecalis has been associated with persistent endodontic infections and its complete removal of crucial importance for endodontics therapy outcome. In recent years, Er:YAG laser has gained increasing popularity among clinicians due to its highly bactericidal properties in endodontic procedures [13, 15].

To date, very few studies have compared different laser parameters regarding their bactericidal capabilities. An exception is the work by Schoop et al. [13], which assessed the effects of Er:YAG laser irradiation on 220 extracted human teeth in vitro, and concluded that irradiation at an intensity of 0.5 W was sufficient to achieve a distinct reduction in bacterial

Table 1 Diameter of IA in relation to different laser parameters

Parameter	Mean ± SD (mm)	*P* value
Exposure time		
5 s	2806 ± 492.6	0.047
10s	3045 ± 391.5	
Tip size		
1 mm	3637.16 ± 718.76	0.0001
0.4 mm	3045 ± 391.5	
Tip distance		
1 mm	3045 ± 391.5	0.0001
10 mm	1324 ± 260	
Intensity		
0.5 W	3045 ± 391.5	n.s.
1 W	3273.39 ± 527.98	

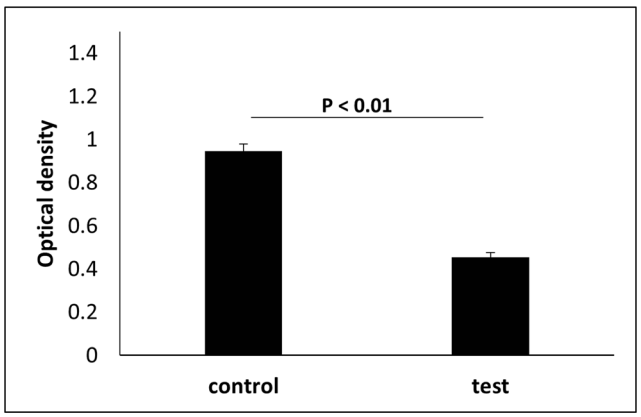

Fig. 2 Quantitative analysis of polled tetrazolium salt reduction valued (in optical density values) of all laser-irradiated groups vs. negative control. Results are expressed as mean and SD

Table 2 Biofilm viability (MTT) in relation to different laser parameters

Parameter	Mean ± SD (optical density)	*P* value
Exposure time		
5 s	0.58 ± 0.14	n.s.
10 s	0.57 ± 0.09	
Tip size		
1 mm	0.57 ± 0.09	0.008
0.4 mm	0.44 ± 0.14	
Tip distance		
1 mm	0.57 ± 0.09	0.047
10 mm	0.68 ± 0.21	
Intensity		
0.5 W	0.57 ± 0.09	0.001
1 W	0.39 ± 0.07	

counts, whereas specimens irradiated at 1 W did not show bacterial growth at all. In the present study, the development of biofilms was conducted in vitro by using 24-well plates as substrates for bacterial biofilm growth. Ideally, in vitro studies should simulate in vivo conditions, thus the ideal substrate is dentin from extracted human teeth. On the other hand, using extracted teeth for biofilm growth is more laborious in use and cannot be fully standardized due to variations in canal sizes, volumes, microanatomy, and in the quality and quantity of the microbial infections. Therefore, we decided to use standard plates for biofilm growth owing to their ready availability, high capacity for rapid biofilm formation, and standardization of the experimental conditions for a large series of tests.

The results presented here demonstrate that *E. faecalis* is fully capable of biofilm formation after short-term incubation periods of 72 h. Furthermore, a high density of bacterial growth was evidenced by the microscopic images at the end of the incubation period.

Considering the results, we can draw several conclusions: first, irradiated areas completely free of bacteria were observed in all experimental groups, indicating Er:YAG's capability of inducing a focal bactericidal effect at the target site. This is in accordance with Hibst et al. [16] who stated that the heat effect of a single Er:YAG laser pulse is little and limited to the vicinity of the impact site. Secondly, longer duration of irradiation as well as the use of a larger tip and irradiating at closer proximity to the biofilm may augment the laser's bactericidal effect at the target site. However, even when the laser tip was placed 10 mm away from the biofilm, a certain decrease in viable mass as well as irradiated areas free of bacteria were observed. This is perhaps the most remarkable feature of Er:YAG treatment, in that the laser aperture does not need to be close to the target site in order to be effective. This is in accordance with Redenski et al. [17], who demonstrated that low-energy laser irradiation with Er:YAG laser can affect bacterial behavior, even when the laser beam is emitted from a large distance (4.5 cm from the biofilm). Nevertheless, in endodontic treatment, a side-firing fiber tip design would be more suitable due to root canal anatomy.

Finally, analysis of the measurements obtained from the MTT assay revealed a substantial decrease in viable biofilm mass following irradiation with Er:YAG laser in all experimental groups. The most significant decrease was observed when the biofilms were lased at an effective power of 1 W, as opposed to 0.5 W. This finding is in agreement with in vitro data reported by Schoop et al. [18], who demonstrated a significant reduction in *E. coli* and *E. faecalis* strains following irradiation with Er:YAG at an intensity of 1 W and 1.5 W, respectively.

Conclusions

Within the limitations of this particular laboratory setup, using a relatively young *E. faecalis* biofilm grown in in standard 24-well plates, Er:YAG proved effective in biofilm elimination.

A focal bactericidal effect can be achieved using all parameters tested. Nevertheless, increasing the effective power (W), irradiating at closer proximity to the biofilm or using a larger size tip, may induce a collateral bactericidal effect and reduce bacterial viability within the biofilm.

Authors' contribution All authors have made a substantial contribution to the manuscript, and have read and approved the final version

Funding information The study was conducted with the department's sources.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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