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The Photobiomodulation Effect of 940nm Laser Irradiation on *Enterococcus faecalis* in Human Root Dentin Slices of Varying Thicknesses

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Abstract

Introduction: An increase in dentine thickness could result in an inadequate depth of laser energy penetration. This study aimed to evaluate the effect of a 940 nm laser on *Enterococcus faecalis* through varying thicknesses of human root dentin slices.

Methods: Thirty-five dentin slices of root dentin with thicknesses ranging between 500 and 3000 µm were produced. Six experimental groups (500, 1000, 1500, 2000, 2500, and 3000 µm (n=5 each) were lased and the seventh, non-lased group served as the positive control with a dentine thickness of 2000 µm. The slices were inoculated with 2 µL of *E. faecalis* suspension of 1.5×10^8 *E. faecalis* cells/mL. All the lased slices were lased from the opposing side of the inoculation. A non-initiated 200 µm bare end fibre at the power of 1 W, in a continuous wave was used. Four doses of laser irradiation of 5 seconds with a side to side movement with the tip held at a 5º angle to the dentine slice were performed. The colony-forming units of *E. faecalis* were determined and the bacterial photobiomodulation effect analysed using one-way ANOVA with a Bonferroni and Holm post hoc test at a significance level of $P>0.05$.

Results: There were statistical differences between the dentin slices of 500, 1000, and 1500 µm treated with the laser compared to the positive control ($P<0.01$). However, there were no statistical differences between the lased 2000 and 2500 µm slices compared to the positive control. There was significantly more photobiomodulation of the *E. faecalis* for the dentine slices of 3000 µm than the positive control ($P<0.01$).

Conclusion: Laser treatment through dentine slices of 2000 µm and thinner significantly reduced bacterial growth. The photobiomodulation effects started to occur in dentine slices thicker than 2500 µm compared to the positive control.

Keywords: Laser; *Enterococcus faecalis*; Dentine thickness; 940 nm; Photobiomodulation.

Introduction

The main etiological factor of pulpal and periapical infections is the invasion of bacteria into the pulpal and endodontic systems.[1] Successful treatment of these infections would require the removal and/or destruction of these microorganisms and create an effective coronal and apical seal to prohibit the reinfection of bacteria and influx of nutrients into the endodontic system. The ability of bacteria to penetrate deep into the dentin tubules and to form biofilms as well as the adhesion of certain bacteria to the dentin walls makes the destruction and removal of bacteria from the three-dimensional dentinal network a challenging task.[2,3] Due to the complexity of this network, about 30%-45% of the root canal system escapes chemo-mechanical instrumentation[4] as per the normal endodontic procedure with the dentist. Lasers have been used as an additional step to kill bacteria that could not be reached by chemo-mechanical instrumentation. Studies show that in favourable conditions, bacteria can penetrate to a depth of more than 1000 µm into the dentinal tubules.[2]

Numerous previous studies have been conducted to evaluate the bactericidal effect of various wavelengths of lasers, but most of these studies were conducted on dentine slices of 100, 300, 500, and 1000 µm.[5-7] They were conducted with dentine samples up to 1000 µm, therefore limiting our understanding as to the effects of lasers and biocides, with one study beyond 2000 µm.[8] This *in vitro* study aimed to compare the photobiomodulation effect of a 940 nm laser on *Enterococcus faecalis* through varying thicknesses of human root dentin slices.

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Materials and Methods

Sample Preparation

Thirty-five caries-free human first pre-molar teeth were removed for orthodontic purposes and cleaned with a curette. The teeth were individually stored in 5 mL microcentrifuge tubes (Eppendorf Vertrieb, Wesseling-Berzdorf, Germany) containing 1% thymol of a sterile physiological solution at a temperature of 4°C until further use. With the aid of a slow-speed saw (Steurs Minitom, Copenhagen, Denmark) and a cooled 80 × 0.2 mm diamond blade (Horico, Berlin, Germany), the teeth were first sectioned at the cemento-enamel junction. The roots were subsequently sectioned parallel to the root canal lumen at various thicknesses (500, 1000, 1500, 2000, 2500, and 3000 µm). The thickness of the dentin slices after surface roughness standardization was confirmed with a microcaliper, and slices below the tolerance of ±5 µm were excluded from the study.

The size of the dentine slices was standardized to the resulting total number of thirty-five precise slices with a dimension of 10 × 3 mm × specified thickness in µm and surface roughness of 0.4 µm. Root dentin slices with thicknesses of 500, 1000, 1500, 2000, 2500, and 3000 µm (n=5 each) were randomly divided into 6 experimental groups to be leased. The second group of 2000 µm served as the positive control with no laser exposure. Enumeration and subsequent *E. faecalis* recovery from separate dentine slices were confirmed with an additional fifteen dentine slices with a thickness of 2000 µm each. These additional 15 dentine slices were inoculated with 2 µL of the prepared McFarland standard 0.5 inoculum after its preparation described below, to determine the pathogen recovery at each step of the methodology. The additional fifteen dentine slices used for enumeration and recovery assessed the colony-forming units present at various time intervals. The samples were distributed to: five dentine slices immediately after the inoculation of the dentin slice at the start of the 1-hour incubation period (n=5; pathogen recovery: 418.2±60 CFU × 10^4/mL); five after 1-hour incubation at 37°C (n=5; pathogen recovery: 416.9±55 CFU x 10^4/mL) and five after the last dentin slice was lased (n=5; pathogen recovery: 410.6±48 CFU x 10^4/mL).

Sample Surface Roughness

The surface roughness (Ra) of the dentine slices required standardization to allow the *E. faecalis* to have the same opportunity to adhere to the dentin surface. The dentine was polished with wet 2500 grit carbide paper until the surface roughness meter recorded a Ra value of 0.4 µm.

The Ra of the dentin slices was measured with a Leeb surface roughness tester and a standard sensor (Model Leeb432, Chongqing Leeb Instruments Co Ltd, China). The Ra meter had a measuring range for Ra between 0.005-16 µm. The testing parameters were: surface roughness (Ra), Filter set at Gauss, and the assessment length (λc) at 0.8 mm x *n*5 (assessment length (Ln=lr x *n*); Ln=3.2 mm) (International Organization for Standardization. ISO 4288: Rules and Procedures for Assessment of Surface Texture. Geneva: ISO; 1998). The standard stylus had a natural diamond at a 90° cone angle with a 5 µm tip radius. The stylus applied a force to the sample of <4 mN. The travel speed (Vt) for the above parameters was 0.135 mm/s and the measurement accuracy was ±10%. The Ra was measured by recording the Ra on both sides of the dentine slice with two parallel measurements by way of the assessment length recorded 1 mm apart and two lines perpendicular to that (*n*=4) per sample. The aforementioned measurements (*n*=4) were therefore recorded per side, and the average of the eight measurements was used as the mean Ra per specimen.

Dentin Surface Cleaning

Each of the precise slices was cleaned according to the guidelines of the American Association of Endodontists' clinical protocol for the preparation of root canal systems. The slices were immersed in a 17% EDTA solution for 1 minute to remove the inorganic components of the smear layer. The organic components were removed by irrigation with 6% sodium hypochlorite (NaOCl).[9] The slices were then individually stored in sterile glass bottles in 1 ml of phosphate-buffered saline and sterilized in an autoclave at 120°C for 20 minutes. The sterile dentin slices were transferred to sterile Petri dishes and allowed to dry in a temperature-controlled incubator for 24 hours.

Bacterial Inoculation

The broth was made by dissolving 25 g of powder (Luria Broth- EXMix™ powder microbial growth medium) in 1 Litre of de-ionized water and sterilized by an autoclave at 121°C for 20 minutes. *E. faecalis* (ATCC 29212) bacteria were reconstituted in Lysogeny Broth (Luria Broth) for 24 hours at 37°C and streaked on a standard agar contact plate (TSA LTHth-ICR, Merck Life Science GmbH; Eppelheim; Germany; Batch 140477). A single colony was used to prepare a McFarland standard of 0.5 in phosphate-buffered saline (ICN Biomedicals Inc., Ohio, USA). The McFarland standard of 0.5 was standardized with a DensiCheck plus (BioMerieux Inc., Hazelwood, Missouri, USA). The DensiCHECK plus measuring device used a 580 nm wavelength. The 0.5 McFarland standard was equivalent to a concentration of 1.5 × 10^8 *E. faecalis* cells/mL in the phosphate-buffered saline solution. With the use of a calibrated micropipette, the dentin samples were inoculated immediately after McFarland standard production on one side of the dentine slice (10 × 3 mm) with 2 µL of the prepared 0.5 McFarland standard inoculum and allowed to dry in a sterile closed Petri dish for one hour in a 37°C incubator.

Laser Irradiation

The samples were randomly divided into seven groups: six groups with five samples each according to the thickness of the slice and the positive control group consisting of five samples of 2000 µm dentine slices that were inoculated, but not laser-irradiated. The dentine slices requiring laser irradiation was rotated 180º clockwise, thus performing laser radiation on the opposite side to where *E. faecalis* was inoculated. The laser specifications are presented in Table 1. The 940 nm laser (Biolase, San Clemente, CA, USA) with a non-initiated 200 µm bare end fibre at the power of 1 Watt in a continuous wave setting served as the delivery system.

The clinical laser irradiation activation cycle is usually advised at 10 seconds for a 20 mm root length at a coronal withdrawal speed of 2 mm/s. Based on half of the lumen surface area used in this study, it was appropriate to divide the laser irradiation activation cycle time into 5 seconds. The dentine slice used (10 × 3 mm) had a 2-dimensional surface area of half the lumen surface area (i.e. 30 mm^2) compared to the average 3-dimensional lumen surface area of mandibular premolars (i.e. 58.51±16.41 mm^2).[10] The laser irradiation activation cycle for this study was therefore repeated a total of four times with a side-to-side movement at a "withdrawal" speed of 2 mm/s with the optical fiber tip held at a 5º angle to the dentine slice. The total laser irradiation activation cycle time of 5 seconds per application to the dentine slice would take into account the clinical helical nature of irradiating root canals. Additionally, the temperature transfer to the outer surface of the dentine slice posed no risk of thermal insult to the viability of periodontal structures as established in the literature.[11] The 200 µm tip was replaced after the laser irradiation of each group. A single-blinded operator was used to ensure conformity of the laser irradiation following local statutory regulations for laser safety.[12]

Table 1. Laser Parameters

	Laser Parameter
Type of laser	940 nm nGaAsP Semi-conductor diode
Laser company	Biolase, San Clemente, CA, USA
Emission mode	Continuous Wave
Delivery system	Optical fibre
Power	1 W
Time on/Time off	5-second laser irradiation by the operator / 5-second laser off to allow dentine to cool (procedure repeated four times)
Spot diameter at the tip	200 µm
Spot area at the tip	0.0003 cm^2
Power density at the tip	3183 W/cm^2
Total energy	20 Joules (5 J per laser irradiation of 5 seconds; repeated four times)
Speed of movement	2 mm/s
Energy density with movement	249 J/cm^2
Beam divergence	8-22 degrees per side angle

Bacteriological Analysis

To simulate physiological conditions, all the irradiated dentin slices were stored in individual 2 mL Cryotubes containing 1.8 mL of sterile Lysogeny Broth and incubated at a temperature of 37°C for 24 hours. The storage of the irradiated dentin specimens incubated in the Lysogeny Broth provided nutrition to the *E. faecalis* bacteria. After 24 hours, the bacteria were separated from the dentine slices using a 10-second vortex. The vortexed Lysogeny Broth containing a 1 mL suspension of *E. faecalis* underwent serial dilutions in a sterile phosphate-buffered saline solution of log 10^3. Streaks of 10 µL from the serial diluted *E. faecalis* were completed on the agar plates followed by 24-hour incubation at 37°C in a 10% CO_2 atmosphere.

Upon the completion of the incubation period, the colony-forming units of *E. faecalis* were counted with a colony counting system. The statistical analysis was completed with a one-way ANOVA with a Bonferroni and Holm post-hoc test at a significance level of $P > 0.05$. The post-hoc tests would likely identify which of the pairs of treatments are significantly different from each other (Statistical analysis with R Core Team (2013); (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

Results

The colony-forming units of *E. faecalis* revealed the data presented in Table 2. The Bonferroni and Holm interference showed significant differences ($P < 0.01$) between the bactericidal effects for dentine slices of 500, 1000, and 1500 µm. There was a significant reduction in the *E. faecalis* growth with the positive control of 2000 µm thickness that did not receive any laser treatment. The lased dentine slices of 2000, 2500 µm did not present with a significant lower bacterial growth ($P > 0.05$), compared to the positive control dentine slices. The lased slices of 2000 µm had fewer bacteria than the positive control without the laser, but 2500 µm had a non-significantly greater amount of colony-forming units. When the 3000 µm slice treated with the laser and *E. faecalis* colony-forming units were considered, the bacterial growth through photobiomodulation was significantly ($P < 0.01$) more than the positive control tooth that received no laser treatment.

Discussion

Enterococcus faecalis has been identified as the microorganism that is present at root canal failure.[1] Strains isolated from the oral biofilm or saliva revealed high percentages of virulent genes and resistance to antibiotics

Table 2. Average Colony Forming Units (CFUs) of *Enterococcus faecalis* on Different Thicknesses Of Dentine

Slice thickness in Group	CFU × 10^{-4}/mL	±SD CFU × 10^{-4}/mL
No laser positive control 2000 µm	411.6	±25.16
500 µm	195*	±24.75
1000 µm	262.8*	±21.75
1500 µm	330.6*	±27.03
2000 µm	384.4	±30.59
2500 µm	442.6	±27.10
3000 µm	556.8*	±34.21

*Indicates significant difference from the positive control group.

similar to isolates obtained from systemic infections.[13] This is a reason why it has been well established that lasers are bactericidal on *E. faecalis* up to 1000 µm in dentinal tubules.[14]

It had been concluded that the 980 nm laser had a lower penetration depth (based on *E. faecalis* reduction) than the 940 nm laser. The 980 nm laser at 2.8 W in continuous wave provide a 32% reduction in *E. faecalis* on 500 µm bovine dentine slices.[15] The results of this study indicated that there was a photobiomodulation effect on the bacteria, with growth stimulation in thicker dentine slices (2500-3000 µm). The results of this *in vitro* study for dentine slices of 500 µm revealed a 53% reduction of *E. faecalis* colony forming units compared to 2000 µm positive control where no laser application was performed. Similarly, the log kill rates of dentine slices exposed to laser irradiation were 36.84% (1000 µm), 19.67% (1500 µm), and 6.6% (2000 µm) compared to the positive control. Even though the complete bacterial reduction was not achieved in dentine slices below thicknesses of 2000 µm, sub-lethal damage to the bacteria inhibited cell growth.[16] The results of decreasing cell death as the dentine thickness increases are important if the consideration is made to the photobiomodulation (photobiostimulation) effect in thicker dentine that may be present. The clinician should consider the thickness determination of the apical root third from the radiographs. The thickness of the root surface is important not only for *E. faecalis* but also for the laser irradiation effect on *Streptococcus mutans*. A study with an 810 nm diode laser at 7 Watt was found to reduce the efficacy of the kill in the *Streptococcus mutans* by nearly 50% as the thickness of the tooth doubled.[8] It was noted that as the dentine slices became thicker, the log kill rate was reduced. The 2000 µm positive control dentine slices simulated the growth of *E. faecalis* when no laser irradiation was present. This positive growth determined in the 2500 µm dentine slices of +7.53% and +35.27% for the 3000 µm dentine slices was attributed to the photobiomodulation growth effect on the *E. faecalis*.

Conclusion

Laser irradiation treatment significantly reduced *E. faecalis* growth through thicknesses of 500, 1000, and 1500 µm. Once the tooth thickness achieved 2000 and 2500 µm, the bacterial growth was not significantly more (2500 µm) or less (2000 µm) than the positive control slices that received no laser. Once the tooth thickness of 3000 µm was lased, the *E. faecalis* growth was stimulated significantly by the laser compared to the *E. faecalis* growth of the positive control. This trend is seen for an increase in bacterial growth in Table 2 for dentine slices from 2500 and 3000 µm. Future studies would need to investigate and compare results to determine the ideal laser irradiation to achieve significant pathogen reduction and prevent photobiomodulation (photobiostimulation) compared to the thickness of the tooth structure without a thermal insult on the periodontal complex.

Limitations of the study

Like other studies, this study design did not measure the power of the laser through the dentine slices (with a thermopile-detector) nor the energy penetrating through the slices (with a photodiode detector or a pyroelectric detector).This is an area of study that could advance the understanding of the anti-bacterial effect through various dentine slice thicknesses.

Ethical Considerations

Ethical approval was obtained from the research ethics committee of The University of Western Cape (Ethical approval: BM19/7/5).

Conflict of Interests

The authors declare no conflict of interest.

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