Effects of 980 nm diode laser application protocols on the reduction of *Enterococcus faecalis* intracanal biofilm: An in vitro study

Efekty zastosowania promieniowania laserowego o długości fali 980 nm na redukcję wewnątrzkanałowego biofilmu bakteryjnego *Enterococcus faecalis* – badania *in vitro*

Ewa Joanna Prażmo[1,A–F], Alicja Renata Godlewska[2,B,D], Monika Sałkiewicz[2,B], Agnieszka Beata Mielczarek[1,D–F]

[1] Department of Conservative Dentistry, Medical University of Warsaw, Warsaw, Poland
[2] Department of Bacterial Genetics, Faculty of Biology, University of Warsaw, Warsaw, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

Dental and Medical Problems, ISSN 1644-387X (print), ISSN 2300-9020 (online) *Dent Med Probl.* 2017;54(4):333–338

Address for correspondence
Ewa Prażmo
E-mail: ewagg@interia.eu

Funding sources
None declared

Conflict of interest
None declared

Received on August 5, 2017
Reviewed on August 29, 2017
Accepted on September 22, 2017

Abstract

Background. *Enterococcus faecalis* is associated with a great number of refractory endodontic infections. There is a need to investigate antibacterial properties of laser radiation and create a new alternative technique for root canal disinfection.

Objectives. The purpose of the study was to investigate the effectiveness of a single and repeated high-power diode laser irradiation protocol on the elimination of a 1-week-old *Enterococcus faecalis* intracanal biofilm.

Material and methods. A total of 46 single-rooted human teeth were subjected to the in vitro observation. They were chemomechanically prepared, sterilized, infected with a clinically isolated strain of *Enterococcus faecalis* and subjected to 1-week incubation under microaerobic conditions. The experimental procedures included: a single cycle of 980 nm laser diode irradiation, second application of the same laser protocol, 5.25% NaOCl irrigation, and 2 control groups. Quantitative evaluation of bacterial colonies in the root canals was performed based on the CFU/mL method, after different sterilization methods had been applied.

Results. A statistically significant reduction in the number of intracanal *Enterococcus faecalis* colonies, after a single and repeated 980 nm diode laser application, was confirmed. The first cycle of laser irradiation eliminated 52.5% of *E. faecalis* colonies, whereas the second application increased disinfection effectiveness to 87.6%.

Conclusions. The 980 nm diode laser demonstrated statistically significant antibacterial activity. High-power diode laser treatment might be considered as an adjunctive to conventional chemomechanical endodontic treatment.

Key words: *Enterococcus faecalis*, diode laser, 980 nm, endodontic disinfection

Słowa kluczowe: *Enterococcus faecalis*, laser diodowy, 980 nm, dezynfekcja endodontyczna

DOI
10.17219/dmp/77898

Enterococcus faecalis is associated with a great number of refractory endodontic infections.[1] It has developed elaborated mechanisms of antibiotic resistance, as well as the ability to organize in biofilm and overcome low-nutrient conditions. These adaptations cause the modulation of a host's immune response and make *E. faecalis* very difficult to eradicate by available medications and disinfectants applied as part of endodontic therapy.[2] To eliminate bacteria not affected by the traditional chemomechanical debridement, new antibacterial strategies should be investigated.

Diode lasers are electrically pumped semiconductor lasers. They are manufactured with many designated applications that also include medicine and dentistry. In contrast to the other laser systems they are characterized by relatively low purchase and maintenance costs, great versatility and compact size. Diode lasers equipped with small irradiation tips, open up new fields of application in endodontics. Thin flexible fibers easily reach even the curved shaped root canals. Diode laser wavelengths have good penetration potential, high absorption peaks in melanin and hemoglobin, and low interaction with water and hydroxyapatite,[3,4] which result in photothermal interaction with root canal dentine. These properties seem to be adequate for the purpose of root canal disinfection. The photothermal effect of high-power laser radiation strictly depends on the power density, irradiation frequency, wavelength, duration of a cycle and dentine thickness.[5] Proper selection of the laser parameters yields a therapeutic effect, but also helps to avoid injury to the periodontal ligament cells and alveolar bone. The photothermal effect, that leads to a temperature increase of 10°C for longer than 1 minute, can cause irreversible changes to the root dentine and the surrounding tissues.[6]

The aim of the study was to assess the effectiveness of a single and repeated high-power diode laser application in the elimination of intracanal *Enterococcus faecalis* biofilm. To date, the potential of 980 nm diode laser in root canal disinfection has seldom been addressed and the most efficient protocol of irradiation has not been determined. Tissue safe application parameters have been chosen for the purpose of the study. To avoid morphological alterations of the dentine and thermal damage of the surrounding tissues, the fiber was moved constantly in a circular motion during activation in the root canals. Microbiological assessment was performed after a single and repeated cycle of laser irradiation. A clinically isolated strain of *E. faecalis* was used for the purpose of the study.

Material and methods

Isolation of *Enterococcus faecalis* clinical strain

A patient, age 31 was admitted to the Department of Conservative Dentistry, Medical University of Warsaw. A panoramic radiograph was taken to establish the further treatment plan. The lower left first molar was diagnosed with chronic periapical inflammation, based on the presence of an osteolytic lesion surrounding the root apex. An endodontic filling material was visible in the coronal part of the roots and the tooth was asymptomatic. The patient agreed to the proposed revision of endodontic treatment and a microbiological analysis of the intracanal pathogenic microflora.

Tooth 36 was isolated with a rubber dam and an access cavity was performed. The root canals were initially chemomechanically prepared with the use of hand K files (VDW) to ISO #25. Sterile 0.9% NaCl was the only irrigant used. After the preliminary removal of residual remnants of filling material, the roots were filled with sterile 0.9% NaCl, and ISO #25 K file was introduced to perform scrubbing motions and to collect dentine shavings. Subsequently, ISO #25 paper points were placed into the canals for 60 s to adsorb material for microbiological analysis. Revision of root canal treatment was continued according to the accepted standards of endodontic therapy with the use of complete irrigation protocol.

Paper points were transferred to tubes containing 3 mL of brain heart infusion broth (BHI) and were subjected to 24 h of incubation at 37°C. The serial dilutions were performed and aliquots of 100 µL were inoculated on plates with BHI (Oxoid) agar and on Slanetz and Bartley LAB-AGAR (Biocorp) plates (selective-differential medium for quantitative determination of *Enterococci*, on which *E. faecalis* forms from dark pink to dark brown colonies).

Strain identification

With the use of a commercial kit (A&A Biotechnology, Poland), chromosomal DNA was isolated. According to standard protocol, polymerase chain reactions (PCRs) were performed with PrimeStar HS DNA Polymerase (TaKaRa). The universal primers F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R1492 (5'-TACGGYTACCTTGTTACGACTT-3'),[7] which target universally conserved regions and permit the amplification of an approximately 1,500-bp fragment, were used to obtain 16S rRNA gene in the course of PCR. Oligonucleotide synthesis and DNA sequencing were performed by Genomed SA, Warsaw, Poland. The nucleotide sequences were analyzed using BLAST and compared to the nucleotide database on the NCBI website. The highest identity, 99%, was related to the nucleotide sequence of the 16S rDNA gene of *E. faecalis* JF85 (GeneBank KT343158.1). The taxonomic position of the isolated clinical strain was identified as *E. faecalis*.

Specimens' preparation

A total of 46 single-rooted human teeth were extracted based on the periodontal of orthodontic referral. All of the teeth were collected after the adult patients gave

consent. The experiment included only mature, intact teeth with a single canal, and no signs of root resorption. The teeth were stored in 0.1% sodium azide solution. All samples were decoronated with a diamond bur (Meisinger 859L.016) to acquire 15 mm long roots and the working length was established at 1 mm. Initial chemomechanical preparation was performed with the use of hand K files to ISO #25 with 5.25% NaOCl as irrigation solution. Further canal enlargement was performed to an apical size 40 (R40) using Reciproc rotary instruments (VDW, Munich, Germany). Irrigation protocol included 5.25% NaOCl and 17% EDTA for final smear layer removal. The root apex of each sample was sealed with glassionomer cement, and the outer surface was covered with 2 layers of nail varnish to prevent reverse contamination of the dentinal tubules. All specimens prepared according the described protocol were autoclaved at 121°C for 15 min. A group of 10 roots was subsequently used to evaluate the effectiveness of the sterilization process. They were subjected to irrigation with sterile 0.9% NaCl. The liquid was plated on blood agar and after 24 h of incubation no bacterial growth was observed.

Root canals contamination

The clinically isolated strain of *Enterococcus faecalis* was cultured overnight in Tryptic Soy Broth at 37°C, under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Subsequently, dilutions of OD_{600} 0.7–0.8 were prepared and plated on BHI agar. This allowed us to determine the number of bacterial colonies to be 1–3 × 10^8 CFU/mL. Each sterile experimental unit was placed in a screw-cap plastic vial with 1 mL of TSB and 1 mL of bacterial suspension. To obtain biofilm formation on the walls of root canals, specimens were incubated for 7 days (37°C, microaerobic conditions).[8] Every 48 h TSB medium was replaced. Kishen et al. investigated the process of *E. faecails* biofilm formation on the internal surface of a root canal dentine.[8] They have confirmed that after 1 week of incubation, bacterial cells aggregate on the dentine surface, forming a characteristic interconnecting network of polymer strands. In this stage of biofilm development, signs of dentine structure dissolution were also detected.

Experimental protocol

The study included 5 groups (Table 1). The specimens from group 0 (n = 10) were used for the assessment of the sterilization process, and were excluded from the experiment. The positive control group (control) was irrigated with sterile 0.9% NaCl to determine the number of *E. faecalis* colonies before the application of any disinfecting methods. The CFU/mL microbiological method was used for the quantitative assessment of the bacterial growth.

Table 1. Experimental groups

Group	Number of specimens	Experimental procedure
0	10	negative control group-evaluation of sterilization process
Control	12	positive control group-irrigation with 0.9% NaCl
1 × 980 nm	12	single 980 nm laser radiation protocol – 3 W/100 Hz, 20 s
2 × 980 nm	12	double 980 nm laser radiation protocol – 3 W/100 Hz, 20 s
NaOCl	12	5.25% NaOCl irrigation – 5 min

The second group (1 × 980 nm) was subjected to a single 980 nm laser radiation protocol. The laser source was a semiconductor 980 nm diode laser (Smart M, Lasotronix, Poland). Radiation was delivered to the root canal walls with the use of 200-μm-diameter flexible optical fiber. The working length of the fiber was adjusted to 13 mm with the use of a rubber stopper. The device was activated to deliver a beam with a power of 3 W, and 100 Hz frequency. Helicoidal forward-backward movements of the optical fiber were performed in contact with the root canal dentin. The duration of each laser cycle was 20 s. To eliminate any changes in the laser light distribution from the used optic fiber tip, 1 mm of the fiber was cut off after each application.

In the third group (2 × 980 nm), specimens from the group 1 × 980 nm were used to repeat the same protocol of laser irradiation. There was a 1 minute break between protocols to reduce the temperature of dentin.

The last experimental group (NaOCl) was subjected to the chemical disinfection with the use of 5.25% NaOCl solution. Irrigation was performed with 10 mL of the disinfectant, which was subsequently left in the roots for 5 min. The chemical disinfection was followed by 0.9% NaCl irrigation.

In each of the groups, the number of *E. faecalis* colonies remaining in the root canals after the completion of the experiment was determined based on the CFU/mL method. Canals were filled with a sterile 0.9% NaCl solution. Hand K files size 30 (VDW, Munich, Germany) were placed into each canal for 15 s to perform scrubbing motions. Sterile paper points R40 (VDW, Munich, Germany), used as absorbents of microbiological material, were introduced into canals for 60 s to be finally transferred to Eppendorf type probes with 1 mL of TSB. All probes were kept in a container filled with ice before being transported to the laboratory.

Tubes with paper points were agitated for 60 s to perform 10-fold serial dilutions. Aliquots of 0.1 mL were incubated on TSB agar plates in 37°C. After 72 h of incubation, *E. faecalis* colonies were counted. Based on the known dilutions, the actual number of bacterial colonies was calculated and given as CFU/mL (colony-forming units per milliliter).

Statistical analysis

Experimental data (Table 2) was subjected to statistical analysis with the use of Statistica 12 software and based on Shapiro-Wilk test, Cochrane's C test, the Mann-Whitney U test and Wilcoxon test. A < 0.05 was considered to be statistically significant.

Table 2. The count of *E. faecalis* colony-forming units after experimental procedures

Measurement conditions	M	SD	Min	Max
Control	$1.3 * 10^6$	$1.0 * 10^6$	$1.0 * 10^5$	$3.2 * 10^6$
1 × 980 nm	$0.6 * 10^6$	$1.2 * 10^6$	$0.4 * 10^5$	$4.6 * 10^6$
2 × 980 nm	$1.6 * 10^5$	$1.4 * 10^5$	$1.5 * 10^4$	$4.9 * 10^5$
5% NaOCl	$0.2 * 10^3$	$0.6 * 10^3$	0.0	$2.0 * 10^3$

M – mean value; SD – standard deviation; min – minimum value; max – maximum value; PDT – 1 cycle of photodynamic therapy; 2PDT – 2 cycles of photodynamic therapy.

Results

Based on the Shapiro-Wilk test, the distribution of the parameters between research groups was verified. In unrelated experimental groups with normal distribution, Cochrane's C test was applied. It allowed us to confirm a statistically significant difference between the control and 2 × 980 nm group (p = 0.003; t = 3.8).

To compare the data without normal distribution, a non-parametric Mann-Whitney U test was applied. Statistically significant differences were observed between the control group and 1 × 980 nm group (p = 0.043; Z = –2.0), as well as between control conditions and NaOCl irrigation (p = 0.000; Z = 4.2). Statistically significant differences were also confirmed between 2 laser applications and NaOCl irrigation (p = 0.000; Z = 4.2), as well as between a single laser protocol and chemical disinfection with the use of NaOCl (p = 0.000; Z = 4.2) (Fig. 1).

The observations in 1 × 980 nm and 2 × 980 nm groups were performed on the same specimens. The resulting measurements without normal distribution were subjected to a Wilcoxon test (p < 0.05). A statistically significant difference between the number of bacterial colonies between 1 × 980 nm and 2 × 980 nm (p = 0.012; t = 7.0) was confirmed.

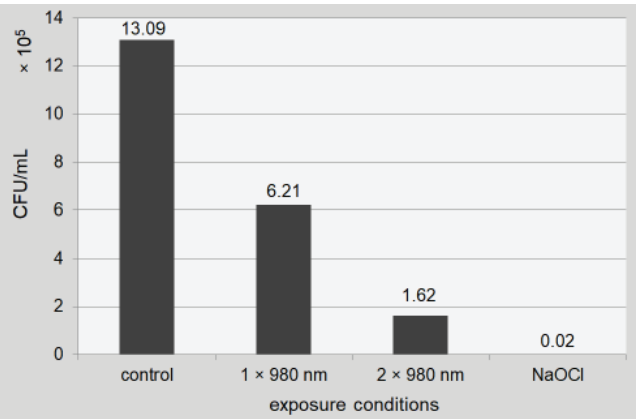

Fig. 1. Mean value of CFU/mL for each of the groups subjected to observations

A single cycle of 980 nm laser application eliminated 52.5% of the *Enterococcus faecalis* colonies from infected root canals (Fig. 2). After a second cycle of laser disinfection, significantly more bacterial colonies were eradicated, and the number of bacteria was reduced to 12.4% of the initial number. Chemical irrigation with 5.25% NaOCl recorded the highest level of disinfection with an efficiency of 99.7%.

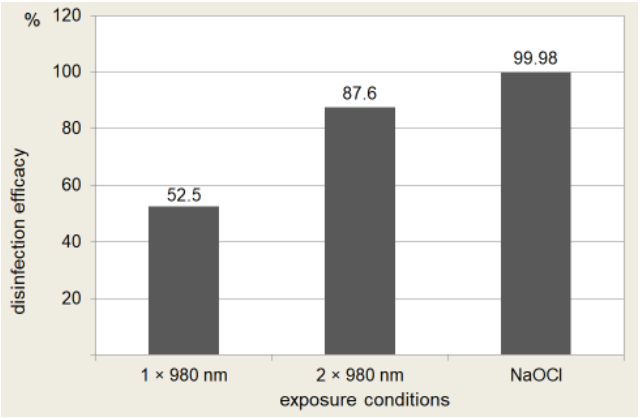

Fig. 2. The efficacy of different antibacterial procedures presented by percentage of eliminated *E. faecalis* colonies

Discussion

Sodium hypochlorite is the most common chemical disinfecting substance used during root canal preparation. Its antimicrobial properties[9] and ability to dissolve organic tissues[10] are crucial for the effectiveness of endodontic therapy. However, improper application may lead to very harmful side effects. Sodium hypochlorite is very cytotoxic when inadvertently injected to periapical tissues,[11] since it promotes dentine deproteination[12] and adversely affects bond strength of adhesive material to dentine tissue.[13] In rare cases of NaOCl allergy,[14,15] the use of this substance is contraindicated.

The described experiment confirmed the high dentine disinfecting potential of 5.25% NaOCl. However, results of in vitro experimental model should be considered as overvalued. When treatment is performed under in vivo conditions, the properties of hypochlorite are weakened by the presence of the organic matter.[16] Pulpal tissue fragments, dentinal collagen, bacterial debris and inflammatory exudates are present within infected root canals. They consume NaOCl and impair its disinfecting action. It was also proved that the penetration depth of the irrigants is limited to a small area close to the surface of the root canal[17] and, a maximum penetration of sodium hypochlorite is 130 μm.[18] To improve the decontamination of root canals, new alternative disinfection techniques need to be tested.

In this experiment the high-power diode laser radiation resulted in a clinically significant reduction of *Enterococcus faecalis* colonies from the infected specimens. Based on other published studies, 980 nm diode laser at the parameters of 3 W/100 Hz, which were applied in the experimental model, not only disinfects but also changes the dentine structure. Modified organic matrix layer with an amorphous form, the melting of the intracanal dentine surface and tubule visibility were observed in Marchesan et al. experiment.[19] Jhingan et al. confirmed diode laser treatment leads to excellent removal of smear layer and dentinal debris from the root canal surfaces.[20] In the same study, the authors attempted to measure the size of dentinal tubules resulting from 5.25% NaOCl irrigation and 980 nm laser radiation. The mean width after chemical disinfection was 2.4701 μm, whereas after laser therapy 0.1975 μm. The authors concluded that 0.1975 μm width of dentinal tubule is difficult for the microorganisms to pass through and in consequence reduces the possibility of reinfection. Changes of the dentine structure can be attributed to the thermal effect caused by laser energy.[21] 980 nm diode laser is located in the near infrared region of electromagnetic spectrum.[22] When laser radiation is applied at this wavelength, some energy is absorbed by dentine mineral content, like phosphate and carbonate. This results in a crystalline arrangement and hard tissues melting.[22] As a consequence, laser parameters of application have to be carefully chosen for the purposes of endodontic disinfection.

The mechanisms regarding the antibacterial properties of a high-power diode laser are based on the thermal and photodisruptive effect of the emitted radiation.[23] 980 nm diode lasers are characterized by high-energy radiation. When the energy is absorbed by the water molecules in the tissues, it is converted to heat. As a consequence, evaporation of water causes cell destruction and death of microorganisms. But there are also other published theories regarding bacterial cell death caused by laser radiation. A possible assumption is that after irradiation temperature rises momentarily to an extremely high level and the intensive heat results in the immediate destruction of the bacteria.[23] Another theory[24] assumes that immediate cell death may not occur during laser irradiation, but sublethal damage. Sublethal damage is explained as a disruption of cell wall integrity and the accumulation of denaturated proteins. Those changes cease the cell growth and result in cell lysis. This last thesis was also described by Moritz et al.[25] They infected dentin slices with *E. faecalis* stain and used high- power laser radiation to eliminate the bacteria. Single cycle of 1.0 W 15 pps and 1.5 W 15 pps did not cause degenerative alterations of the bacterial cells. The repetition of 1.5 W 15 pps laser radiation cycle resulted in substantial morphologic changes, and a significant decrease of colony forming units. The authors concluded that the lethal effect of laser irradiation on *E. faecalis* is based on a cumulative effect. Heat, caused by the laser application, is a stress factor that causes nonlethal reversible damage, which might be transformed into lethal damage after repetition of the stress.[26] This theory explains the results of the conducted experiment and significant differences in the number of bacterial colonies after single and repeated laser irradiation protocols.

The effectiveness of a high-power diode laser in root canal disinfection has seldom been addressed. The parameters of irradiation power, such as frequency and time of a single cycle, have to be chosen carefully, to avoid damaging the root canal dentine, and to remain effective. Kanumuru et al. investigated the bacterial efficiency of $Ca(OH)_2$ against *E. faecalis* compared with 3 dental lasers.[27] 980 nm diode laser proved to be 2 times more effective than the chemical agent with a high pH. However, researchers did not include information about the power and time of irradiation of the laser device. Mithra et al. investigated the bactericidal effect of 980 nm diode laser, 3% NaOCl, 2% CHX, and their combination with laser in *E. faecalis* infected root canals.[28] The power of 2.5 W in continuous mode was applied in 3 cycles of 5 s. Chemical disinfection with 3% NaOCl was the most effective antibacterial protocol. 980 laser radiation was significantly more effective than 2% CHX, and in combination with 2% CHX it was as effective as 3% NaOCl irrigation. An in vitro research conducted by Souza et al. was also focused on the effectiveness of a high-power diode laser in association with chemical auxiliary substances on bacterial decontamination of root canal system.[29] A total power of 3 W was applied in 4 short cycles of 6 s each. Four cycles of laser radiation were able to reduce 65.4% of *E. faecalis* colonies. In combination with 2% CXH, 93.48% of bacteria was eliminated. The best results of disinfection, 99.42%, were observed after combining the action of 980 nm laser radiation and 2.5% NaOCl solution. Grącka-Mańkowska et al. also investigated the bactericidal efficacy of different diode laser operation modes against *Enterococcus faecalis* under in vitro conditions.[30] Bovine teeth were infected with the bacterial strain and a high-power diode laser was applied with the parameters of 1.5 W CV and 3 W impulse irradiation. In both experimental groups, the mean number of bacterial cells was reduced: in 1.5 W group by 97%, and in 3 W by 93%. However, authors did not reveal the time of single 980 nm diode laser irradiation protocol.

All cited experiments and the described in vitro study confirmed the effectiveness of antibacterial properties of the high-power diode laser. However, parameters of application differ between the studies, and it is difficult to establish the most effective and still safe protocol of irradiation. The power of a laser exceeding 3 W in continuous mode may cause irreversible damage to the dentine structure. Marchesan et al. after 20 s of 3 W/CW laser radiation observed sparse lava-like melting and a scaly surface of root canal dentine.[19] Alfredo et al. confirmed that the power of 3 W, in the pulse mode, applied for 20 s is a safe threshold for the 980 nm diode laser application.[31]

It does not cause a dangerous increase of temperature in the targeted tissues. The extended time of 1 irradiation cycle may be damaging for the dentine and surrounding soft tissues because of the heat dispersion. The results of the described experiment have shown that repeating the laser irradiation is an effective modulation of the therapy. To minimize dangerous thermal effects of 980 nm diode laser application, and to increase the effectiveness of the therapy, the cycle should be repeated. Further studies with multiplied number of laser applications could bring new significant data.

Conclusions

In the study, we confirmed a positive impact of the repeated 980 nm laser radiation in the elimination of intracanal *Enterococcus faecalis* biofilm. The disinfecting potential of the applied protocol did not allow for the complete elimination of the pathogenic cells from infected specimens. Irrigation with 5.25% NaOCl was the most effective method of root canal disinfection. However, the high-power diode laser therapy might be considered as an adjunctive application to chemomechanical endodontic treatment.

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