

EWA JOANNA PRAŻMO^{1, A–D}, RENATA ALICJA GODLEWSKA^{2, B–D},
MONIKA SAŁKIEWICZ^{2, B}, AGNIESZKA BEATA MIELCZAREK^{1, C, E, F}

Evaluation of Photodynamic Therapy Efficacy in Eliminating *Enterococcus faecalis* Biofilm: A Preliminary Study

Ocena skuteczności terapii fotodynamicznej w eliminacji biofilmu *Enterococcus faecalis* – badania wstępne

¹ Department of Conservative Dentistry, Medical University of Warsaw, Poland

² Department of Bacterial Genetics, Faculty of Biology, University of 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 article

Abstract

Background. Photodynamic therapy is a new treatment protocol used in many areas of medicine. It has the potential to eliminate bacteria, fungi and parasites that may cause different pathological states.

Objectives. The aim of the study was to evaluate the efficacy of a specific photodynamic protocol in the reduction of *Enterococcus faecalis* biofilm inside the canals of extracted teeth.

Material and Methods. Thirty two extracted human, single-rooted, single-canal, fully developed teeth were subjected to observation under *in vitro* conditions. After chemomechanical preparation, the specimens were infected with a clinical strain of *E. faecalis* and divided into four groups. Two served as a positive and a negative control group. The first of the experimental groups was subjected to a single photodynamic protocol with the use of toluidine blue as a photosensitizer and laser light for the induction of reaction. In the second experimental group, photodynamic protocol was repeated on the previously irradiated specimens. The last group was disinfected with 5.25% NaOCl. The CFU method was used to determine the number of viable bacterial colonies inside the root canals after completion of the experiment.

Results. The single application of PDT eliminated 78% of the initial *Enterococcus faecalis* colonies. The repeated photodynamic protocol reduced 86% of the bacteria colonizing the root canal dentine. Irrigation with 5.25% NaOCl completely disinfected the internal surface of the root canal.

Conclusions. Photodynamic therapy is an effective antibacterial protocol and can be used as a complementary disinfecting method after conventional endodontic treatment (**Dent. Med. Probl. 2016, 53, 4, 490–495**).

Key words: photodynamic therapy, *Enterococcus faecalis*, biofilm, disinfection of root canals.

Słowa kluczowe: terapia fotodynamiczna, *Enterococcus faecalis*, biofilm, odkażanie kanałów korzeniowych.

The purpose of endodontic therapy is to eradicate bacterial cells that exist in the tooth's root canal system and cause inflammation. The complex anatomy of root canals makes it difficult to completely eliminate pathogenic micro-organisms [1]. Regardless of the instruments used, mechanical treatment of the canal inner walls does not result in even preparation and removal of infected dentine [2]. The use of chemical agents

during irrigation, between intermediate visits and during the final obturation of the canal to disinfect the dentine areas not mechanically prepared is an essential part of complete endodontic therapy. The most common irrigant is NaOCl. However, its ability to penetrate into dentinal tubules is limited. In one of the experiments, its penetration did not exceed 130 µm [3], which, compared to the penetration ability of bacteria of

1000 μm [4], is not sufficient to completely disinfect the infected area.

Photodynamic therapy was developed as an alternative cancer treatment protocol. It is based on the effect of a photosensitizing substance having affinity to pathogenic and pathologically changed cells, and light. Under aerobic conditions, the appropriate wavelength activates a photosensitizer and leads to the formation of reactive oxygen species that are cytotoxic to the target tissues. Contemporary endodontics has made an attempt to use this therapy as an alternative method to disinfect the root canal system. Different types of photoactive substances and light sources have been investigated. The most effective and safe PAD protocol has not been established so far. The experiment described uses toluidine blue as the photosensitizer and a diode laser with a compatible wavelength as the light source initiating the reaction. Application conditions that are safe for human tissues and show a high efficacy potential have been developed on the basis of scientific reports available in medical databases [5]. Extracted human teeth and a clinical strain of *Enterococcus faecalis* obtained from a patient were used to reproduce the environment of the oral cavity. The study was designed to evaluate the efficacy of laser photodynamic therapy in eliminating bacterial biofilm.

Material and Methods

Study Material

Thirty two root canals were observed under *in vitro* conditions. The study material obtained by extraction for medical reasons included human single-rooted, single-canalled, fully developed teeth with no signs of root resorption. The material was obtained during the period of 3 months when the teeth were stored in 0.1% sodium azide. The study material was standardized by cutting the root to a length of 15 mm using a diamond bur for turbine (Meisinger 859L.016).

Chemomechanical Root Canal Preparation

The working length of each canal was established using steel hand canal instruments. A size 10 K-file (VDW) was inserted to the full length of the root until the instrument tip appeared in the apical foramen. Then it was retracted by 1 mm to establish the working space. The canal lumen was widened to size 25 with a series of hand K-files (VDW). The canal was alternately irrigated with 2 mL of 40% citric acid and 2% NaOCl between

using the subsequent instruments. The next preparation step involved using size R 25 and R 40 Reciproc instruments (VDW) to further widen the canal. The preparations were sterilized in an autoclave at 121°C for 15 minutes. Eight preparations were randomly selected for the evaluation of the sterilization efficiency and excluded from further experimental protocol.

Obtaining *E. faecalis* Cells From a Patient

The study used a clinical strain of *E. faecalis* obtained from a patient. A 31-year-old male patient reported to the Department of Conservative Dentistry of Warsaw Medical University for the diagnosis of caries. A panoramic dental X-ray showed an osteolytic inflammatory lesion around the distal root of tooth 36. Revision of the endodontic treatment was required because half of the root canal was filled with a non-homogeneous substance. The patient did not report any pain. He expressed his consent to endodontic retreatment and collection of microbiological material from the canal lumen.

Before initiating the treatment, tooth 36 was isolated with a dental dam. The composite material and lining securing the root orifices were removed. Using manual K-files (VDW), the non-homogeneous filling material was removed and the canal lumen was widened to size 25. Only 0.9% NaCl was used for irrigation. Then, the tooth crown was cleansed with an isopropyl alcohol solution. The canals were filled with 0.9% NaCl, sterile K-files were inserted, then an up-and-down scrubbing motion was continued for 30 s. The material was collected on paper points by inserting them in the canals for 60 s. Further chemomechanical canal preparation was conducted according to the established standards using full irrigation protocol.

The microbiological material obtained from the patient was placed in test tubes containing 3 mL of BHI liquid medium and was incubated at 37°C for 24 h. Dilutions were prepared and inoculated on plates with Oxoid BHI agar and on Biorcorp Slanetz and Bartley LAB-AGAR plates (medium for the detection and enumeration of *Enterococci*, on which *E. faecalis* forms from dark pink to dark brown colonies).

Strain Identification

Chromosomal DNAs were isolated using a commercial kit and protocol (A&A Biotechnology, Poland). Polymerase chain reactions (PCRs) were performed with PrimeStar HS DNA Polymerase (TaKaRa) or HotStar HiFidelity Polymerase

(Qiagen) under standard conditions. The amplified 16S rRNA gene was obtained from isolate by PCR with the universal primers F27 (5'-AGAGTTT-GATCMTGGCTCAG-3') and R1 492 (5'-TACGGY-TACCTTGTTACGACTT-3') [6], which are targeted to universally conserved regions and permit the amplification of an approximately 1,500-bp fragment. DNA sequencing of the PCR product spanning the 16S rDNA gene was performed by the DNA Sequencing and Oligonucleotide Synthesis Service at IBB PAS in Warsaw, using a ABI377 sequencer (Applied Biosystems). Then the nucleotide sequences were analyzed using BLAST against the nucleotide database on the NCBI website.

Bacterial Culturing

Overnight cultures of *E. faecalis* were prepared (TSB liquid medium, 37°C, without shaking, microaerobic conditions – 5% O₂, 10% CO₂, 85% N₂) and then diluted to an OD₆₀₀ of 0.7–0.8. At the same time, the solution was inoculated on dishes to determine the actual bacterial titer, which was ca. $1-3 \times 10^8$ /mL. The experimental preparations were obtained in 15 mL Falcon type test tubes. A sterile root, 1 mL of the fresh TSB medium and 1 mL of *E. faecalis* culture were placed in each test tube. An incubation time of 7 days was needed to obtain the structure of bacterial biofilm on the surface of the canal dentine (37°C, microaerobic conditions). The TSB growth medium was exchanged every 48–72 h.

Study Protocol Course

The tooth preparations infected with *E. faecalis* were divided into four equal groups (n = 8). The control group, which was not subjected to further preparation, was used to evaluate the growth of the *E. faecalis* clinical strain in the lumen of the chemomechanically prepared root canals.

In the first experimental group, photodynamic therapy was conducted. The photosensitizer was 13–15 mg/mL toluidine blue placed in the canal for 120 s to achieve accumulation in the target tissues. The light source was a diode laser with a wavelength of 635 nm. During the experiment, the laser light emitting device was set to 120 mW, 12 J, 2 min. Before the PDT cycle, each preparation was irrigated with 2 mL of 0.9% NaCl sterile solution. Then the canal was dried with a R 40 paper point and filled with toluidine blue. After 2 min, a laser fiber was inserted into the canal, 1 mm short of the working length established during the preparation step. In each irradiation cycle, the laser tip was moved in a spiral motion towards the canal orifice, then towards the canal apex for

an even distribution of radiation. After each PAD cycle, the fiber was cut at 1 mm and the tip was cleaned with a liquid disinfectant. After completion of the photodynamic therapy, the canal interior was again irrigated with 0.9% NaCl to remove the deactivated photosensitizer.

In the second study group the same roots were exposed to the second cycle of photodynamic therapy using the parameters selected for the first study group. The break between the cycles was 2 min. Laser radiation was preceded by reapplication of the photosensitizer into the root canal.

In the third experimental group, the tooth preparations were chemically disinfected with 5.25% NaOCl. This substance, commonly used in conventional endodontic therapy, was applied to the infected canals for 5 min. After this time, the preparations were cleaned by irrigation with 2 mL of 0.9% NaCl.

Microbiological Material Collection and Quantitative Analysis

In each of the preparations, the microbiological material for quantitative evaluation of the growth and survival of *Enterococcus faecalis* was collected using the same procedure. The canals were filled with 0.9% NaCl. Then, the up-and-down scrubbing motion with size 40 manual K-files (VDW) inserted to the working length was continued for 15 s. The microbiological material was collected using size R 40 sterile paper points placed in the root canals for 30 s. The paper points with the infectious material were placed in 1 mL of sterile TSB medium using Eppendorf type 1.6 mL conical test tubes. The test tubes were placed in a container with ice and handed over to the laboratory 30 min after the experiment completion.

The solution with the bacteria cells was shaken for 60 s. Then, a series of dilutions were made to obtain the material for inoculation on plates with the TSB medium (Oxoid). 0.1 mL of the preparation was inoculated at a time and then incubated for 48 h at 37°C. The number of *E. faecalis* colonies remaining in the root canals after completion of the experiment was calculated using the CFU method and taking into account the dilution of the test solutions. The results obtained were statistically analyzed.

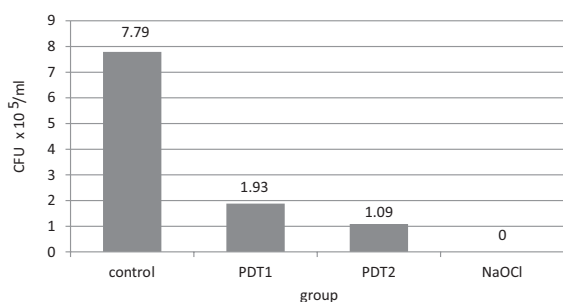
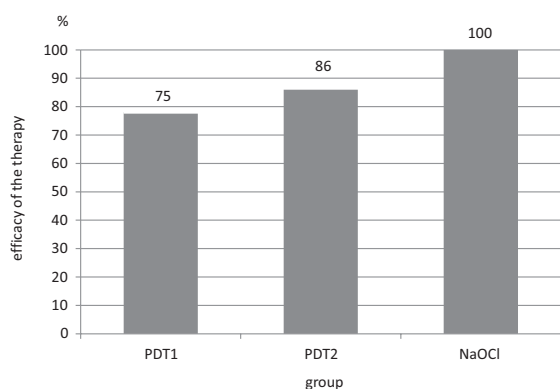
Results

Statistical analysis of the obtained data (Table 1) was performed with the use of Statistica 12 software. The Shapiro-Wilk test was used to veri-

Table 1. Descriptive statistics for bacterial titer after the experiment

Group	M	SD	Min.	Max.
Control	7.79×10^5	6.02×10^5	2.4×10^5	20×10^5
PDT	1.93×10^5	1.38×10^5	0.025×10^5	3.9×10^5
2PDT	1.09×10^5	1.13×10^5	0.055×10^5	3.5×10^5
NaOCl	0	0	0	0

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

**Fig. 1.** Mean bacterial titers under different experimental conditions**Fig. 2.** Percentage elimination of bacterial strains from root canals after using individual disinfection methods

fy, if the distribution of the parameters between research groups is normal. Experimental observation in groups PDT1 and PDT2 were performed on the same specimens, so the groups were statistically related and student's t-test ($p > 0.05$) was used to calculate correlation between them. As a result no statistically significant difference between one and two applications of PDT was observed ($p = 0.117$; $t = 1.8$). The other research groups were not experimentally related. To compare data from control, PDT1 and control, PDT2 groups Cochran's C test ($p < 0.05$) was used because each individual data series was normally distributed. Statistically significant difference was observed between control and PDT1 ($p = 0.029$; $t = 2.7$) as well as between control and PDT2 ($p = 0.016$; $t = 7.5$) (Fig. 1). To compare the data without normal distribution, nonparametric Mann-Whitney U test was applied

($p < 0.001$). In the group of preparations treated with sodium hypochlorite, bacterial colonies were not observed and as a result there was statistically significant difference between both PDT1, NaOCl and PDT2, NaOCl ($p = 0.000$; $Z = 3.5$).

A single cycle of photodynamic therapy eliminated 75% of bacterial colonies from the lumen of the infected root canals (Fig. 2). Two cycles of PDT resulted in a further reduction of *E. faecalis* biofilm. Disinfection efficiency in this study group was 86%. Irrigation with NaOCl was most efficient and resulted in complete elimination of the bacterial strain observed in this experiment.

Discussion

The purpose of endodontic therapy is to eliminate pathogens colonizing the root canal dentine. Chemomechanical preparation of the canal walls is followed by obturation of the obtained surface. However, conventional treatment techniques are associated with the risk of leaving persistent bacteria within accessory canals, stenoses and dentinal cracks. Tight three-dimensional filling of the root canal deprives bacteria of access to nutrients, however, the risk of re-infection is always present. Therefore, it is necessary to seek new disinfection methods that would replace or complement the existing treatment protocols. The above research project was designed to evaluate the bactericidal potential of photodynamic therapy in the elimination of *E. faecalis* biofilm.

The experimental strain of *E. faecalis* was isolated from a patient. The strain grew as small white colonies on BHI and TSB agar medium and as dark purple colonies on Slanetz and Bartley LAB-AGAR plates. The morphological observations were confirmed by molecular identification. Nucleotide analysis revealed the highest identity, i.e. 99%, to the nucleotide sequence of the 16S rDNA gene of *E. faecalis* JF85 (GeneBank KT343158.1), thus identifying the taxonomic position of the strain as *E. faecalis*.

E. faecalis is detected in 24 to 77% of cases of persistent endodontic infection accompanied by in-

flammatory changes in periapical tissues [7, 8]. It is isolated 9 times more frequently from root canals requiring revision of endodontic treatment than from primary endodontic infections [9]. *E. faecalis* is a pathogen with many virulence factors and complex virulence mechanisms [10]. This hinders its elimination from the root canal and adversely affects treatment outcomes. The results of the experiment confirm that photodynamic therapy is efficient in eliminating *E. faecalis* bacterial biofilm. One PDT cycle eliminated 75% of bacterial colonies. After the second application of the photosensitizer and subsequent irradiation cycle, the number of colonies was reduced by another 11%. The *in vitro* experimental conditions did not allow for complete eradication of the infectious factor.

In an aerobic environment, rich in nutrients, *E. faecalis* penetrated the dentinal tubules to a mean depth of 1483.33 μm [11]. Under anaerobic conditions, the penetration depth decreased to 1166.66 μm , and the smallest depth of tubule colonization of 620 μm was achieved in an anaerobic environment additionally deprived of nutrients [11]. NaOCl dentinal tubule penetration ability is significantly lower. In a study by Berutti et al. [3], the depth was found to be 130 μm after the removal of the smear layer from the surface of the dentine. Therefore, the absence of bacterial cells in the collected material after the experiment and irrigation with NaOCl does not indicate complete elimination of the infectious factor throughout dentinal tubules. Photodynamic therapy is a new treatment protocol in dentistry and the number of corresponding studies is limited. Bumb et al. [12] evaluated the depth of dentinal tubules at which PDT is effective in eliminating *E. faecalis*. Scanning electron microscope images confirmed the absence of infection at a depth of 890–900 μm . These values indicate that photodynamic therapy shows significantly higher efficacy in eliminating *E. faecalis* from the depth of dentinal tubules than conventionally used NaOCl.

Irrigation with sodium hypochlorite results in the removal of dead and damaged bacterial cells from the root canal lumen. In the course of PDT, pathogens are damaged, however, in this disinfection method they remain in the canal. This ad-

versely affects the therapy's efficacy and hinders the access of the photosensitizer, oxygen and light to other target cells. This mechanism partially explains the results obtained in the described experiment. The canal was irrigated with NaCl between the two cycles of photodisinfection. The removal of damaged bacterial cells and inactive particles of the photosensitizer resulted in improvement of the second PDT cycle effect.

Photodynamic therapy is based on mutual correlation between three elements: light, photosensitizer and oxygen. Thus, the disinfection protocol efficacy depends on application parameters used for each of the factors. The light wavelength should be compatible with the photosensitizer used. Other parameters such as radiation density and power also affect the reaction mechanism. Studies on PDT in tumor treatment have confirmed that power exceeding 100 mW/cm^2 reduces the potential to destroy pathologically changed cells [13]. This leads to excess consumption of oxygen in tissues, and therefore to the depletion of the substrate for the production of reactive oxygen species. Inappropriate selection of light source parameters may additionally lead to gradual photobleaching of the photosensitizer. There is a need for studies on the effect of exposure time, density and power of radiation on the activity of the photosensitizer. The observation of their mutual correlations is crucial for developing an optimal PDT protocol in endodontic treatment.

NaOCl irrigation and PDT show high efficacy in eliminating *E. faecalis* biofilm. Both methods are based on different mechanisms of action that do not show adverse interactions. NaOCl showed a greater bactericidal efficacy in the experiment; whereas photoactive disinfection has a high potential for penetration into dentinal tubules. Under *in vivo* conditions, it does not show toxicity against periapical tissues, effectively eliminates micro-organisms organized in the biofilm structure and is not associated with the risk of resistance development, contrary to antibiotic therapy. Taking into consideration the results of the experiment and other published results, it can be concluded that photodynamic therapy can be used to complement conventional endodontic treatment.

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Address for correspondence:

Ewa J. Prażmo
Department of Conservative Dentistry
Medical University of Warsaw
Miodowa 18
00-246 Warsaw
Poland
E-mail: ewagg@interia.eu

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