

The Disinfecting Efficacy of Root Canals with Laser Photodynamic Therapy

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Abstract:

Introduction: Infecting microorganisms of the root canals are difficult to eliminate during endodontic treatment. In this study the effect of root canal disinfection with photodynamic therapy (PDT) at different time intervals in comparison to 2.5% sodium hypochlorite (NaOCl) irrigation and passive ultrasonic irrigation (PUI) in extracted teeth colonized with *Enterococcus faecalis* and *Candida albicans* was tested to assess which treatment reaches the best disinfection rate.

Methods: One hundred and fifty-six extracted single-rooted teeth were collected, sterilized, and incubated with *Enterococcus faecalis* (ATCC 29212) and *Candida albicans* (ATCC 60193). The two groups were further divided into 6 groups depending on the treatment mode; HELBO[®]Endo Blue photosensitizer dye application followed by HELBO laser irradiation, with the output power 100 mW and emission of 660 nm, for a 1, 3 and 5 minutes, irrigation with 2.5% NaOCl, 10 second PUI with 2.5% NaOCl and control group. Flow cytometry and scanning electron microscopic (SEM) analysis were used to determine the effectiveness of the different disinfecting methods.

Results: The different disinfecting methods had a significantly different effect on the percent of dead cells ($p < 0.001$). A statistical significance of dead cells between organisms ($p < 0.001$) was observed. Interaction between the disinfecting method and both of organisms had shown the statistical significance ($p = 0.045$). Percent of dead cells in treatment groups were significantly higher compared to control group for both organisms ($p < 0.001$).

Conclusions: PUI still remains the most effective method for disinfection of infected root canals in endodontics compared to hand instrumentation for both microorganisms. SEM analysis only confirmed the results. Other results *ex vivo* suggested that prolonging the time from 1 to 5 minutes of PDT increased the number of killed microorganisms significantly, therefore longer times of photodynamic therapy were recommended. Irrigation with 2.5% NaOCl showed similar results to 5 min irradiation.

Keywords: enterococcus faecalis; Candida albicans; infection; root canal

Please cite this article as follows:

Xhevdet A, Stubljär D, Kriznar I, Jukic T, Skvarc M, Veranic P, Ihan A. The Disinfecting Efficacy of Root Canals with Laser Photodynamic Therapy J Lasers Med Sci 2014;5(1):19-26

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Introduction

The goal of endodontic treatment is to eliminate pathogenic bacteria which are the major problems in

therapy treatment of infected root canals and periapical healing^{1,2}. Because of the complex structure of root canal system, the complete elimination of microorganisms still presents a major challenge and enables resistance to

irrigation and mechanical cleaning of root canals. Bacteria also produce biofilm that represents safe habitat against antibiotics³. Existing treatment procedures include mechanical treatment with rotary files accompanied by chemical cleaning and irrigation with irrigants such as sodium hypochlorite (NaOCl) or chlorhexidine (CHX) following application of medications and sealing of the root canal⁴.

Although during endodontic treatment, irrigation with NaOCl removes the majority of infecting microorganisms, it is still possible to regain infection of the root canal because of a smear layer that reduces effectiveness of disinfecting agents². On the other hand, according to the small diameter of root canal it is difficult to irrigate the whole surface. The use of chelator substances such as ethylenediaminetetraacetic acid (EDTA) that removes smear layer and passive ultrasonic irrigation (PUI) with NaOCl have been suggested as enhanced method of irrigation⁵.

Photodynamic therapy (PDT) has been firstly introduced for elimination of cancer cells and treatment of cancer² and lately as an innovative alternative method for disinfecting root canals⁶. It is based on activation of photosensitizing molecule attached to bacterial or fungal membrane with light of an appropriate wavelength⁷. Photoactivation of a photosensitizer in the presence of oxygen produces highly reactive oxygen species (singlet oxygen, superoxide, hydroxyl radicals) that are able to destroy microorganisms^{7,8,9}.

Enterococcus faecalis is one of the most frequent microorganisms that cause post-treatment diseases^{5,10,11}. In the same way some cases have shown that occasionally *Candida albicans* has been associated with endodontic failures¹².

The objective of this ex vivo study was to evaluate the effectiveness of different disinfecting methods namely PDT, irrigation with NaOCl and PUI with NaOCl in extracted teeth infected with *Enterococcus faecalis* and *Candida albicans*. PDT and PUI can be used to enhance the disinfection compared to standard irrigating methods with NaOCl.

Methods

One hundred and fifty-six freshly extracted single-rooted teeth were collected with the consent of the adult patients and stored in saline solution. Approval was obtained by the Ethics Committee of the University Dental Centre Prishtina, Kosovo (process number 205/2013). The crown of each tooth was removed using

a water cooled diamond blade in a low speed saw Isomet 1000 (Buehler GmbH, Germany) obtaining 15 mm long root specimens. Optical microscope analysis of the cross section of the roots was performed and only teeth with round canals were included in the study. A #10 Kerr file (Maillefer Instruments SA, Switzerland) was used to determine the working length, which was 0.5 mm shorter than the length of the specimen. The canals were then enlarged to an apical size of #35 (F3) using Protaper files (Maillefer Instruments, Switzerland)¹³. Between each file copious, irrigation with 2.5% NaOCl was performed. After root canal instrumentation, teeth were rinsed with 17% EDTA and then sterilized with absolute alcohol. The canals were dried with paper points (Dentsply Maillefer). Confirmation of sterilization was carried out by incubation of the rinsed liquid from the root canals on blood agar for 24 h at 37 °C, after which no bacterial growth was observed.

After sterilization 156 specimens were divided into two test groups (n=78). One group was inoculated with *Enterococcus faecalis* strain ATCC 29212 and one with *Candida albicans* strain ATCC 10231. The root canals were filled with 30 µl of microbial suspension in thioglycolate broth (TIO) in concentration of 5 McFarland (1.5×10^9 CFU) with an automatic pipette. The specimens were incubated at 37 °C for one week so that microorganisms could penetrate the dentinal tubules¹⁴⁻¹⁶. Every second day 30 µl of fresh bacterial or fungal suspension in TIO broth was added in concentration of 5 McFarland.

The two groups were then further divided into 6 groups depending on the treatment mode (n=13); HELBO laser irradiation followed dye application for a 1, 3 and 5 minutes, irrigation with 2.5% NaOCl, 10 second PUI with 2.5% NaOCl and positive controls (Figure 1).

The Erbium-Doped Yttrium Aluminum Garnet (Er:YAG) laser light is highly absorbed, what represents a more efficient mode. The laser seems to have better potential with providing the irradiation for the root canal system. Although in the past, the forward, direct emission of most fiber optic tips has been a major problem in delivery of laser irradiation, fiber modifications have been induced that result in a cone-shaped tip with lateral emission and provide a much better exposure of the root canal walls¹⁷. In this investigation, optical system with optical fiber for direct irradiation was used. Smear layer was flushed out before the microorganisms had been inoculated into root canal due to easier penetration of bacteria and photosensitizing dye or irrigating agent⁶. The root apex was pierced through for easier flushing.

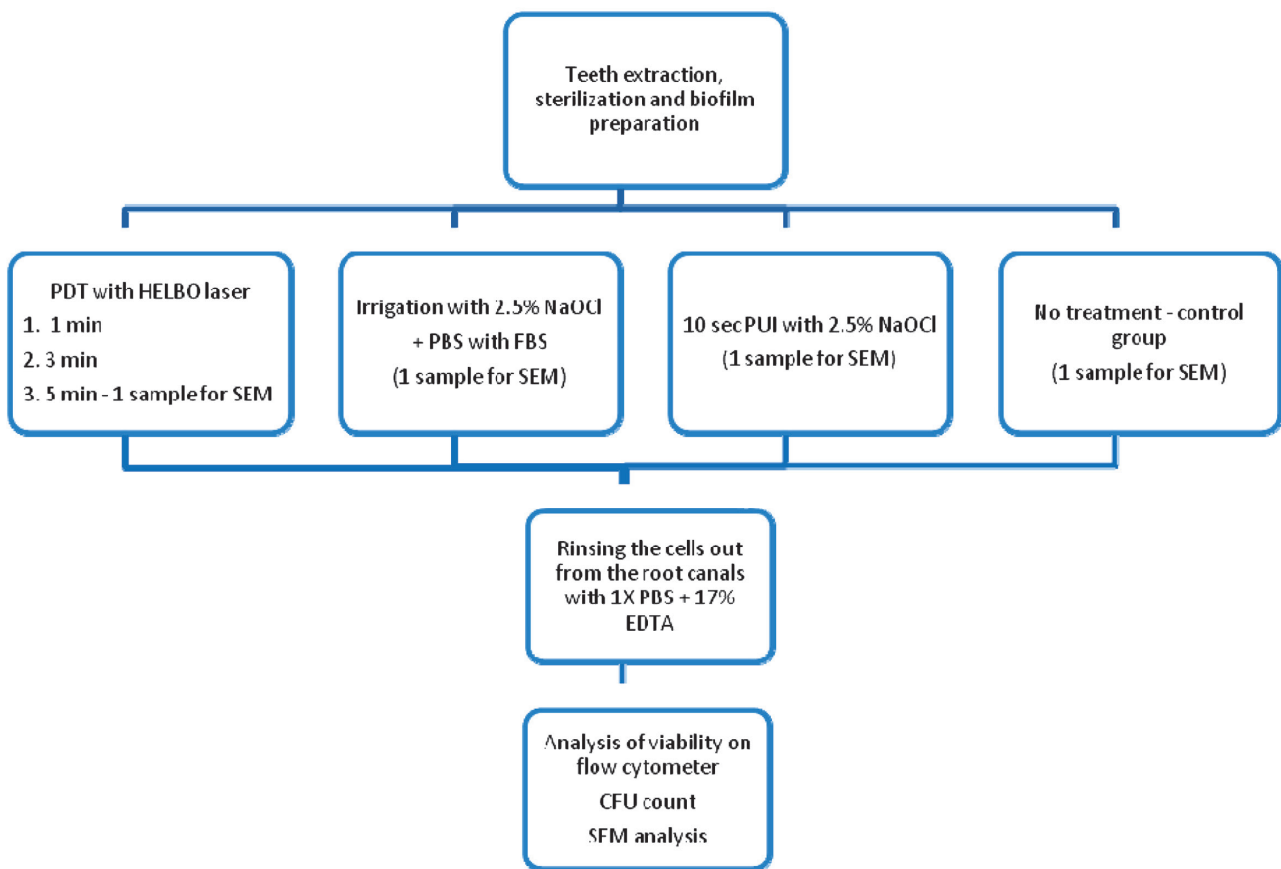


Figure 1. Work flow of experimental design and group distribution.

Group 1-3

In the first three groups, disinfection using HELBO Minilaser 2075 F dent (HELBO Bredent, Germany) diode laser with power output of 100 mW/cm² and 660 nm of wavelength was tested. Firstly, the canals were filled with a photosensitizer dye phenothiazine chloride at concentration 10 mg/ml and absorbance peak at 670 nm (HELBO®Endo, HELBO Photodynamic Systems GmbH, Austria) that was kept in the root canal for 1 min so it could attach to the target organisms ^{14, 15}. Afterwards the application of laser for 1, 3 and 5 minutes was performed following the instructions of manufacturer. The cells were rinsed out of the canal using 2.4 ml of 1X phosphate buffer solution (PBS) with addition of 1 mM 17% EDTA, pH=8.3. To determine the viability, flow cytometry was applied. Flow cytometry provides quick quantitative analysis of cells in suspension. For the determination of cell viability with flow cytometry a simple method which involves the use of a pair of fluorescent dyes; thiazole orange (TO) and propidium iodide (PI) was used. Both dyes bind to nuclear DNA. The

cell membrane is permeable for the TO and so it enters the living and the dead cells. Membrane of living cells is impermeable for PI dye, which enters only in cells with damaged membranes (necrotic cells). The combination of these dyes for flow cytometry allows for distinguishing between living and dead cells and thus determines their viability. The Cell Viability Kit with Liquid Counting Beads (BD Biosciences, USA) was used. The procedure was performed according to the manufacturer instructions. The combination of these dyes is a quick and ready method for discrimination of live and dead cells. The advantage of the flow cytometer to growing bacteria on agar plates is the speed of results. Results are available in less than an hour compared to 24 hour growing on plates. Flow cytometry is to be preferred because of easier handling, more accurate counting, smaller turnaround time and better specificity and sensitivity ¹⁸.

Group 4

In group four, infected root canals were irrigated for a 5 seconds with 1.2 ml of 2.5% NaOCl. Before analyzing the

cell viability on flow cytometer, the toxic effectiveness of NaOCl on damaged cells was neutralized using 1.2 ml of irrigant 10X PBS with added fetal bovine serum (FBS).

Group 5

In group five, PUI using 500 µl of 2.5% NaOCl was performed for 10 seconds after which the cells were rinsed out of the root canal using 2.4 ml 1X PBS with 1 mM 17% EDTA.

Group 6

The sixth group consisted of positive controls. After one week incubation period the canals were rinsed with the mentioned irrigant.

Before flow cytometry four representative samples from each treatment group were randomly chosen. Root canals were rinsed out with 2.4 ml 1X PBS and 17% EDTA, after which 100 µl of the rinsed liquid was inoculated on blood agar plates. After 24 h incubation at 37 °C counting of colony forming units (CFU/ml) was performed.

One representative specimen from each *E. faecalis* treatments groups was chosen for scanning electron microscopic (SEM) analysis.

A two-way ANOVA with post-hoc Tukey analysis were performed with the percentage of dead microorganisms as the dependant variable and type of microorganism and treatment mode as factors. The level of significance was set at $p < 0.05$. Statistical analysis was performed using SPSS 20 (IBM, USA).

Results

Average percent of dead cells measured with flow cytometry analysis are presented in Table 1. Two factor

ANOVA showed statistically significant differences in mean percentage of dead cells between tested organisms ($p < 0.001$), between treatment methods ($p < 0.001$), as well as in interaction between tested organisms and treatment methods ($p = 0.045$). The percent of dead cells was significantly higher for *C. albicans* compared to *E. faecalis* in all laser treatment groups, but not in the irrigation and PUI group (Table 1). However, when comparing disinfecting methods, PUI was significantly more effective ($p < 0.001$) compared to other groups, followed by 5-minute, 3-minute laser irradiation and NaOCl irrigation, while the lowest percent of dead cells was detected in the 1-minute laser irradiation group.

SEM analysis revealed the differences between the surface of root dentin and morphology of the microbial cells after treatments. The experiment was carried out with *E. faecalis* only. Examination of the root canals after incubation with *E. faecalis* (control group) showed the formation of a thick biofilm on the surface of root dentin with bacteria penetrating the dentin tubules (Figure 2A). After irrigation with 2.5 % NaOCl a high number of burst bacterial cells could be seen (Figure 2B). 5-minute laser irradiation resulted in the recession and dispersion of bacterial cells (Figure 2C), while PUI caused a significant elimination of bacteria from the dentin walls with no remaining live bacterial cells (Figure 2D).

Discussion

This study was conducted to estimate efficacy of PDT compared to different antibacterial methods in therapy of infected root canals. Chemo-mechanical irrigation or only instrumentation techniques for cleaning the infected root canals does not ensure complete removal of microorganisms due to anatomy of root dentin, where microorganisms can form complex biofilm or

Table 1. Mean percentage of dead microbial cells with standard deviation after treatment application with p-values for both organisms. Different index letter denote different statistical difference at the post hoc test (Tukey test).

| Variable | Enterococcus faecalis | | Candida albicans | | p-value |
|--------------------------------|-----------------------|--------|------------------|--------|---------|
| | % dead cells | CFU/ml | % dead cells | CFU/ml | |
| PUI ^{b, c, d, e, f} | 85.05 ± 5.48 | 5 | 92.82 ± 2.40 | 0 | 0.150 |
| 2.5% NaOCl ^a | 70.77 ± 21.45 | 64 | 79.78 ± 10.20 | 2 | 0.087 |
| Laser 5 min ^{a, c} | 71.59 ± 8.56 | 34 | 87.55 ± 7.22 | 2 | 0.005 |
| Laser 3 min ^{a, c} | 69.45 ± 11.63 | >300 | 84.65 ± 7.45 | >300 | 0.007 |
| Laser 1 min ^{a, c, d} | 54.35 ± 14.87 | >300 | 82.36 ± 12.40 | >300 | <0.001 |
| Positive control | 41.94 ± 8.48 | >300 | 45.99 ± 1.94 | >300 | 0.482 |

^a PUI was statistically different compared to all groups.

^b NaOCl irrigation showed statistical difference only compared to PUI and positive control.

^c 5-minute laser irradiation had no difference in disinfection effectiveness compared to 3-minute laser irradiation or NaOCl irrigation.

^d 3-minute laser irradiation was equally effective as 5-minute irradiation and irrigation with NaOCl.

^e 1-minute laser irradiation was at least effective and had statistical difference to all treatment groups except to NaOCl group.

^f positive control

can penetrate into dentin tubules. Repeated infections are possible ^{6, 13}. It is assumed that currently used hand instrumentation methods and substances in endodontics could not be able to destroy the remaining bacteria after primary therapy or could only damage cells on the surface of formed biofilm ^{6, 19}. The results of our study proved those indications. All therapy methods used showed a statistically significant increase in percent of dead microbial cells compared to control group ($p < 0.001$). There were also significant differences between all tested methods, microorganisms and interaction between the two variables.

Our findings were also confirmed with the microbiological control on blood agar plates. After each treatment there were a decreased number of mean values of CFU/ml on blood agar plates compared to control group, where more than 300 CFU/ml were detected. For

instance 34 CFU/ml after laser irradiation in the case of *E. faecalis* showed that laser had a significantly higher effect on the mortality of microorganisms compared to irrigation alone, where 64 CFU/ml were counted (Table 1).

SEM analysis of the canal walls proved the biofilm formation and showed estimated visual amount and distribution of bacterial cells on the surface of dentin before and after application of each treatment ⁵. Only 5-minute PDT application of root canal infected with *Enterococcus* was scanned. Canal walls were noticed to be different compared to control group, where whole microbial cells were observed. Altered morphology, decreased number and damaged cells with large spaces between them after treatments were indicated (Figure 2). Thus again, the PUI with almost complete removal of microbes indicated to be the most efficient treatment. Despite this, bacterial cells were also assessed in the

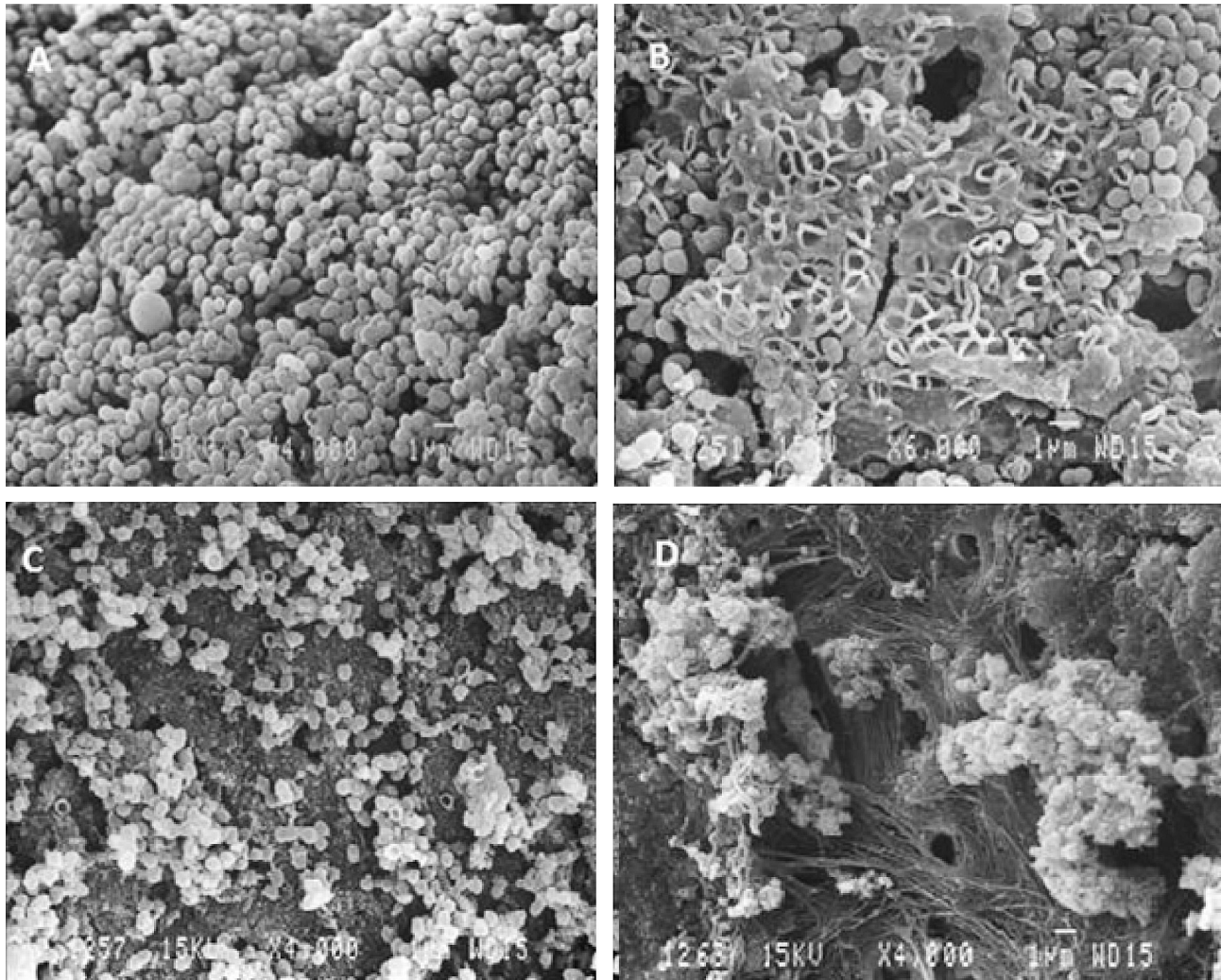


Figure 2. SEM analysis of *E. faecalis* colonization on root canal walls. A – *E. faecalis* control group displaying biofilm, B – burst cells of *E. faecalis* after irrigation with 2.5% NaOCl, C – recessed and dispersed *E. faecalis* after 5-minute laser irradiation, D – displaying canal wall after PUI.

dentinal tubules after application of each therapy method because of the anatomical structure of dentin ⁶.

E. faecalis as facultative anaerobe is able to survive long period without nutrients ⁶. Thus, it invades dentinal tubules, which provides protection against irrigating agents and it makes it difficult to eliminate ^{6, 8, 19}. In order to eliminate those microorganisms PDT with low-power lasers as antibacterial therapy was introduced ^{6, 20}. Toluidine blue as one of the components of PDT is much more effective at producing reactive oxygen species (ROS) than NaOCl, respectively ²¹. The ROS are free oxygen radicals that disrupt a wide range of bacterial membrane and cause rapid death of the microorganisms ²². The results of Bouillaguet et al. suggest that common red and blue dental-light sources are useful for activating photosensitive disinfecting dyes against dental infections ²¹. Each of the PDT components is not toxic itself. It represents safe treatment that does not result in any alterations or excessive damage of neighbour tissue when the light is not too strong ^{23, 24}.

Low-power lasers are the most frequently used light sources because they are in a wavelength specific for activation of the photosensitizer in PDT. In our investigation, low-power small HELBO Minilaser 2075 F dent (HELBO Bredent, Germany) diode laser with direct irradiation was used. It produces red light at 660 nm with the output power of 100mW. That amount of laser energy initiates specific light-induced chemical reactions in the target tissue, causing cell dissolution or destruction. Energy density is one of the most important parameters in laser surgery ²⁵. During irradiation, direct irradiation with the optic fiber that was placed within the root canal at its total length, permitted an emitting light at the tip and from the lateral sides, thus leading to even light distribution for disinfection effect both vertically and horizontally.

Results of different PDT studies have shown variable disinfecting efficacy regarding to different laser and dyes used and to success of the therapy ^{2, 6, 13, 21, 22, 26}. Despite this, the therapy has never achieved 100% reduction in microbial cells, so that repeated infections were still possible ^{6, 24}. To treat the post-treatment infections the use of PUI has been introduced as an alternative to increase disinfection of the root canals ^{5, 27, 28}. In this study, significant differences were observed between group that used PUI and all test groups for *E. faecalis* but not for *C. albicans*. Only PUI and irrigation for *Candida* groups showed some statistical significance ($p=0.014$). It can be explained by the different type of biofilm which *Candida* makes. Cells are grown together and adhere to the

surface, while hyphal cells form external layer of biofilm ^{29, 30}. *Candida* was equally susceptible to all treatment methods. Nevertheless *Enterococcus* forms slightly different, more complex biofilm, where cells are grown together and with no hyphen ^{31, 32}. Prolonged 5-minute time of PDT was compared to 1-minute ($p=0.334$) and 3-minute ($p=0.589$) showed no statistical differences for *Candida*, because in this case even 1-minute exposure was powerful enough to kill over 82% of *Candida* cells. Analysis with *Enterococcus* showed statistically relevant differences between 5-minute and 1-minute ($p=0.003$), but not with 3-minute exposure ($p=0.709$). According to these results of statistical difference in PDT group, it was recommended to prolong irradiation time for better disinfection. 1-minute irradiation had no mentionable effect on the cells mortality compared to control group. Reason for incomplete elimination of bacteria could be the short irradiation time and consequently low concentration of arisen ROS in the canals, in dentinal tubules, where the photosensitizer agent might not diffuse well into deep dentinal tubules. In the same time the ability of *E. faecalis* to form a bacterial biofilm was a major factor that contributes to the bacterial persistence after endodontic treatment ²¹.

PUI group with 85.05% of dead *Enterococcus* and 92.82% of dead *Candida* and p -value of 0.150 showed no statistical difference between organisms. That indicated a good cleaning effect and it was proved as the best disinfecting agent of mixed-infected root canals. The superiority of this method could be explained with inaccessible areas of dentinal tubules in root canals for endodontic instruments or the action of the irrigating solutions ⁵. Moreover PDT and PUI were more efficient than 2.5% NaOCl to reduce bacteria. To increase efficiency a higher concentration of NaOCl or longer exposure time should be applied ^{5, 13, 32}. Meire et al. demonstrated that the treatment with 2.5% NaOCl solution for at least 5 min resulted in complete elimination of the biofilm. When they were using a 0.5% solution, 30 min were required ³¹. Meanwhile, Yao et al. showed that despite the proteolytic effect of irrigation by 5.25% NaOCl, there were still bacteria in the deep tubules and canal irregularities because of the complexities of root canal system, invasion of microorganisms into dentinal tubules and the formation of bacterial biofilms on the surface of the root canal walls ²².

Nevertheless, NaOCl is highly toxic to vital tissues so higher concentrations of NaOCl in vivo could cause unpleasant and irritating connective tissue reactions such as inflammatory response ²⁴. The safe concentration

of NaOCl should not exceed 0.025% which has no significant antimicrobial effect for endodontic treatment. At the moment, there is no optimal concentration that is safe and effective for NaOCl use in endodontics. A low and a high concentration of NaOCl can all provide tissue dissolution and antimicrobial effects. However, both can cause NaOCl accidents. Therefore, great care or an alternative irrigation solution should be considered. Unlike NaOCl, the components of PDT are nontoxic to vital tissues²¹. However, the time and concentration of NaOCl were not variables in our study so the results were not consistent with the findings of other studies^{5, 13, 17, 34}, who found no difference between conventional NaOCl irrigation and PUI. Laser treatment has not yet replaced the conventional NaOCl irrigation³⁵. For further research, it would be possible to test PDT as an adjuvant to the conventional irrigating treatment, what may result in better reduction of pathogens in a shorter period of time. Meire et al. investigated the effect of laser disinfection after a NaOCl treatment. Their results showed that laser further reduced the number or remaining cells³¹. Till now, PDT was proved to be an efficient adjunct or an alternative antimicrobial therapy to NaOCl in oral cavity in vitro^{2, 4}.

Conclusion

The study results indicate that PDT is suitable as a disinfecting agent in a tooth model contaminated with *E. faecalis* or *C. albicans*, because they showed significant reduction in cell viability. However it did not totally eradicate the contaminating microorganism in root canals ex vivo. The outcome results indicate promising usage in in vivo conditions despite the fact that PUI showed even better loss in microbial load. Despite comparing our results to other studies, further investigations of PDT should be carried out for clinical use.

Acknowledgments

The authors deny any conflicts of interest.

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