Dental Journal
Majalah Kedokteran Gigi

Dental Journal
(Majalah Kedokteran Gigi)
2020 June; 53(2): 71–75

Research Report

Effectiveness of light-emitting diode exposure on photodynamic therapy against *Enterococcus faecalis*: in vitro study

Nanik Zubaidah, Agus Subiwahjudi, Dinda Dewi Artini and **Karina Erda Saninggar**
Department of Conservative Dentistry,
Faculty of Dental Medicine, Universitas Airlangga
Surabaya – Indonesia

ABSTRACT

Background: *A successful root canal treatment eliminates pathogenic bacteria from infected root canals. The most common bacteria in root canal infections is Enterococcus faecalis (E. faecalis), due to its resistance to medicament and root canal irrigation. A photodynamic therapy (PDT) is a method of root canal disinfection that uses a combination of photosensitisers and light activation to eliminate bacteria in the root canal. The duration of the PDT irradiation results in the production of singlet oxygen and reactive oxygen species (ROS) to eliminate the E. faecalis bacteria.* ***Purpose:*** *To analyse the differences in the duration exposure of photodynamic therapy against the E. faecalis bacteria.* ***Methods:*** *The E. faecalis bacteria culture was divided into seven eppendorf tubes. Group I was a control group, and group II, III, IV, V, VI and VII were treated using PDT consisting of Toluidine Blue O (TBO) photosensitiser and light source irradiation for ten, 20, 30, 40, 50 and 60 seconds, respectively. After incubation, the number of bacteria was calculated by the Quebec Colony Counter and analysed using the Kruskal–Wallis test and the Mann–Whitney test ($p < 0.05$).* ***Results:*** *There was a significant difference between the number of E. faecalis bacteria colonies in each treatment group ($p < 0.05$). Group VI and VII, which had a longer exposure to PDT, showed a smaller amount of E. faecalis bacteria.* ***Conclusion:*** *The longer exposure of PDT results in a smaller amount of E. faecalis bacteria. The light irradiation of 50 seconds is the most effective to eliminate E. faecalis bacteria.*

Keywords: *Enterococcus faecalis; irradiation time; light-emitting diode; photodynamic therapy; root canal treatment*

Correspondence: Nanik Zubaidah, Department of Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Jl. Mayjen Prof. Dr. Moestopo 47, Surabaya – Indonesia. Email: nanik-z@fkg.unair.ac.id

INTRODUCTION

Endodontic infections occur due to the invasion of bacteria in the root canal. *Enterococcus faecalis* (*E. faecalis*) is the most common pathogenic bacteria that is found in the root canal (4–40 per cent) and causes a 20–70 per cent failure of endodontic treatment.[1,2] In several research studies, *E. faecalis* was found in the treated root canals, and this bacteria is reported to be resistant to some medicaments and antimicrobial irrigation during the root canal treatment.[3–5] In dentinal tubules, *E. faecalis* can survive the intracanal medicament of calcium hydroxide ($Ca(OH)_2$) for more than ten days.[6]

The elimination of the pathogenic bacteria that is present in the root canal affects the success of root canal treatment. The complex structure and shape of the root canal is a major problem when cleansing the root canal to eliminate the pathogenic bacteria. The bacteria that is left in the root canal can penetrate the root dentinal tubules to a depth of 1000 µm, while irrigation disinfection materials can only reach a depth of 100 µm.[4,5,7]

Over the last few decades, photodynamic therapy (PDT) was developed. PDT is a disinfection method that uses a light source (light-activated disinfection) of a specific wavelength, which consists of two components: a light source in the form of a light-emitting diode (LED) or laser diode as photoactivation, and a photosensitising agent (photosensitiser), which causes photoinactivation against the bacteria. There is an energy transfer from the photosensitiser, which is activated by a light source, to the available oxygen. This results in the formation of a singlet oxygen reactive, which has a cytotoxic effect

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 32a/E/KPT/2017.

DOI: 10.20473/j.djmkg.v53.i2.p71–75

against bacteria and can damage the structure of bacterial cells. The use of PDT after the root canal preparation and mechanical irrigation could effectively eliminate the pathogenic bacteria in the root canal. The light source of PDT can reach the root canal area, which is difficult to reach using conventional irrigation because the light can reach a depth of 0.5–1.5 centimetres of root dentinal tubules. In addition, the light source is reported to be non-toxic and has a high degree of selectivity to eliminate the bacteria through the reaction of photosensitisers and oxygen without damaging the host cell. In vivo studies also reported that PDT could effectively eliminate the bacteria that is resistant to several types of medicaments. FotoSan, which is a PDT method, has been reported to eliminate gram-positive and negative bacteria, such as *Streptococcus mutans* and *E. faecalis*.[8,9]

FotoSan is a photodynamic therapy that utilises a red LED with a 630-nanometre wavelength and a Toluidine Blue O (TBO) photosensitiser agent. When using FotoSan in endodontic treatment, the photosensitiser agents are inserted into the root canal for 60 seconds so that the liquid comes into contact with the root canal wall. The endodontic tip from the device is then inserted into the root canal and irradiated for 30 seconds. This is consistent with Schlafer's in vitro and ex vivo research study, which shows that the use of FotoSan for 30 seconds reduces the number of pathogenic microorganisms that cause endodontic infections (*Escherichia coli, E. faecalis, Fusobacterium nucleatum, Streptococcus intermedius*), compared to ten seconds and 20 seconds. However, Poggio's study found a decrease in the number of E. *faecalis, S. mutans* and *Streptococcus sanguinis* bacteria with a longer exposure time of 90 seconds. Xhevdet *et al*.[4] also reported that the irradiation time of five minutes against *E. faecalis* bacteria has a greater effect than irradiation of one minute and three minutes. However, there is no significant difference between the time exposure and the reduced number of bacteria.[4,10,11]

The effectiveness of PDT depends on the strength, duration, absorption of light in the tissue, geometry of the root canal and the distance of the tip to the target cell. The light absorption phenomenon by the photosensitiser is a photophysical process when a photosensitiser molecule that has an electron configuration in stable state (ground state) absorbs photon light. After absorbing the light, the molecular electron configuration changes to an unstable (excited state). From the excited state, the electron photosensitiser molecule can either return to a ground state if it loses energy or become a triplet state if it continues to get enough energy. This triplet state is a reactive state. A chemical interaction occurs between the electron molecules and oxygen, which have a stable state electron configuration. This results in the oxygen molecule becoming excited (unstable). The excited oxygen tends to flow towards the stable electron conditions, which means that it will interact with the surrounding biological systems. The interactions that occur between the excited oxygen and biological systems, such as the bacterial cells, will damage the cell's system and structure. The major concept of irradiation time is to produce reactive oxygen to reduce the number of bacteria.[12–14] The aim of this study was to analyse the differences in the duration exposure of PDT using red LED lights and TBO photosensitisers against the number of *E. faecalis* bacteria.

MATERIALS AND METHODS

This study was approved by the ethics committee of the Faculty of Dentistry at Airlangga University with the reference number 160/HRECC.FODM/VIII/2017. This research was a laboratory experimental study with a post-test only control group design. The sample that was used in this study was *E. faecalis* ATCC 29212 bacteria. The determination of the number of samples using Lemeshow et al.'s (1990) formula obtained 42 total samples. The samples were divided into seven groups; group I (I-C) was a control group without light exposure; group II (II-10) had PDT irradiation for ten seconds; group III (III-20) had 20 seconds; group IV (IV-30) had 30 seconds; group V (V-40) had 40 seconds; group VI (VI-50) had 50 seconds; and group VII (VII-60) had 60 seconds.

The preparation of the *E. faecalis* bacterial culture was carried out by taking the *E. faecalis* bacterial culture preparations with the osse wire and placing it in a test tube, which contained Brain Heart Infusion (BHI) broth I. It was then stirred and incubated at 37 degrees Celsius (^{o}C) for 48 hours in an anaerobic atmosphere.[15] A 0.5 millilitre culture from the BHI broth I tube then was taken with a micropipette and inserted into a test tube that contained BHI broth II and equalised with the Mc Farland scale to obtain a 1.5 x 108 CFU/ml bacterial suspension.

The final samples were obtained from 0.5 ml bacterial suspension test tubes, which were taken with a micropipette to be put into 42 eppendorf tubes each. The eppendorf tube was coated with black tape[15] to ensure that during the irradiation, the PDT light was not transmitted outside the tube wall. These 42 samples in the eppendorf tubes were divided into seven groups, with each group consisting of six eppendorf tubes.

Group I was the control group without light exposure and photosensitisers and only contained the *E. faecalis* bacteria sample. Group II was added with photosensitisers in the form of TBO liquid 0.5 ml, and after 60 seconds, it was irradiated with the LED light for ten seconds. Groups III to group VII also were treated like group II with the irradiation time of the LED light 20 seconds, 30 seconds, 40 seconds, 50 seconds and 60 seconds, respectively.[10,11]

After the irradiation was carried out to all groups, a 0.1 millilitre sample was taken with a micropipette from each eppendorf tube (groups I–VII), cultured in a petri dish containing agar nutrient and incubated for 48 hours at 37^{o}C in an anaerobic atmosphere. The number of bacteria

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 32a/E/KPT/2017.

DOI: 10.20473/j.djmkg.v53.i2.p71–75

colonies in the petri dish was calculated using the *iuebec colony counter* with *colony-forming unit (CFU)* method and used for the data analysis.[9]

RESULTS

A statistical calculation was conducted to get the average results and standard deviation of the number of *E. faecalis* bacteria colonies after irradiation, as shown in Table 1. From the average results, a normality test was performed using the *Shapiro–Wilk* test and a significance value or p value > 0.05 was obtained. This shows that all the groups have a normal data distribution.

Subsequently, a homogeneity test was conducted on the data using the *Levene* test and obtained a significance value of homogeneity 0.007 (p <0.05) with *Levene* Statistics of 3.640. This shows that all the groups' data did not have a homogeneous variance. After the normality and homogeneity tests were conducted, a *Kruskal–Wallis Test* then was applied to assess the differences of the whole groups. It was obtained a significance value of 0.000 (p <0.05) for chi-square 40.038. This shows that there is a significant difference between the number of *E. Faecalis* colonies in all treatment groups.

The *Mann–Whitney* test has a requirement of p <0.05 to show that there are significant differences in each group. There are significant differences between group I (control) and other treatment groups (groups II, III, IV, V, VI and VII). The majority of p is less than 0.05; however, there are some groups that show p > 0.05: group VI (50 seconds) compared to VII (60 seconds), which is 1.000, and group VII (60 seconds) compared to VI (50 seconds), which is 1.000. This shows that there were no significant differences between groups VI and VII. It has been suggested that both groups could eliminate all the *E. faecalis* bacteria.

DISCUSSION

From the results, it was obtained that the mean number of *E. faecalis* bacteria after irradiation is significantly different in all treatment groups (control, ten seconds, 20 seconds, 30 seconds, 40 seconds, 50 seconds and 60 seconds). It has been suggested that the PDT method can significantly eliminate *E. faecalis* bacteria. In accordance with Rios's study, which states that PDT in combination with LED light and TBO fluid has an antibacterial effect against *E. faecalis* bacteria, there is potential for it to be used as microbial disinfection for conventional endodontic treatment.[16]

For the irradiation times of ten seconds, 20 seconds, 30 seconds and 40 seconds, there was still a small amount of *E. faecalis* bacteria that was calculated by CFU (the mean was 33.67; 23.33; 16 and 12.50, respectively). This is not in accordance with Schlafer's study, which found that the use of FotoSan for 30 seconds, according to the protocol for endodontic treatment, could effectively decrease the number of *E. faecalis* bacteria by 99.7 per cent compared to ten seconds and 20 seconds. However, the difference in the results of this study is due to the different research methods that were used, as well as the use of different fibre tip sizes for irradiation on the eppendorf tubes that contained bacterial suspension. A study reported that the use of the optical fibre tip size gives better results than when the light is used directly on the cavity of a tooth or root canal because the longer and smaller fibre tip size can help to emit the light of PDT that reaches the apical end root canal, which is difficult to access.[9,10,17]

In the groups with 50 seconds and 60 seconds irradiation, we found zero *E. faecalis colonies*, which suggests that 50 seconds of irradiation is effective enough to eliminate all *E. faecalis* bacteria. This is different to Poggio's study, which stated that the number of *E. faecalis* bacteria was reduced after 90 seconds of irradiation by 91.49 per cent

Table 1. Statistical analysis data relating to the quantity of *E. faecalis* colonies after irradiation in each treatment group

Group	Mean ± SD	Normality test	Homogeneity test	Kruskal-Wallis test	Mann-Whitney test						
					I-C	II-10	III-20	IV-30	V-40	VI-50	VII-60
I-C	116.67 ± 4.67	0.896				0.004*	0.004*	0.004*	0.004*	0.002*	0.002*
II-10	33.67 ± 4.32	0.06					0.004*	0.004*	0.004*	0.002*	0.002*
III-20	23.33 ± 3.72	0.096						0.004*	0.004*	0.002*	0.002*
IV-30	16.00 ± 2.19	0.783	0.007	0.000					0.044*	0.002*	0.002*
V-40	12.50 ± 2.73	0.357								0.002*	0.002*
VI-50	.00 ± .000	-									1.000
VII-60	.00 ± .000	-									

*) There is a significant difference (p <0.05)

Note: a normality test score of p>0.05 means the data follows normal distribution; a homogeneity test score of p<0.05 means the data did not have a homogeneous variance; Kruskal-Wallis test score of p<0.05 means that significant difference exists; Mann-Whitney test score of p>0.05 in group VI (50 seconds) compared to VII (60 seconds) and group VII (60 seconds) compared to VI (50 seconds) means that there were no significant differences between groups VI and VII.

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 32a/E/KPT/2017.
 Available at http://e-journal.unair.ac.id/index.php/MKG
DOI: 10.20473/j.djmkg.v53.i2.p71–75

compared to 30 seconds by as much as 87.72 per cent. Prolonged exposure to FotoSan could significantly reduce the percentage of bacteria compared to a short exposure. However, Poggio's study only compared 30 seconds and 90 seconds irradiation time and did not investigate the effect of 50 seconds irradiation, whereas this study conducted ten to 60 seconds of irradiation at ten second intervals and found that 50 seconds is adequate time to eliminate the bacteria.[11]

This study used FotoSan as the PDT method for the root canal disinfection, as FotoSan utilises a red LED light with a wavelength of 628 nanometres and TBO photosensitisers. This is in accordance with Hopp's research, which states that a red light with a wavelength of 628 nanometres can activate TBO fluid to produce a singlet oxygen reactive that causes oxidative damage to bacterial cells. These light rays can reach up to 0.5–1.5 centimetres into the depths of the root dentinal tubules, which are difficult to reach by irrigation disinfecting materials and have a high degree of selectivity to eliminate the *E. faecalis* bacteria without damaging the host cell.[13,18]

The photosensitiser that was used in this study was TBO. TBO photosensitisers contain phenothiazine, which is a cation that will bind to the cell wall of the *E. faecalis* bacteria and is anionic. The bonding results in an electrostatic interaction, which further increases the bacterial cell wall's permeability and causes the photosensitising cation to enter the cytoplasmic membrane of the bacteria and further disorganise the barrier's permeability. From Kikuchi's research, TBO was reported to have antibacterial power because it can interact with the bacterial cell membrane lipopolysaccharides without irradiation. Irradiation with a wavelength of 630 nanometres leads to the maximum absorption of photosensitiser fluid, which results in PDT photoinactivation. This kills the bacteria more effectively than photosensitiser fluid without irradiation. This is consistent with Arneiro's findings that the use of TBO without irradiating kills a smaller number of bacteria than TBO with irradiation.[19,20]

The LED light in FotoSan is a red light with a wavelength of 628 nanometres, an output power of 1000 mW and 30 J energy. The rays will cause the light absorption phenomenon by the photosensitiser, which is called the photophysical process. The first phase in this process is the ground state. In this phase, each electron is in a stable and paired state in its orbitals. After being exposed to the irradiation, there is an energy transfer, which causes the photosensitiser electron molecule in the ground state phase to change into an excited state. The paired electrons begin to become unstable and then increase to the triplet state phase where the electrons have separated from their pairs. Therefore, they become reactive and look for pairs with other molecules. The triplet state is a reactive state, which occurs when there are chemical interactions between the electron molecules and oxygen that have electron configurations in a stable state, which results in the oxygen molecule becoming excited (unstable). The excited oxygen flows towards stable electron condition and interacts with the surrounding biological systems. The interactions that occur between the excited oxygen and biological systems, such as the bacterial cells, will damage the cell system and structure of bacteria cell.[12,14]

These interactions result in two types of mechanisms. In type I, there is an electron transfer between the photosensitiser and the substrate, which produces radical ions called ROS. These consist of superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2). These ions are oxidative to the cells. In type II, there is an electron transfer between the photosensitiser and the oxygen receptor (O_2), which will produce a singlet oxygen ($^1O_2$). This singlet oxygen is a reactive form of oxygen and a powerful oxidative agent. ROS and singlet oxygen will cause damage to lysosomes, mitochondria and bacterial plasma membranes.[14]

That damage occurs because ROS and singlet oxygen cause oxidative stress, which results in lipid peroxidation of the plasma membrane and organelles. The fatty acids that bond with the unstable free radicals can cause severe membrane damage, as well as the oxidation of amino acid chains, the formation of covalent protein bonds and protein oxidation. Consequently, this will damage the structure of the protein by increasing the proteasomal protein degradation. In addition, it can cause prolonged DNA chain crosslinking, inactivate the NaDH succinate enzymes and lactate dehydrogenase, damage the balance of K^+ ions and other ions and damage the bacterial cell DNA, which will cause the death of the bacteria.[4,14]

From the test results in each group, group II (irradiation of ten seconds) is the group that has the weakest ability to kill the *E. faecalis* bacteria. This is because the lack of irradiation time will result in a smaller concentration of radical ion formations and singlet oxygen. Group VI (irradiation of 50 seconds) and group VII (irradiation of 60 seconds) had the best ability to kill the *E. faecalis* bacteria. In group VI, no bacteria colonies were found. Therefore, it could be concluded that 50 seconds was the most effective time of irradiating the PDT to kill all the *E. faecalis* bacteria. This suggests that the importance of the irradiation time will result in the numerous concentration of photosensitiser molecules of the excited state and triplet state, so that produce reactive oxygen to kill the bacteria. The radical ions and singlet oxygen will damage the lysosomes, mitochondria and plasma membranes of the bacterial cells and kill more bacteria.[12,14] In conclusion, the longer irradiation exposure during photodynamic therapy results in a smaller number of *E. faecalis* bacteria. The irradiation time of 50 seconds is the most effective time to eliminate all the *E. faecalis* bacteria.

REFERENCES

1. Alagl AS, Bedi S, Almas K. Phytosolutions for Enterococcus faecalis in Endodontics: An Update. Oral Health Dent Manag. 2016; 15(5): 332–6.

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 32a/E/KPT/2017.

DOI: 10.20473/j.djmkg.v53.i2.p71–75

2. Pourhajibagher M, Kazemian H, Chiniforush N, Hosseini N, Pourakbari B, Azizollahi A, Rezaei F, Bahador A. Exploring different photosensitizers to optimize elimination of planktonic and biofilm forms of Enterococcus faecalis from infected root canal during antimicrobial photodynamic therapy. Photodiagnosis Photodyn Ther. 2018; 24: 206–11.
3. Filipov I, Markova K, Boyadzhieva E. Efficency of photoactivated disinfection on experimental biofilm - scaning electron microscopy results. J IMAB - Annu Proceeding (Scientific Pap). 2013; 19(4): 383–7.
4. Xhevdet A, Stubljar 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: 19–26.
5. Lins CCSA, Melo ARS, Silva CC, Oliveira JB, Lima GA, Castro CMMB, Diniz FA. Photodynamic therapy application in endodontic aerobic microorganisms and facultative anaerobic. Formatex. 2015; : 559–63.
6. de la Maza LM, Pezzlo MT, Shigei JT, Tan GL, Peterson EM. Color atlas of medical bacteriology. 2nd ed. Washington: ASM Press; 2013. p. 67–90.
7. Yildirim C, Karaarslan E, Ozsevik S, Zer Y, Sari T, Usumez A. Antimicrobial efficiency of photodynamic therapy with different irradiation durations. Eur J Dent. 2013; 7(4): 469–73.
8. López-Jiménez L, Fusté E, Martínez-Garriga B, Arnabat-Domínguez J, Vinuesa T, Viñas M. Effects of photodynamic therapy on Enterococcus faecalis biofilms. Lasers Med Sci. 2015; 30(5): 1519–26.
9. Bago I, Plečko V, Gabrić Pandurić D, Schauperl Z, Baraba A, Anić I. Antimicrobial efficacy of a high-power diode laser, photo-activated disinfection, conventional and sonic activated irrigation during root canal treatment. Int Endod J. 2013; 46(4): 339–47.
10. Schlafer S, Vaeth M, Hørsted-Bindslev P, Frandsen EVG. Endodontic photoactivated disinfection using a conventional light source: an in vitro and ex vivo study. Oral Surgery, Oral Med Oral Pathol Oral Radiol Endodontology. 2010; 109(4): 634–41.
11. Poggio C, Arciola CR, Dagna A, Florindi F, Chiesa M, Saino E, Imbriani M, Visai L. Photoactivated disinfection (PAD) in endodontics: An in vitro microbiological evaluation. Int J Artif Organs. 2011; 34(9): 889–97.
12. Lestari WP, Astuti SD, Setiawatie EM. Potensi inaktivasi Streptococcus mutans dengan penambahan fotosensitiser ekstrak daun kelor pada aplikasi fotodinamik light emitting diode (LED). Thesis. Surabaya: Universitas Airlangga; 2016.
13. Kishen A, Shrestha A. Photodynamic Therapy for Root Canal Disinfection. In: Endodontic Irrigation. Springer International Publishing; 2015. p. 237–51.
14. Dai T, Fuchs BB, Coleman JJ, Prates RA, Astrakas C, St. Denis TG, Ribeiro MS, Mylonakis E, Hamblin MR, Tegos GP. Concepts and principles of photodynamic therapy as an alternative antifungal discovery platform. Front Microbiol. 2012; 3: 1–16.
15. Alison MR, Poulsom R, Forbes S, Wright NA. An introduction to stem cells. J Pathol. 2002; 197(4): 419–23.
16. Rios A, He J, Glickman GN, Spears R, Schneiderman ED, Honeyman AL. Evaluation of photodynamic therapy using a light-emitting diode lamp against enterococcus faecalis in extracted human teeth. J Endod. 2011; 37(6): 856–9.
17. Garcez AS, Fregnani ER, Rodriguez HM, Nunez SC, Sabino CP, Suzuki H, Ribeiro MS. The use of optical fiber in endodontic photodynamic therapy. Is it really relevant? Lasers Med Sci. 2013; 28(1): 79–85.
18. Hopp M, Biffar R. Photodynamic therapies – blue versus green. Laser. 2013; 1: 10–25.
19. Kikuchi T, Mogi M, Okabe I, Okada K, Goto H, Sasaki Y, Fujimura T, Fukuda M, Mitani A. Adjunctive application of antimicrobial photodynamic therapy in nonsurgical periodontal treatment: A review of literature. Int J Mol Sci. 2015; 16(10): 24111–26.
20. Usacheva MN, Teichert MC, Biel MA. Comparison of the methylene blue and toluidine blue photobactericidal efficacy against gram-positive and gram-negative microorganisms. Lasers Surg Med. 2001; 29(2): 165–73.

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 32a/E/KPT/2017.
 Available at http://e-journal.unair.ac.id/index.php/MKG
DOI: 10.20473/j.djmkg.v53.i2.p71–75