ISSN 0974-3618 (Print)
0974-360X (Online)

www.rjptonline.org

RESEARCH ARTICLE

Extracellular Polymeric Substance (EPS) Degradation of *Enterococcus Faecalis* biofilm after irradiation with 405nm diode laser

Kun Ismiyatin[1]*, Mochamad Mudjiono[1], Sri Kunarti[1], Maria Liliana Santoso[2], Dalhar Hakiki[2], Windi Irsya[2]

[1]Lecturer of Conservative Dentistry Department, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

[2]Resident of Conservative Dentistry Specialist Program, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

*Corresponding Author E-mail: **kun-is@fkg.unair.ac.id**

ABSTRACT:

This research aimed to determine whether irradiation of 405 nm diode laser with chlorophyll as photosensitizer could degrade the extracellular polymeric substance (EPS) of *Enterococcus faecalis* (*E. faecalis*) biofilm. The material for this study needs 25 biofilm formed by *E. faecalis* was divided equally into five groups. The control negative group (C-) consisted of *E. faecalis* biofilm, the control positive group (C+) consisted of *E. faecalis* biofilm and chlorophyll photosensitizers, and the other three treatment group (T1, T2, T3) consisted of *E. faecalis* biofilm and chlorophyll photosensitizers. Each treatment groups were irradiated for 90 second (s) for T1 group, 105 s for T2 group, and 120 s for T3 group with 405nm diode laser. The degradation EPS of *E. faecalis'* biofilm was determined using Confocal Laser Scanning Microscope (CLSM). Irradiation duration affected the degradation EPS of *E. faecalis'* biofilm. Chlorophyll with 120 s laser irradiation showed significant degradation EPS of *E. faecalis'* biofilm compared to other groups ($p < 0.05$). Irradiation of diode laser 405nm with chlorophyll photosensitizer 120 s could degrade EPS of *E. faecalis* biofilm up to 97.51%.

KEYWORDS: Diode laser, *Enterococcus faecalis*, Extracellular Polymeric Substance, Chlorophyll, Biofilm.

INTRODUCTION:

The presence of microorganisms after root canal treatment, especially facultative bacteria such as Enterococcus faecalis (E. faecalis), can lead to failure.[1] In that case, conventional retreatment, surgery or extraction should be carried out.[2] Previous research reveal that the prevalence of *E. faecalis* infections ranges from 24% - 77%. This number occurs due to various endurance and virulence factors of *E. faecalis*, including its ability to compete with other microorganisms in its invasion into dentinal tubules and its ability to survive even in a low nutrition state.

What make this bacteria even more resilient is its ability to persist in an alkali environment (pH=9.6), detergents, heavy metals, ethanol, and hydrogen peroxide.[3] Some strain even become highly resistant to phagocytosis, antibodies, and antimicrobials.[4]

Biofilm is a microbial aggregate from a diverse type and strain that attaches to the surface of biological and nonbiological substrates through an extracellular polymeric substance (EPS) matrix.[5] It is formed as a defense mechanism of microorganism. Biofilms maintain its structure and spread by binding into each other and form a molecular chain to develop a complex dimensions with EPS expression.[6] Nowadays laser has been widely used and has produced satisfactory results. In dentistry, there are some frequently used types of lasers such as YAG lasers, Diode lasers, CO_2 lasers, and Erbium lasers. When this laser device is used, a process called Photodynamic Inactivation (PDI) will occur. PDI is an in vitro approach to microorganisms' inactivation. It activates nontoxic photosensitizers by using a specific

Received on 06.07.2020 Modified on 22.08.2020
Accepted on 16.09.2020
Research J. Pharm. and Tech. 2021; 14(7):3869-3873.
DOI: 10.52711/0974-360X.2021.00671

wavelength of visible light to incapacitate microorganism.[7] previous study have shown that combination of root canal treatment and PDI can effectively reduce the number of bacteria and biofilms.[8] Another studies also shown PDI effectivity on specific bacteria. Araujo, et al reported that 405 nm Light Emitting Diode (LED) exposure for 60 seconds combined with exogenous photosensitizers can significantly reduce *Lactobacillus acidophilus* up to 70.4%.[9] The use of *Alfalfa chlorophyll* as photosensitizer combined with irradiating process using a 405 nm laser also can reduce the viability *of E. faecalis*.[10]

However, it is unknown whether diode laser exposure with a wavelength of 405 nm and chlorophyll photosensitizer can affect the EPS of *E. faecalis'* biofilm. This research was an experimental laboratory study to investigate the EPS degradation of *E. faecalis'* biofilm after 90 second (s), 105 s and 120 s irradiation of 405nm diode laser with a wavelength of 405nm combined with chlorophyll photosensitizer.

MATERIALS AND METHODS:

Experimental design:

Before conducting the research, ethical clearance was approved by the Health Research Ethical Clearance committee of the Faculty of Dental Medicine, Universitas Airlangga, with a certificate No.122/ HRECC.FODM/VIII/2017. This research was an experimental laboratory study using biofilm formed by *E. faecalis.* 25 biofilm samples from *E. faecalis* were equally divided into five groups. Each group consisted of five samples with different treatments. The control negative group (C-) consist of *E. faecalis* biofilm. The control positive group (C+) consist of *E. faecalis* biofilm and chlorophyll photosensitizers. The other three treatment group (T1, T2, T3) consist of *E. faecalis* biofilm and chlorophyll photosensitizers with90 second (s) irradiation of 405 nm diode laser for T1 group; 105 s irradiation for T2 group, and 120 s irradiation for T3 group.

Culture E. faecalis:

E. faecalis was cultured in *Leuria Bertani* media and incubated in an incubator with 5% CO2 at 37ºC for 3 days. Identification of *E. faecalis* was by looking at colony morphology, indole production, nitrate reduction, positive catalase reaction, negative fermentation when tested with lactose.

E. faecalis biofilm:

E. faecalis (*American Type Culture Collection* (ATCC, 29212) was cultured by inserting the bacteria into falcon tube with 9ml Tryptic Soy Broth (TSB) media (Hardy Diagnostics, USA; Cat No: U38) and supplemented with 2 ml of sucrose (Tocris Bioscience, USA; Cat No. 5511). Then It was incubated overnight at 37ºC with 10% CO2. 100µL of the solution was taken from falcon tube with 9ml TSB media and supplemented with 2ml of sucrose and added to micro titer plate. After 24 hours, a biofilm was formed.

Chlorophyll inserted into the *E. Faecalis* biofilm:

1 mL of chlorophyll (World Organics, Huntington Beach, USA Product code: WOR-59013) was diluted by adding 4mL of sterile aqua and 1mL of dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA, CAS No: 67-68-5) until 20% concentration of chlorophyll photosensitizers was obtained. Each biofilm-filled microtiter plate was given 20µL of the solution. Afterwards, it was incubated for 2 hours.

Irradiation of 405 nm diode laser:

Irradiation of 405nm diode laser was given for 90s, 105 s, and 120 s to each group. Two milliliters of Dextran reagent, Alexa-Fluor ™ 647 (Thermo Fisher Scientific, Waltham, MA USA; catalog number: D22914) was also added, then incubated again at 37ºC for 24 hours. Afterward, the contents of each plate were aspirated and washed 4 times with 0.2mL phosphate-buffered saline (pH 7.3) using a pipette then air dried. Test material was labeled according to the amount of concentration and light exposure period. The biofilm sample was cut transversely 0.5mm thick and placed in the glass slab. Later, The EPS biofilm was observed using inverted Olympus TCS-SPE Confocal Laser Scanning Microscope (CLSM) (CLSM Fluoview FV 3000, Olympus Scientific Solutions America Corp. Wattham, MA, US), under 400x in 1024x1024 pixels. The EPS biofilm of *E. faecalis* that absorbs Dextran Alexa-Fluor will show the red colour, and the graph will show the intensity of fluorescence.

Statistical analysis:

Data were tested for normal distributions and homogenous variances. After that, statistical significance was assessed using *Kruskal-Wallis* test and *Post-hoc Tukey HSD test.* Probability value of $p< 0.05$ was considered statistically significant. All statistical analysis was done using Statistical Package for the Social Sciences (SPSS) version 21.0 (IBM, New York, USA),

RESULTS:

The Results of CLSM observation reveals a decreased intensity of fluorescence in EPS expression of *E. faecalis* biofilm after irradiation of diode laser combined with photosensitizer (Figure 1, 2, 3, 4, 5). Table 1 shows that Degradation EPS of *E. faecalis'* biofilm after irradiation of 405 nm diode laser for 120 seconds accompanied by chlorophyll photosensitizers (T3) resulted in a significant decrease of EPS biofilm compared to other groups ($p <0,05$).

Table 1: The mean, Standard Deviation (SD)and data distribution of degradation EPS of the *E. faecalis* biofilm observed in the negative control group (C(-), positive control group (C(+) ,and treatment groups (T1,T2, and T3).

Group	Degradation EPS Mean ± SD (arb.unit)	Degradation EPS Mean (%)
C (-)	1150.9496[a]± 54.34959	0
C (+)	831.2774[b]± 17.32299	27.77
T1	740.9260[c] ± 28.63790	79.07
T2	143.7894[d] ± 25.50267	87.50
T3	28.6932[e]± 5.59029	97.51

Note: [a,b,c,d,e] Different superscript denotes a significant difference using Post hoc Tukey HSD Test ($p<0.05$). SD : Standard deviation, arb. unit : arbitrary unit

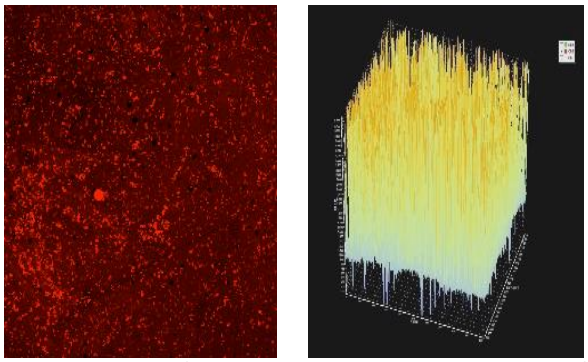

Figure 1: Results of CLSM observations in C(-) group. Left: Description of EPS biofilm that absorbs Dextran Alexa-Fluor (red). Right: Graph of fluorescence expression intensity.

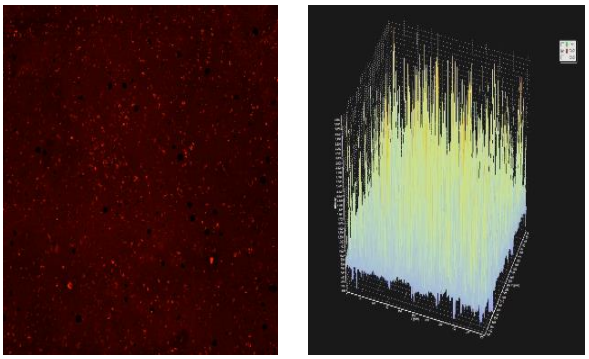

Figure 2: Results of CLSM observations in the C(+) group. Left: Description of EPS biofilm that absorbs Dextran Alexa Fluor (red). Right: Graph of fluorescence expression intensity.

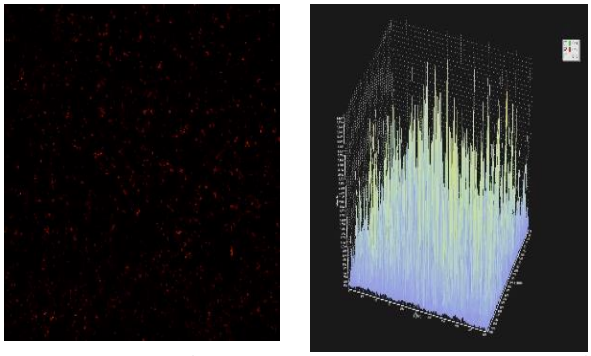

Figure 3: Results of CLSM observations in T1 group. Left: Description of EPS biofilm that absorbs Dextran Alexa-Fluor (red). Right: Graph of fluorescence expression intensity.

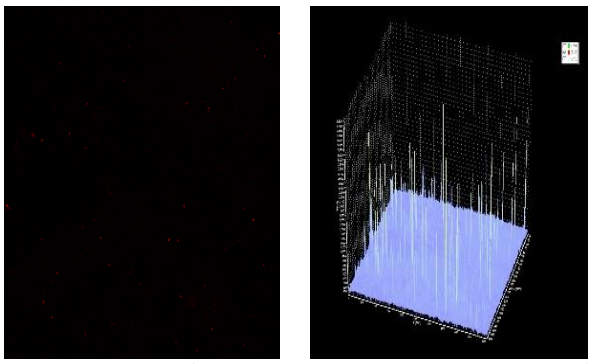

Figure 4: Results of CLSM observations in T2 group. Left: Description of EPS biofilm that absorbs Dextran Alexa Fluor (red). Right: Graph of fluorescence expression intensity.

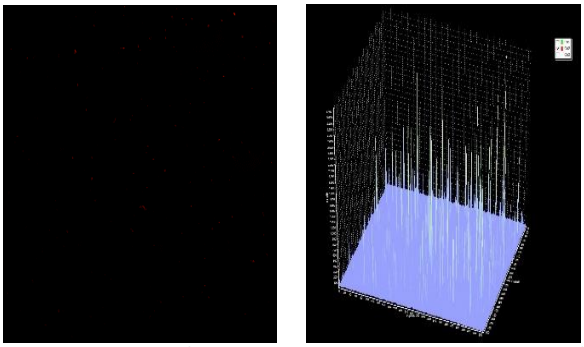

Figure 5: Results of CLSM observations in T3 group. Left: Description of EPS biofilm that absorbs Dextran Alexa-Fluor (red). Right: Graph of fluorescence expression intensity.

DISCUSSIONS:

Diode laser has been used in several areas of dentistry with promising disinfection outcomes. This laser has diodes that emit photons with a coherent nature in a certain wavelength. The active material of diode laser is a semi-conductor, which is similar to semi-conductor in light emitting diode.[11]

Photodynamic Inactivation (PDI) is a method of inactivating microorganisms through light, which will form free radical particles. This process is possible due to chemical interactions at the molecular level, which can damage microorganisms. PDI itself is part of photodynamic therapy to incapacitate microbes by utilizing photosensitizer molecules produced by bacteria through a photophysical procedure in the form of absorption. Inhibition of cell metabolic activity in photodynamic therapy is due to damage of the bacteria's cytoplasmatic membrane. The light can cause a per oxidation reaction with lipid and protein in cell membrane that results in disturbance of transport system or even cell lysis.[12]

Bacterial biofilms maintain the unity of its shape by bonding to each other via polymer chain called EPS, which make stronger adhesion and resistance to anti-microbial, phagocytosis and host immunity.[13] Its main

chemical compound is polysaccharide. This polysaccharide provides nutrition also bind and protect bacterial cell from toxic. In gram-positive bacteria like *E. faecalis*, the chemical component of its EPS can be quite differ. The deposit of this coagulated bacteria consisted of teichioc acid diluted in low concentration proteins.[3,13] In current study, diode laser is able to induce photoinactivation in bacteria with the help of chlorophyll photosensitizer. Chlorophyll is the pigment that plays important role in the primary photochemical reaction in photosynthesis. The main functions of chlorophyll in photosynthesis process are light absorbing, energy transfer and charge separator on the photosynthetic membrane. As an exogenous photosensitizer, it is capable to absorb 99.51% of light at a wavelength of 405 nm as it has a long excitation stage (≤ 10-8 seconds).[10] The process of bacterial photoinactivation can be explained as follows: First, photophysical process will occur. Photosensitizer molecule in a stable state will absorbs photon light. As the configuration becomes unstable, the configuration will then excite to triplet state. In this state, the molecule interacts with oxygen whose electron configuration is stable. It will then lead to the oxygen molecules to become unstable. This oxygen is called Reactive Oxygen Species (ROS), ROS will try to stabilize itself with the surrounding molecules, in this case the *E. faecalis* bacteria. The next stage was photochemical processes that produce radical ions, which consist of superoxide anion (O2-), hydroxyl radical (OH-) and hydrogen peroxide (H_2O_2). This ion is highly oxidative to cells. The results was prolongation of the plasma membrane protein of bacterial cells, inactivation of the NADH enzyme and lactated hydrogenase, damaging the balance of K ions, damaging bacterial DNA, and ultimately inhibiting bacterial growth, which results in cell death of *E. faecalis* bacteria.[4,9,14] Hydroxyl radicals can penetrate cell membranes easily and cause cell damage. Hydroxyl radicals are the most reactive ROS. These radicals can abstract hydrogen atoms from carbon bonds and form carbon radicals. Carbon radicals will form a glyosidic bond and an β-scission reaction occurs which causes the chain break of the polysaccharide.[15] In addition to proteins and polysaccharides damage, free radicals or ROS formed will also cause per oxidation in unsaturated lipids and DNA destruction. We have seen that proteins, polysaccharides, lipids and DNA are the structures that make up the EPS matrix.[16,17] Al ternatively, excess energy at the excitation triplet level can causes chlorophyll to transfer energy to the surrounding oxygen molecules to produce ROS. Oxygen singlets can react with protein molecules to cause oxidation, fragmentation, and inactivation of bacterial enzymes.[18,19] From that statements, it can be concluded that photodynamic therapy can degrade the matrix of bacterial EPS biofilm.[16,17]

This study collected and conclude that degradation of *E. faecalis* EPS biofilm after exposure to diode lasers 405 nm for 120 seconds accompanied by chlorophyll photosensitizers (group 3) showed a more significant decrease of *E. faecalis* EPS biofilm than the other study groups. This is in accordance with theory that the longer laser exposure period, the more excitation of chlorophyll molecules, the greater the conversion of electronic energy from the base level to the excitation level. However, the single EPS biofilm used in this study could not represent the polymicrobial biofilm in oral cavity. Thus the author considered it as a limitation.

CONCLUSIONS:

Based on the results of the study, it can be concluded that exposure to 405nm diode lasers and chlorophyll photosensitizers can degrade *E. faecalis* EPS biofilm. The time of diode laser exposure for 120 seconds caused the degradation of *E. faecalis* EPS biofilm up to 97.51%.

CONFLICTS OF INTEREST:

There are no conflicts of interest.

REFERENCES:

1. Pecora CN, Baskaradoss JK, Al-Sharif A, Al-Rejaie M, Mokhlis H, Al-Fouzan K, Pecora GE. Histological evaluation of the root apices of failed endodontic cases. Saudi Endod J. 2015; 5: 120-4.
2. Vineet R, Nayak M, Kotigadde S. Association of endodontic signs and symptoms with root canal pathogens: A clinical comparative study. Saudi Endod J. 2016; 6(2): 82-6.
3. Chatterjee Rajshekhar, Venugopal P, Jyothi K N, Jayashankar C M, Kumar S Anil, Kumar P Sarath. Effect of Sonic Agitation, Manual Dynamic Agitation on Removal of Enterococcus Faecalis Biofilm. Saudi Endod J. 2015; 5(2): 125-8.
4. Neelakantan P, Cheng CQ, Ravichandran V, Mao T, Sriraman P, Sridharan S, Subbarao C, Sharma S, Kishen A, Photoactivation of curcumin and sodium hypochlorite to enhance antibiofilm efficacy in root canal dentin, Photodiagnosis and Photodynamic Therapy. 2015; 12(1): 108-14.
5. Rosen E, Tsesis I, Elbahary S, Storzi N, Kolodkin-Gal I. Eradication of Enterococcus faecalis biofilms on human dentin. Front Microbiol. 2016; 7(16): 1-9, Article 2055.
6. Li Y, Pan J, Wu D, Tian Y, Zhang J, Fang J. Regulation of Enterococcus faecalis Biofilm Formation and Quorum Sensing Related Virulence Factors with Ultra-low Dose Reactive Species Produced by Plasma Activated Water. Plasma Chem Plasma Process. 2019; 39(1): 35-49.
7. Lin Ching-Hsuan, Chien Hsiung-Fei, Lin Ming-Hsuan, Chen Chueh-Pin, Shen Mandy and Chen Chin-Tin. Chitosan Inhibits the Rehabilitation of Damaged Microbes Induced by Photodynamic Inactivation. Int. J. Mol. Sci. 2018; 19(9): Article 2598
8. De Aguiar Coletti, TMSF., de Freitas, L. M., Almeida, A. M. F., and Fontana, CR. Optimization of Antimicrobial Photodynamic Therapy in Biofilms by Inhibiting Efflux Pump. Photomed. Laser Surg. 2017; 35: 378-85.
9. Araújo N.C., Fontana C.R., Gerbi M.E., and Bagnato V.S. Overall-mouth disinfection by photodynamic therapy using curcumin. Photomed. Laser Surg. 2012; 30: 96-100.
10. Suryani Dyah Astuti. An in-vitro antimicrobial effect of 405 nm laser diode combined with chlorophylls of Alfalfa (Medicago sativa L.) on Enterococcus faecalis. Dental Journal. 2018; 51(1): 47-51.

11. Derikvand N, Chinipardaz Z, Ghasemi S, Chiniforush N. The versatility of 980 nm diode laser in dentistry: A case series. J Lasers Med Sci. 2016; 7(3): 205-208. doi:10.15171/jlms.2016.36
12. Hamblin MR. Antimicrobial photodynamic inactivation: a bright new technique to kill resistant microbes. Curr Opin Microbiol. 2016; 33: 67-73.
13. Ramirez-Mora T, Retana-Lobo C, Valle-Bourrouet G. Biochemical characterization of extracellular polymeric substances from endodontic biofilms. PLoS ONE. 2018; 13(11): Research Article (7 pages).
14. Maria Garcia-Diaz, Ying-Ying Huang, Michael R. Hamblin. Use of fluorescent probes for ROS to tease apart Type I and Type II photochemical pathways in photodynamic therapy. Methods. 2016; 109: 158-66.
15. Möllers KB, Mikkelsen H, Simonsen TI, et al. On the formation and role of reactive oxygen species in light-driven LPMO oxidation of phosphoric acid swollen cellulose. Carbohydr Res. 2017; 448: 182-186.
16. Hu X, Huang Y, Wang Y, Wang X,Hamblin MR. Antimicrobial Photodynamic Therapy to Control Clinically Relevant Biofilm Infections. Front. Microbiol. 2018; 9: 1299.
17. Khoobi, M., Farkhonde Masoule, S., Pourhajibagher, M., Safari, J. Photodynamic Inactivation of Endopathogenic Microbiota Using Curcumin- mediated Antimicrobial Photodynamic Therapy. J of Sciences, Islamic Republic of Iran, 2018; 29(3): 205-9.
18. Ehrenshaft M, Deterding LJ, Mason RP. Tripping up Trp: Modification of protein tryptophan residues by reactive oxygen species, modes of detection, and biological consequences. Free Radic Biol Med. 2015; 89: 220-8
19. Cieplik F, Deng D, Crielaard W, et al. Antimicrobial photodynamic therapy–what we know and what we don't. Crit Rev Microbiol. 2018; 44(5): 571-589.