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Submission date: 08-Nov-2018 03:24PM (UTC+0800)
Submission ID: 1035206692
File name: in-vitro_antimicrobial_effect_of_405_nm_laser_diode_combined.pdf (202.77K)
Word count: 3533
Character count: 19534

Dental Journal
Majalah Kedokteran Gigi

Dental Journal
(Majalah Kedokteran Gigi)
2018 March; 51(1): 47–51

Research Report

An in-vitro antimicrobial effect of 405 nm laser diode combined with chlorophylls of Alfalfa (*Medicago sativa L.*) on *Enterococcus faecalis*

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ABSTRACT

Background: *Enterococcus faecalis (E. faecalis) is a bacterium commonly detected in the root canals of teeth with post-treatment apical periodontitis or advanced marginal periodontitis. It has the ability to live in an extreme environment and survive as an organism with its virulence factor possibly contributing to the pathogenesis of post-treatment apical and marginal periodontitis. Photodynamic therapy (PDT) is an urgently required alternative method of improving therapy effectiveness. Photodynamic therapy combined with conventional endodontic treatment decreases the number of antibiotic-resistant bacteria and biofilms. Chlorophyll is one of the photosensitizers added to enhance the absorption of light in photodynamic therapy.* ***Purpose:*** *The purpose of this study was to determine the antimicrobial effect of the combination of photodynamic laser therapy and Alfalfa chlorophyll in E. faecalis.* ***Methods:*** *In vitro study using E. faecalis distributed between negative control (C-) and positive control (C+), treatment groups using various energy doses of a 405 nm diode laser (2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 J/cm^2) with (G1) and without alfalfa chlorophyll as organic photosensitizer (G2). The suspension was inoculated on Tryptocase Soy Agar (TSA) and incubated at 37° C for 24 hours. The number of colony-forming units per milliliter (CFU/ml) was determined. The results were analyzed by ANOVA with p value Ý0.05.* ***Results:*** *A 405 nm irradiating laser with or without a photosensitizer can decrease E. faecalis viability percentage through the administering of various energy doses. The highest decrease (42%) was obtained in the group without a photosensitizer using 20 J/cm^2, while 10 J/cm^2 in the group with a photosensitizer proved the most effective dose (25%).* ***Conclusion:*** *The results of this study showed a decrease in the viability of E. faecalis exposed to a 405 nm (40 mW) laser. An irradiating process using a 405 nm laser without a photosensitizer (Alfalfa chlorophyll) resulted in the highest percentage decrease (42%) in E. faecalis bacterial viability.*

Keywords: *antimicrobial Photodynamic therapy; Enterococcus faecalis; diode laser 405 nm; Alfafa chlorophyll*

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INTRODUCTION

Enterococcus faecalis (*E. faecalis*), a Gram-positive bacterium commonly found in the root canal, is ovoid in shape with a diameter of between 0.5 and 1 µm. This bacterium is a facultative anaerobe and possesses the ability to survive in an extreme environment such as in a highly alkaline pH and high salt concentrated condition.[1]

E. faecalis is resistant to calcium hydroxide and antibiotics.[2] Tetracycline produce a poor antimicrobial effect on periodontal *E. faecalis*, with more than 50% of the *E. faecalis* periodontal isolates showing resistance. Furthermore, *E. faecalis* isolates from root canals demonstrate a high prevalence of the genetic determinant of tetracycline resistance (*tetM*). Recent studies have indicated that *tetM* genes were detected in approximately 50% of isolates from the root canal.[1]

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E. faecalis demonstrate high frequency in cases of poor response to either endodontic or periodontal treatment.[3] Therefore, alternative methods are urgently needed to improve the therapy effectiveness, one such method being photodynamic therapy which is a medical treatment that utilizes light to activate photosensitizer agents. Exposure of the photosensitizer to light produces a wide range of oxygen species and free radicals that cause localized damage and the death of bacteria.[3] Photodynamic therapy is also known as an antimicrobial photodynamic therapy (aPDT) and photodynamic inactivation (PDI).[4]

Photodynamic therapy uses light sources and light-sensitive photosensitizer agents. The suitability of the light spectrum and photosensitizer agent to photodynamic therapy will produce ROS. Light sources such as Light Emitting Diodes (LED) and a variety of lasers can be used to activate the photosensitizer agent.[3] These lasers generally have complicated systems resulting in a high cost. Currently, diode lasers are widely used because they offer the advantages of simple systems, portability and low cost.[5] Research results showed that photodynamic therapy combined with conventional endodontic treatment decreases the prevalence of bacteria and biofilms.[6,7]

Certain photosensitizer agents have been tested for their ability to efficiently produce reactive oxygen species (ROS) formed through photochemical type I or II that inactivate microbial cells.[8] Antimicrobial photodynamic therapy success relies heavily on the type of photosensitizer, the light source wavelength and output power and the irradiation time used.[9] The use of low power laser photodynamic therapy with different exogenous photosensitizers, toluidine blue (TBO),[10,11] methylene Blue (MB),[12–14] and organic photosensitizer curcumin[15] has been investigated. For these reasons, chlorophyll is more appropriate for development as a photodynamic therapy in cases of tumors and cancer.[16]

Chlorophyll is a substantial bioorganic molecule with the core function of absorbing light and transferring excitation energy to the reaction center in photosynthetic devices. High absorption energy during the photosynthesis process is caused by a relatively long excitation process of chlorophyll (Ý 10^{-8} seconds). The longer the process of singlet excitation of chlorophyll, the greater the electronic energy converted. The conversion process may occur from a basic level to ones of triplet excitation. The excess energy produced by chlorophyll at the level of triplet excitation provides an opportunity to transfer energy to oxygen molecules, a process producing reactive singlet oxygen.[17]

In this study, chlorophyll was extracted from the leaves of Alfalfa (*Medicago sativa L*), a type of perennial leguminous plant with high chlorophyll content.[18] Other research showed that chlorophyll a and b, in addition to pheophytin are all powerful antioxidants that can reduce ROS and prevent lipid peroxidation and DNA damage.[19] The purpose of this study was to determine the antimicrobial photodynamic effect of the photodynamic therapy laser diode in combination with alfalfa chlorophyll in *E. faecalis* bacteria.

MATERIALS AND METHODS

The sample strains used in this research consisted of purely cultured bacteria from *E. faecalis* ATCC 29212. The bacteria strains were grown in Tryptic soy agar (Oxoid, England, UK), subsequently inoculated in Triptic Soy Broth (TSB) solution (Merk, Darmstadt, Germany), incubated for 24 hours and, finally, diluted to a value of optical density of OD_{600nm} = 1.6/0.142, which is equal to ~ 10^3 CFU/mL.

Alfalfa chlorophyll (K-Link liquid chlorophyll, Jakarta Selatan, Indonesia) at a concentration of 1.6 mg/ml was diluted in normal saline. The absorption spectrum of chlorophyll was measured using a Shimadzu UV-VIS 1800 spectrometer (Shimadzu, Tokyo, Japan).

Laser irradiation was carried out using Sony diode lasers with output wavelengths of 405 nm. The power outputs were 40 mW with a focal spot ®xed: 0.3 cm^2

This research was conducted using post-test only control group design. The samples were distributed into four groups: (1) *E. faecalis* exposed to neither a 405 nm diode laser nor Alfalfa chlorophyll as the Negative Control (C-); (2) *E. faecalis* exposed only to 1.6 mg/ml Alfalfa chlorophyll as the Positive Control (C+); (3) *E. faecalis* exposed to various energy doses of 405 nm diode laser (2.5; 5.0; 7.5; 10.0; 12.5; 15.0; 17.5; 20.0) and 1.6 mg/ml Alfalfa chlorophyll as Group 1 (G1); (4) *E. faecalis* exposed to various energy doses of diode laser 405 nm (2.5; 5.0; 7.5; 10.0; 12.5; 15.0; 17.5; 20.0 J/cm^2) without Alfalfa chlorophyll as Group 2 (G2). After treatment, the suspension was inoculated on TSA and incubated at 37° C for 24 hours. The number of colony-forming units per milliliter was counted manually to determine the antimicrobial effect on *E. faecalis*. The percentage of decrease bacterial viability was defined as:

$$\left[\frac{(\Sigma \text{ control colony} - \Sigma \text{ treatment colony})}{\Sigma \text{ control colony}} \times 100\%\right]$$

A broth microdilution method was applied to determine the minimum inhibitory concentration (MIC) of Alfafa chlorophyll against *E. faecalis*. Testing was conducted using a 96-well flat-bottomed microplate (Nunc, Sjælland, Denmark). Each well contained 90 µl TSB, 90 µl Alfafa chlorophyll at various concentrations (0.8, 1.0, 1.4, 1.6 mg/ml), and 20 µl of *E. faecalis* culture at a concentration of 10 CFU/ml[1]. The microplates were incubated for two hours at 37° C under microaerophilic conditions. The MIC was defined after two hours of incubation. All tests were repeated at least four times. The number of colony-forming units per milliliter (CFU/ml) was calculated manually to determine the MIC test results which were log-transformed and analyzed with SPSS version 10.05 (SPSS Inc., Chicago, Illinois, USA) using ANOVA (p=0.05).

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RESULTS

The photosensitizer used in this study was Alfalfa chlorophyll, the absorption spectrum of which[20] is shown in Figure 1.The MIC test for Alfalfa chlorophyll confirmed that the dye had no toxic effects on *E. faecalis*. Statistical results showed that the chlorophyll concentrations (0.8, 1.0, 1.4, 1.6 mg/ml) did not significantly differ from each other at p=0.12 (p>0.05).

Figure 2 shows the viability of *E. faecalis* exposed to 405 nm diode laser at the same concentration of photosensitizer (chlorophylls). The 405 nm diode laser treatment group resulted in statistically significant increases and decreases of CFU p=0.00 (p<0.05) compared to the control group. The statistical test results showed the largest percentage of reduction in bacterial viability to be in the treatment of 405 nm 20.0 J/cm^2 laser exposure to be 41.75%.

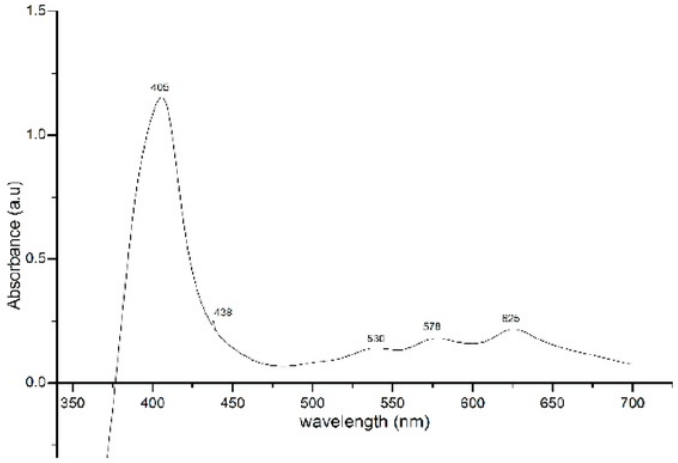

Figure 1. The absorption spectrum of chlorophyll Alfalfa (*Medicago sativa L.*)

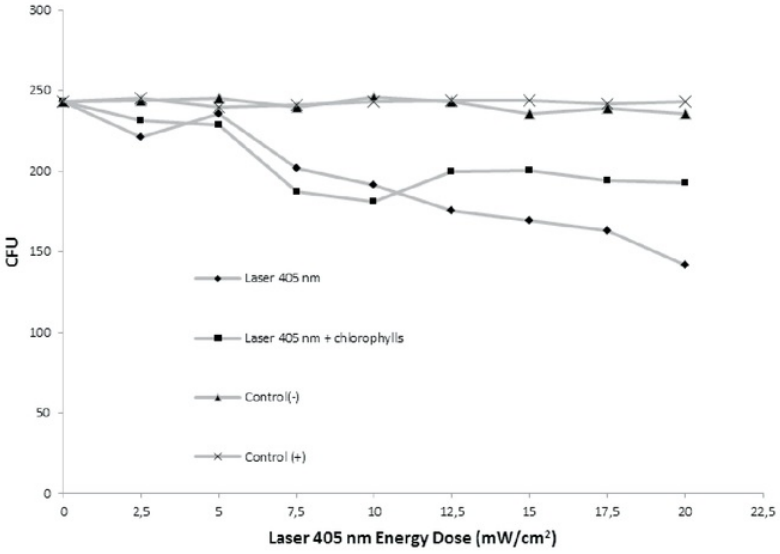

Figure 2. The viability of *E. faecalis* exposed to diode laser 405 nm.

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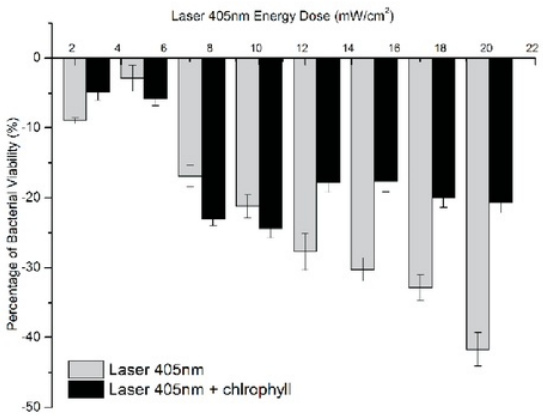

Figure 3. The percentage of the *E. faecalis* decrease exposed to laser 405 nm.

Figure 3 shows the percentage (%) of decreasing bacterial viability at various irradiating laser energy doses with chlorophyll as a photosensitizer. The results in figure 3 indicate that the irradiating process using a 405 nm laser with and without chlorophyll can reduce the viability of *E. faecalis* bacteria. A 405 nm laser without a photosensitizer (Alfalfa chlorophyll) produced the highest decrease in the *E. faecalis* bacterial viability percentage (42%) and that of the photosensitizer (25%).

DISCUSSION

Antimicrobial photodynamic therapy requires a light source at a specific wavelength that activates the PS. This study used a laser diode at an output power wavelength of 405 nm with a range of 40 mW. For medical applications, the mechanism of photochemical interaction plays a significant role in photodynamic therapy. Similarly with biostimulation, photochemical interactions occur at a very low power density (1 W/cm^2) and an exposure time of one second.[21] The results of temperature measurements in figure 3 showed that during irradiation the temperature stablized below 45°C, which remained in the optimum growth range of the *E. faecalis* bacterium. This suggested that bacterial death was not caused by an increase in temperature but, rather, was due to irradiation.

The photosensitizer used in this study was Alfalfa chlorophyll at a concentration of 1.6 mg/ml. The absorption spectrum of exogenous photosensitizer in figure 4 indicated effective absorption at a blue and red wavelength. Based on the data, the quantum yield of laser diode with a wavelength of 405 nm could be calculated. The chlorophylls absorption stood at 92.97%.[22] The absorption percentage of photosensitizer affects the production of ROS. The concentration of photosensitizer and conformity of the light wavelength with the absorption spectrum of the photosensitizer leads to successful antimicrobial photodynamic therapy.[23]

This study used a diode laser with a 405 nm wavelength and the same focal spot. Table 1 shows the energy doses and time duration of laser irradiation applied in this study. Irradiation at different wavelengths produces a variety of quantum yields. The similarity of the wavelength of the light source with the photosensitizer absorption spectrum will produce a photophysical mechanism, i.e. the absorption of light energy. Absorption of light energy will excite photosensitizer molecules that trigger the occurrence of photochemical reactions resulting in radical oxygen species. Another deciding factor was the radiation energy dosage. The appropriate dose of energy activates a chemical reaction producing a wide range of reactive oxygen species that causes photoinactivation in bacteria. The energy dose of laser irradiation per total area of irradiation (power density, unit J/cm^2) is the magnitude of radiation energy (power multiplied by the longer exposure time) divided by the total area of irradiation. This determines the time duration of the laser irradiation adjusted to the energy dose and quantum yield.[7]

The results of this study showed a decrease in the viability of *E. faecalis* that were exposed to a 405 nm laser with a power output of 40 mW. The irradiating process using a 405 nm laser at an energy dose of 20 J/cm^2 without a photosensitizer (Alfalfa chlorophyll) resulted in the highest decrease in the *E. faecalis* bacterial viability percentage (42%). An irradiating 405 nm 10 J/cm^2 laser with a photosensitizer resulted in the highest percentage decrease (25%) in the bacterial viability of *E. faecalis*.

Singlet Dioxygen is capable of causing permanent damage to various parts of the cells, including: the plasma, mitochondria, lysosomes, nuclear membranes, in addition to the modification of proteins. Photodynamic therapy within various photosensitizer molecules is more effective in immobilizing Gram-positive than Gram-negative

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bacteria.[24,25] Differential susceptibility to photodynamic therapy arises because of contrasting cell wall structure of the respective groups. The outer wall (15–80 nm thick) of Gram-positive bacteria consists of more than 100 layers of peptidoglycan-related lipotechoic and teichuronic acids. This layer is relatively porous allowing macromolecules with a molecular weight of 30,000–60,000 Da to diffuse across the plasma membrane. Therefore, a photosensitizer with a molecular weight of 1500-1800 Da can be diffused. Gram-negative bacterial cells consist of the cytoplasmic membrane and outer membrane separated by peptidoglycan-containing periplasm. Moreover, the latter is of heterogeneous composition, including: proteins that function as porin, lipopolysaccharide and lipoprotein. The outer membrane forms an effective barrier to permeability. Only hydrophilic compounds with a low molecular weight of 600-700 Da can diffuse and limit the penetration of photosensitizer. Type 1 photochemical reactions occur in Gram (+), while type II occur in Gram (-). Photoinactivation in bacteria consists of several processes: (a) photosensitizer translocation to the plasma inner membrane, (b) photoinactivation of the photosensitizer, (c) the generating of ROS, (d) oxidative modification of the target, (e) a decrease in cell function and metabolism and (f) inhibition of cell growth and cell death.[25]

The results of this study showed a decrease in the viability of *E. faecalis* that had been exposed to a 405 nm (40 mW) laser. An irradiating process using a 405 nm laser without a photosensitizer (Alfalfa chlorophyll) resulted in the highest percentage decrease (42%) in *E. faecalis* bacterial viability. The use of photosensitizer (Alfalfa chlorophyll) decreases bacterial viability to 25%.

ACKNOWLEDGEMENT

The research reported here was funded by a 2016 grant from the Ministry of Research, Technology, and Higher Education.

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