Effectiveness Photodynamic Inactivation with Wide Spectrum Range of Diode Laser to *Staphylococcus aureus* Bacteria with Endogenous Photosensitizer: An *in vitro* Study

Suryani Dyah Astuti[1,2]*, Indira Wastu Widya [1], Deny Arifianto[2], Retna Apsari [1]

1. Department of Physics, Faculty of Science and Technology, Airlangga University, Surabaya 60115, Indonesia.
2. Department of Biomedical Engineering Post Graduate School, Airlangga University, Surabaya 60115, Indonesia.

Abstract

Infection is a disease caused by the invasion of pathogenic bacteria or microorganisms that breed on the wound. Infectious bacteria such as *Staphylococcus aureus* can cause wound infections. Systemic therapy using antibiotics leads to bacterial resistance. Meanwhile, bacteria naturally produce light-sensitive porphyrins, as endogenous photosensitizer. Light irradiance with suitable wavelength spectrum to the spectral absorption of porphyrin with the proper irradiation energy density can cause inactivation of bacterial cells. This study aims to determine effectiveness photodynamic inactivation with wide spectrum range of diode laser 405 nm to inactivate the *Staphylococcus aureus* bacteria with endogenous photosensitizer.

In order to determine the role of energy density of diode laser to activate the endogenous photosensitizer porphyrin in bacteria, the study was divided into two parts: 1) characterization of diode laser light source and absorption spectrum of endogenous photosensitizer *Staphylococcus aureus*, 2) determine the antimicrobial effect of diode laser with endogenous photosensitizer. Combination treatment were divided into the following experimental groups: C group for the control group without laser irradiation treatment and T group for groups with laser irradiation at various exposure time (120; 150; 180, 210; 240; 270) s. The results were analyzed by Anova and the Tukey test with P value ≤0.05.

The statistical analysis showed that there was no significantly difference between the control group and the laser treatments at 120 s exposure time, as well as on treatments with 180 s and 210 s. Treatment of laser exposure resulting in significant differences in bacteria survival of 0.00 ($P < 0.05$) at 150 s, 270 s and 240 s laser exposure time. 240 s laser exposure time with energy density of 55.02 J/cm^2 resulted in the lowest bacteria survival of 5.89 log CFU/ml with a 55.22% reduction in bacterial reduction, which was significantly different from the other treatment groups.

The suitability between wavelength of the diode laser with photosensitizer's absorption spectrum affect the effectiveness of bacterial inactivation. The more precisely the absorption of photon light energy, the more photosensitizer will be activated to produce various reactive oxygen species that have an effect on the number of bacterial deaths.

Experimental article (J Int Dent Med Res 2019; 12(2): 481-486)

Keywords: Wavelength, Absorption, Porphyrin, Diode laser, Staphylococcus aureus.

Received date: 16 July 2018 **Accept date:** 21 October 2018

Introduction

Staphylococcus aureus (*S.aureus*) is a bacterium that lives commensally on human skin. under pathological conditions, *S.aureus* can cause a number of illnesses from minor skin diseases such as skin infections, acne vulgaris, to severe diseases such as pneumoni and meningitis [1]. *S.aureus* infection can also be caused by direct contamination of the wound, e.g. in postoperative wound infections or post-traumatic infection [2]. Generally, dermatologists use systemic therapy in the form of antibiotics such as methicillin to reduce the number of infectious bacteria, so the mechanisms against inflammation can be formed and skin irritation can be corrected. However, this method often causes problems, since long-term antibiotics cause bacteria to become resistant [3-4].

Some bacteria naturally produce

Corresponding author:
Suryani Dyah Astuti
Department of Physics,
Faculty of Science and Technology, Universitas Airlangga,
Surabaya, Indonesia.
E-mail: suryanidyah@fst.unair.ac.id; suryanidyah@gmail.com

endogenous porphyrins as photosensitivity molecules which are light-sensitive [5-6]. The ability of porphyrin to absorb light is specific. Theoretically, the porphyrins have 2 specific intensities, namely Soret Band and *Q Band* [7]. The presence of porphyrins as endogenous photosensitizer can be used for photodynamic inactivation (PDI) bacteria [8-10]. PDI is a method of bacterial inactivation utilizing light sources, photosensitizing agents, and oxygen [11-13]. PDI can be applied for antimicrobial photodynamic therapy in various cases of skin, dental and oral diseases[14-15]. otherwise photodynamic activation produces a photobiomodulating effect. Photobiomodulation is a therapeutic modality by using light to eliminate inflammatory effects in various cases such as periodontitis[15-18].

The absorption of light by photosensitizer is related to the wavelength of light source and the photosensitizer absorption spectrum [19-20]. This photophysical process will activate the occurrence of photochemical reactions resulting in various reactive oxygen species (ROS) products that inactivate bacterial cells [21]. Photodynamic inactivation is inhibition of cell metabolic activity caused by cytoplasmic membrane damage due to peroxidation by reactive oxygen at lipids and proteins, results in cell lysis or inactivation of membrane transport systems and membrane transport enzyme systems in these bacterial cells [22].

Various studies on the success of photoinactivation in microbes *in vitro* were also performed by Rolim *et al.* (2012) [23] and Tavares *et al.* (2010) [24]. Nitzan *et al.* (2004) [10] investigated photoinactivation with blue halogen lamp (407-420) nm (intensity 20 mW/cm^2), energy dose of 100 J/cm^2 on various strains of bacteria with exogenous ALA. Guffey *et al.* (2006) [25] investigated photoinactivation using light spectrum 405 nm and 470 nm in *Pseudomonas aeruginosa* bacteria *in vitro*.

Some literatures suggest that visible light, particularly blue light in the 400-470 nm spectrum can cause photoinactivation in some bacteria through photostimulation in intracellular endogenous porphyrin [20]. Imamura's research (2014) to photodynamic therapy of *C. albicans* bacteria with 90% bacterial death percentage using laser diode 405nm power 0.2W with 1200s exposure time, whereas in bacteria *P. gingivalis* and *P. intermedia* obtained percentage of bacterial deaths of 60% and 80% with power 0.2 W and 300 s exposure [26]. Complementing the role of light characterizaztion in photodynamic microbial inactivation, this study aims to determine effectiveness photodynamic inactivation with wide spectrum range of diode laser 405 nm to inactivate the *Staphylococcus aureus* bacteria with endogenous photosensitizer, an *in vitro* study.

Materials and methods

Bacterial Culture

The bacterial strain, *S. aureus* ATCC 25923 was inoculated from Tryptone Soy Agar (Oxoid, UK) and taken on Tryptone Soy Broth sterile (Oxoid, UK). The culture of bacteria was incubated in 37^{o}C incubator CO_2 until bacterial colonies reached ~10^8 CFU/mL or 1.0 McFarland Standard. The irradiation of the *E. coli* bacteria was done in vitro in Petri dishes.

Characterization of Light Source

Characterization of the light source aims to determine the role between the wavelength spectrum and energy density of diode laser with the absorbent spectrum of endogenous photosensitizer in *Staphylococcus aureus* bacteria. Characterization of light source was performed using wavelength meter and universal fiber optic detector Thorlabs PM 100D include diode laser spectrum, power stability over time, temperature and file area. Characterization of bacteria porphyryine's resorptive spectrum was performed also.

Antimicrobial Effect of Treatments Against *S. aureus*

Combination treatment were divided into the two groups; group (C) for control group no laser treatment and group (T) for treatment group with diode laser exposure at varying time exposure (120; 150; 180, 210; 240, 270) s.

After treatment, the bacteria suspension was planted and incubated at 37^{o}C for 24 hours, and then the number of colony-forming units per milliliter (CFU/ml) was determined. The decrease in bacterial colony growth (D) was calculated using D(%): $|(C-T)/C|$ x 100%, where C and Y are the total numbers of bacterial colony, under treatment and control, respectively. The data were analyzed by Anova and the Tukey test with P value ≤0.05.

Results

Wavelength characterization was done by a wavelength meter connected to a universal fiber optic detector. The wavelength of diode laser spectrum is shown in Figure 1.

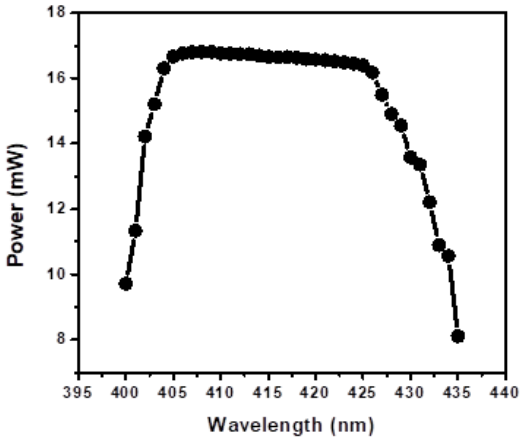

Figure 1. Wavelength spectrograph of diode laser.

The wavelength characterization result show that the diode laser wavelength is in the wide range of (405-425) nm.

The magnitude of laser exposure intensity be affected by exposure distance and the area of the laser spot. Figure 2 shows the laser power at various exposure distances. The diode laser power at a distance of 1.5 cm indicates power of (95.68±0.08) mW. At a distance of 1.5 cm, the characterization was done to check the power stability to the duration of diode laser exposure. The result of power stability characterization of diode laser in the varying time exposure is shown in Figure 3.

The results of power characterization against laser exposure time show stability after 100 seconds of laser exposure, i.e. at power of (82.53±0.06) mW.

Laser diode is a diode that emits a coherent and directional photon at certain wavelength. The photon emitting process in the semiconductor diode laser is due to the electron transition from the conduction band to the valence band and recombine. Light intensity depends on the amount of current used. At low currents, the radiation emitted by the laser diode is the result of spontaneous emission. In the presence of an additional current, a radiation emitted is no longer due to spontaneous emissions, but to the emission of stimulation. The output power of the laser is also strongly influenced by the temperature; hence temperature changes will produce a light spectrum with different wavelengths.

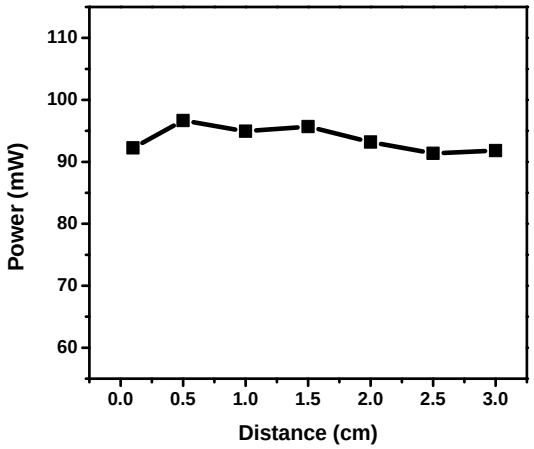

Figure 2. Diode laser power stability at various irradiance distance.

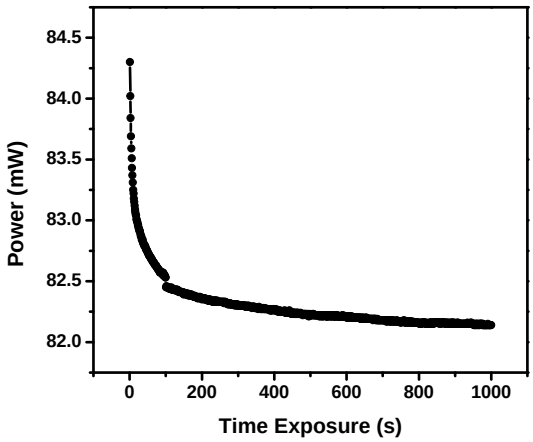

Figure 3. Graph of diode laser stability at various time exposure.

Diode Laser has a narrower spectrum distribution than an LED. Laser light has a mode structure, with peaks separated in wavelength. Lasers are known to have Gaussian-shaped beams, the distribution of light can be viewed in a transverse direction ie perpendicular to the propagation axis. The light beam that has a diameter in the transverse direction, will spread with a spreading angle [27]. If a light beam passes through a gap, then the intensity of light located at a distance from the gap will be oscillated in the transverse direction. The spread of the beam is caused by the diffraction event that is the flexing of light as it passes through a lattice gap that produces wave front along the z-direction and the diffraction angle θ. The laser file formed by the z-

diffraction event is called a Gaussian beam. The Gaussian beam is the ideal and desired beam of a laser because in that shape the highest intensity is at the top and then the smaller at the edges of the beam. A laser beam having a gaussian profile at a particular location will have a gaussian profile for all z positions along the propagation direction. The peak intensity of the z-axis can be attributed to the total power (*P*) propagated by the Gaussian beam. The intensity of the axis of the beam can be obtained through the equation with A is the effective area of the beam at position z [27]:

$$I(0,z) = \frac{P}{A} \quad (1)$$

One of the weakness of the diode laser is each distance traveled will produce a different output that is elliptical with a large divergence angle; this is caused by light diffraction (light bending) when the light comes out of the laser. The further the radiation distance, the output light will be elliptical with a penumbra that tends to increase. The diameter of the beam intensity (A) on the x and y-axes is a distance function (z) according to the formulation of [28]:

$$A = \frac{\pi}{2} d_x d_y \quad (2)$$

With d_x is the ellipse diameter on the x axis, d_y is the ellipse diameter on the y-axis [28]. Therefore, the size of the beam is 0.3617 cm^2. The intensity of laser diode exposure (*I*) in mW/cm^2 is laser exposure power (*P*) divided by the area of the file (A). Whereas the energy density (*E*) of laser exposure in J/cm^2 is the intensity (I) multiplied by exposure time (t).

$$E = \frac{P}{A} xt \quad (3)$$

The magnitude of the energy density at various irradiation times is shown in Table 1.

Treatment	Power (mW)	Area (cm^2)	Exposure time (s)	Energy density (Jcm^{-2})
1	82.53	0.36	120	22.56
2	82.53	0.36	150	34.39
3	82.53	0.36	180	41.27
4	82.53	0.36	210	48.14
5	82.53	0.36	240	55.02
6	82.53	0.36	270	61.89

Table 1. Laser energy density at various time exposure.

The results of the endogenous spectral absorption test of bacterial porphyrin *Staphylococcus aureus* are shown in Figure 4.

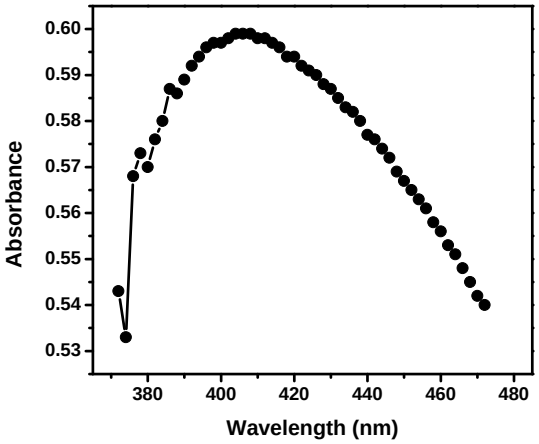

Figure 4. Bacterial absorption spectrum of *Staphylococcus aureus*.

Figure 4 shows the peak of endogenous photosensitizer of *Staphylococcus aureus* bacteria is in the range of 372 nm to 472 nm with peak spectrum at 405 nm.

Treatment results at various laser exposure time are shown in Figure 5. The statistical analysis showed not significantly different between the control group and the laser treatments at 120s exposure time, as well as on treatments with duration of 180 s and 210 s. Treatment of laser exposure resulting in significant differences in bacteria survival of 0.00 (P <0.05) at laser exposure of 150 s, 270 s and 240 s. At treatments of 240 s laser exposure with energy density of 55.02 J/cm^2 resulted lowest bacteria survival of 5.89 log CFU/ml with a 55.22% reduction in bacterial death, which was significantly different from that of the other treatment groups.

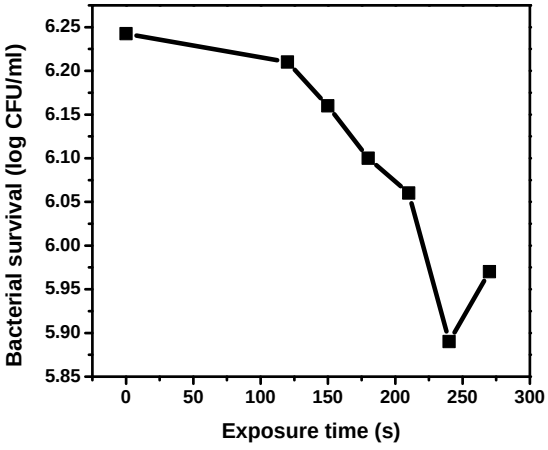

Figure 5. The effect of laser exposure time to bacteria survival.

Discussion

There are various kinds of porphyrins produced by the bacterium [6]. Each bacterium accumulates a particular type of porphyrin with a specific absorptive spectrum [8]. The synthesis of bacterial porphyrins occurs in the cytoplasm with the natural precursors of δ-aminolevulinic acid (dALA) which produces various types of porphyrins namely, coproporfirin III, uroporfirin III and protoporfirin IX [9]. Nitzan *et al*. (2004) [10] reported that Gram-positive Staphylococci bacteria produced coproporfirin III (68-75%). The advantages of porphyrins as endogenous photosensitizers are the strong absorbency at the visible spectrum of light, stable to light exposure, and low toxicity in dark conditions.

The endogenous spectrum of endogenous phorpyrin bacteria *Staphylococcus aureus* lies at the wavelength of 405 nm. This result is consistent with previous research showing that it has 2 specific intensities, i.e. *Soret Band* located at wavelength of 402 nm and *Q Band* located at wavelength 272 nm, 631 nm, 720 nm, and 930 nm [7]. The result of the diode laser wavelength characterization is in spectrum range of (405-425) nm so that the diode laser spectrum is in the porphyrin absorption spectrum of *Staphylococcus aureus* bacteria. Light photon uptake by photosensitic molecules (photophysical process) occurs only in photons of wavelength corresponding to the absorbent spectrum of photosensitizer [21, 29].

Lasers are known to have monochromatic, coherent, and focused beams. The laser light source has an intensity associated with the power per unit area. The larger power with a small surface area produces larger intensity. The intensity of diode laser exposure shows the number of emitted photons that appear at high brightness levels. Therefore, the diode laser energy density is the quantity of photon energy per unit area of light spot. The larger the diode laser energy density, the more photons with high energy can be absorbed by the photosensitizer molecule. The laser energy absorbed by the photosensitizer molecule will then activate the occurrence of photochemical reactions resulting in a radical product that damages the bacterial cell.

The success of the photoinactivation of bacteria involves the process of photosensitization, i.e. the process of light absorption (photophysics) by photosensitizer porphyrins, which further activates the occurrence of chemical reactions resulting in various species of reactive oxygen. The initial mechanism of photosensitization is the absorption of photons by photosensitivity molecules followed by photochemical reactions[21]. Photochemical reactions mediated by porphyrins are most common from excited triplet states. Chemical reactions of type 1, optically excited photosensitive molecules react with substrates such as cell membranes or molecules and transfer a proton or an electron to produce an anion superoxide or radical cation. This radical will then react with oxygen to produce the reactive oxygen species (ROS). Anion superoxide will react with the substrate to produce hydrogen peroxide (H_2O_2). In type 2 reactions, triplet photosensitizer can transfer energy directly to oxygen molecules that are in a triplet excitation state to produce electrophilic singlet oxygen ($_1O^2$). The singlet oxygen reaction with the amino acids will form peroxides that damage cells.

The results of this study indicate that laser exposure of 240 s with energy density of 55.02 J/cm^2 resulted in the lowest bacteria survival of 5.89 log CFU/ml with a 55.22% reduction in bacterial mortality, which was significantly different from the other treatment groups. Photodynamic effects on *Staphylococcus aureus* bacteria are reported by Maclean *et al*. (2009) [19] with a 405 nm blue LED without exogenous photosensitizer molecule at dose of 36 J/cm^2 resulted in a 14% percentage of *Staphylococcus aureus* bacterial death. The results of Imamura [26] showed that the photodynamic effect of 405 nm and laser power of 0.2 W for 300s s decreased the bacterial population of *P. gingivalis* and *P. intermedia* by 60% - 80%, whereas in the 1200 s decreased *C. albicans* population by 90%. Based on research that has been done, the role of photon number and photon energy affect the number of bacterial deaths. The larger the photon intensity and the longer the exposure, the more photosensitizer will be activated to produce various ROS that have an effect on the number of bacterial deaths.

Conclusions

Laser exposure of 240 s with energy density of 55.02 J/cm^2 resulted in the lowest bacteria survival

of 5.89 log CFU/ml with a 55.22% reduction in bacterial death, which was significantly different from the other treatment groups. The suitability between wavelength of the diode laser with photosensitizer's absorption spectrum affect the effectiveness of bacterial inactivation. The more precisely the absorption of photon light energy, the more photosensitizer will be activated to produce various reactive oxygen species that have an effect on the number of bacterial deaths.

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

References

1. Coates R , Moran J , Horsburgh MJ , Staphylococci: colonizers and pathogens of human skin, Future Microbiol, 2014; 9:75–91.
2. Honda H , Doern CD , Michael-Dunne W, Jr , Warren DK , The impact of vancomycin susceptibility on treatment outcomes among patients with methicillin resistant Staphylococcus aureus bacteremia, BMC Infectious Diseases, 2011; 11:335.
3. Anderson DJ, Kaye KS, Chen LF, Schmader KE, Choi Y, Sloane R, et al. (2009) Clinical and Financial Outcomes Due to Methicillin Resistant Staphylococcus aureus Surgical Site Infection: A Multi-Center Matched Outcomes Study. PLoS ONE 2009; 4(12): e8305.
4. Stryjewski ME , Corey GR , Methicillin-resistant Staphylococcus aureus: an evolving pathogen. Clin Infect Dis, 2014; 58(Suppl 1):S10–S19.
5. Nitzan Y. and Kauffman M., Endogenous Porphyrin Production in Bacteria by ?-Aminolaevulinic Acid and Subsequent Bacterial Photoeradication, Lasers Med Sci. 1999; 14:269-277.
6. Bruce-Micah R., Huttenberger D., Freitag L., Cullun J., Foth H-J., Pharacokinetic of ALA and h-ALA Induced Porphyrins in the Models Mycobacterium phlei and Mycobacterium, Journal of Photochemistry and Photobiology, 2009; 97:1-7.
7. Makarska, M. &. Radzki S., Water-soluble Porphyrins and Their Metal Complexes. Annales Universitasis Mariae Curie-Sklowdoska Lublin, Polonia. 2002; LVII:17.
8. Ramberg K., MelØ T.B., Johnsson A., In Situ Assesment of Porphyrin Photosensitizer in P.acnes, Z.Naturforsch, 2004; 59C:93-98.
9. Hamblin MR, Hasan T. Photodynamic therapy: A new antimicrobial approach to infectious disease? Photochem Photobiol Sci. 2004; 3(5):436–450.
10. Nitzan Y., Divon M.S., Shporen E., Malik Z., ALA Induced Photodynamic Effect on Gram Positive and Negative Bacteria, Journal of Photochemistry and Photobiology P: Physics., 2004; 3:430-435.
11. Jori G., Fabris C., Soncin M., Ferro S., Coppelotti O., Dei D., Fantetti L., Chiti G., Roncucci G., Photodynamic Therapy in The Treatment of Microbial Infections: Basic Principles and Perspective Applications, Laser in Surgery and Medicine, 2006; 38: 468-481.
12. Astuti S.D., Wibowo A., Abdurachman, Triyana K., Antimicrobial Photodynamic Effects of Polychromatic Light Activated By Magnetic Fields to Bacterial Viability, J. International Dental and Medical Research 2017; 10(1):111-117.
13. Astuti S.D, Arifianto, D, Drantantiyas N.D.G., Nasution A.M.T. and Abdurachman, Efficacy of CNC-Diode Laser Combine with Chlorophylls to Eliminate Staphylococcus aureus Biofilm, IEEE, 2017; 7803722:57–61.
14. Mustafa E.A and Al Kawas S, The effect of Galium-Arsenide Laser Irradiation on Odontogenis, Journal of International Dental and Medical Research, 2010; 3(2):52-56.
15. Sulijaya B., Soeroso Y., Sunarto H., Suri P.A, Nazar K., The Clinical Management of Nd-YAG LASER Combined with Endodontic Treatment in Localized Severe Periodontitis Patient: Case Evaluation, Journal of International Dental and Medical Research (Special Issue, U.I. 1st International Workshop on Dental Research), 2016; 9:392-397.
16. Kashmoola M.A., Mustafa N.S, Hayati, A.F.K, Idzhar M.I, A Pilot Study on The Use of Low Level Laser Therapy in Treatment of Temporare mandibular Disorder Journal of International Dental and Medical Research, 2018; 11 (2):669-675.
17. Bunjaku V, Popovska M., Grcev A, Mrasori S., Kameri A., Sllamniku Z., Dragidella F., Non-surgical Periodontal Treatment and Low Level Laser Therapy (LLLT) Outcomes for Patients Suffering from Type 2 Diabetes Mellitus, Obesity and Chronic Periodontitis, Journal of International Dental and Medical Research, 2017; 10(2):214-221.
18. Ismaili B. and Bokonjic D, Short Term Low Level Laser Therapy Attenuates Inflamation and Production of Interleukin-1, but Elevates The Level of Matrix Metalloproteinase 9 in Chronic Periodontitis, Journal of International Dental and Medical Research, 2014, 7(1), 7-13.
19. Maclean M., Macgregor S.J., Anderson J.G., Woolsey G.A., High Intensity Narrow spectrum Light inactivation and Wavelength Sensitivity of Staphylococcus aureus, research letter, Journal of Photochemistry and Photobiology B: Biology, Elsevier, 2008; 285:227-232.
20. Lipovsky A., Nitzan Y., Gedanken A., and Lubart R., Visible Light-Induced Killing of Bacteria as a Function of Wavelength: Implication for Wound Healing, Lasers in Surgery and Medicine. 2010; 42:467–472.
21. Plaetzer K., Krammer B., Berlanda J., Berr F., Photophysics and Photochemistry of Photodynamic Therapy: Fundamental Aspects, Journal of Laser Medical Sciences, 2009; 24:259-268.
22. Demidova, T.N., Hamblin, M.R., Effect of cell-Photosensitizer Binding cell Density on Microbial Photoinactivation, Antimicrobial Agents and Chemotherapy, 2005; 49(6):2329-2335.
23. Rolim J. P.M.L., de-Melo M. A.S., Guedes S. F., Albuquerque-Filho F. B., J. R. de Souza, Nogueir N. A.P., Zanin I. C.J., Rodrigues L. K.A., The antimicrobial activity of photodynamic therapy against Streptococcus mutans using different photosensitizers, Journal of Photochemistry and Photobiology B: Biology, 2012; 106: 40–46.
24. Tavares A., Carvalho C. M. B., Faustino M. A., Neves M. G. P. M. S., João P. C. Tomé, Tomé A. C., Cavaleiro J. A. S.,Cunha Â., Gomes N. C. M., Alves E. and Almeida A., Antimicrobial Photodynamic Therapy: Study of Bacterial Recovery Viability and Potential Development of Resistance after Treatment,Marine Drug; 2010; 8(1):91-105.
25. Guffey J.S., Wilborn J., 2006, In Vitro Bactericidal Effect of 405 nm and 470 nm Blue Light, Photomedicine. Laser Surgery, 2006; 24(6):684-688.
26. Imamura, T., Tatehara S., Takebe, Y., Tokuyama, R., Ohshima, T., Maeda, N., Satomura, K, Antibacterial and Antifungal Effect of 405nm Monochromatic Laser on Endodontopathogenic Microorganisms, International Journal of Photoenergy Volume 2014; Article ID 387215:1-7.
27. Quimby, R. S. 2006. Photonics and Lasers: An Introduction. John Wiley & Sons,Inc. New Jersey.
28. Sun H., 2012, Laser Diode Beam Basics, Manipulations and Characterizations: Springer Briefs in Physics. 2012; ISBN 978-94-007-4663-3.
29. Chan Y, Lai CH. Bactericidal Effects of Different Laser Wavelengths on Periodontopathic Germs in Photodynamic Therapy Lasers Med Sci, 2003; 18:51–55.