ORIGINAL ARTICLE

Combination of Blue Laser Exposure with UV-LED to Improve Antimicrobial Effects on *Staphylococcus aureus* Biofilm

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ABSTRACT

Introduction: *Staphylococcus aureus* biofilm is a major mediator of infection. The light based therapy is still not adequate to eradicate biofilm caused by Extracellular Polymeric Substances (EPS). The Ultra Violet (UV) irradiation has high energy and sufficient to penetrate a tissue. This study aims to investigate the effect of combination of UV LED irradiation and blue laser on the biofilm. **Methods:** The pure culture of *Staphylococcus aureus* ATCC 25923 approximated 108 CFU/mL or 1.0 McFarland Standard was used for this study. The biofilm sample was placed onto micro plate for 48 hours. The treatment group was divided into 3 groups, which were blue laser group, UV LED group and UV-Blue laser group. **Results:** The results showed that the highest biofilm reduction (80.57 ± 0.77) % was treated by blue laser irradiation for 4 minutes and UV irradiation for 20 s. **Conclusion:** Thus, the combination UV LED and Blue laser is the best choice to eradicate more biofilm.

Keywords: Blue laser, UV LED, *Staphylococcus aureus*, biofilm, photodynamic inactivation

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INTRODUCTION

The infectious disease caused by biofilm like *Staphylococcus aureus* biofilm is a global problem on the medical field (1). The impact of this biofilm could lead to higher mortality and morbidity (2). Biofilms are collections of microorganisms that attach to a surface and are covered with extracellular matrices produced by these microorganisms from the environment. A biofilm is an ideal place for plasmid exchange, where plasmids can carry genes that regulate resistance to antibiotics so that biofilms play a role in the spread of bacterial resistance to antibiotics. This allegedly due to changes and rearrangement of cell walls so that antibiotics do not easily penetrate it. The conventional therapy like antibiotic drug has failed to eradicate and develop them to be resistant (3-5). Many methods have been developed to control the growth of biofilm, and one of them is the light-based therapy (6). This employs molecules which are able to absorb light with a certain wavelength. The light energy absorption would produce some free radicals which destroy the targeted cells (7). To obtain a high reduction, this method needs a certain dose of energy light. This was caused by matrix layer which enclose the biofilm.

The matrix layer of biofilm consists of high polymeric material which is mostly a product from organisms themselves – known as Extracellular Polymeric Substances (EPS) (8, 9). The EPS provide an excellent adaptability, protective barrier and functional enzyme activity (10). In fact, EPS are able to reduce the production of Reactive Oxygen Species (ROS) from physical and chemical treatments. Therefore, it needs the other methods that are able to destroy the EPS layer. Some bacteria naturally produce endogenous porphyrins as photosensitivity molecules which are light-sensitive (10). The ability of porphyrin to absorb light is specific. The previous research showing that it has 2 specific intensities, i.e. Soret Band located at wavelength of 230-400 nm and Q Band located at wavelength 272 nm, 631 nm, 720 nm, and 930 nm. The result show UV (399.79 nm) were capable of activating other molecules than porphyrin and riboflavin so that the low energy density was able to produce a high biofilm reduction (11-12). The UV light have high energy and easy to obtain in the sun light. The UV light is divided in three part

wavelengths, which are UVA (230 – 400 nm), UVB (290 – 320 nm) and UVC (290 – 100 nm) (13). In addition, the UVA region which have good penetration to the dermis than UVB or UVC. This allowed UVA to damage the outer layer (EPS) in the biofilm to obtain a high biofilm reduction.

Many researches has been developed in the sterilization and medical field using UVA and UVB (14-15). The absorption of light by photosensitizer is related to the wavelength of light source and the photosensitizer absorption spectrum. The blue laser spectrum is in the porphyrin absorption spectrum of *Staphylococcus aureus* bacteria. Some literatures suggest that visible light, particularly blue light in the 400-470 nm spectrum can cause photoinactivation in some bacteria. Imamura's research (2014) to photodynamic therapy of C. albicans bacteria with 90% bacterial death percentage (16). Various studies on the success of photoinactivation in microbes in vitro were also performed by using laser diode 405nm power 0.2W with 1200s exposure time (17). To avoid the adverse effects of UV, the energy density are still unclear for the part of medical because UV leads to damage the skin. Moreover, some studies have shown that biofilms were still capable of reducing the effects of UV (18-19). This was caused by katA and katB enzyme on EPS (20-21). Hamamoto et al reported that high energy UVA allowed to be used as a disinfectant (22).

MATERIALS AND METHODS

Biofilm Development Assay

The pure culture of *Staphylococcus aureus* ATCC 25923 approximated 108 CFU/mL or 1.0 McFarland Standard was used for this study. 100µL bacteria culture was placed in 96-well microplate and was added 20 µL 20% sucrose solutions. The samples were placed to a shaker for 4 hours until appeared suspense. The samples were incubated for 44 hours on 39°C in the incubator.

The samples were treated accordance with each groups. The samples were treated with a crystal violet assay for measuring the survival biofilm. The samples were rinsed by PBS with pH 7.4 three times. The samples were added 100 µL 1% crystal violet solutions and rinsed by saline water. The samples were added 50µL 33% GAA solution and measured using micro plate reader 595 nm.

Light Source

The two light sources, Blue laser and UV LED, respectively, were used to this study. The light source setting was controlled by the CNC module set (23). The output power of the blue laser and the UV LED were measured with PM100D Powermeter (Thorlab). The wavelengths for blue lasers and UV LED were measured with a JASCO CT10 monochromator. The spot diameter on the microplates in this experiment is the same as the diameter of the LED beam (with a focusing Lens). Laser light exposure in various wells is spaced so that it does not have an effect on the samples in the next well. The spectral output of the UV-LED is polychromatic lambertian so that the exposure distance is made as close as possible to the sample.

Sample Treatments

The treatment group was divided into UV LED exposure group, blue laser diode irradiation group and UV-Blue irradiation group. The UV-Blue group treatment was performed with UV LED irradiation then continued blue laser irradiation. The irradiation of both light sources was directly alternated. The UV LED irradiation was performed with time variations of 10 s and 20 s. The UV variations were performed with 2 variations due to the UV irradiation limits on the target cells. The blue diode laser irradiation was treated with variation of time irradiation of 60 s, 120 s, 180 s, and 240 s.

The results of the data were the percentage of ratio of the dead deaths and the survival biofilm or the biofilm reduction (% $CFU.ml^{-1}$). The data measured in OD was converted to log CFU/ mL by using Mc. Farland standard diagram. The biofilm reduction was measured by using equation 1 [11].

$$\% \text{ Biofilm reduction} = \frac{(\sum_{control} - \sum_{treatment})}{(\sum_{control})} \times 100\% \qquad (1)$$

For each treatment, the biofilm reduction percentage was already calculated based on the control group which was the untreated biofilm of *S. aureus*.

The data would be tested statistically using ANOVA one-way test. The significant value p = 0.05 was used as a determinant of statistical conclusion results. The response of biofilm reduction used Statistical test uses factorial ANOVA test. The number of samples is 4 for each treatment. The p value of the statistical test results is 0.00 (<0.05) which means that there are significant differences in the results of treatments.

RESULTS

Spectral Output of Light Sources

The output light source was important for determining the effectiveness of the treatment. Fig. 1 was the output power of light sources, which were UV LED and blue laser, respectively. The output power of the blue laser and the UV LED were 34.35 ± 0.004 mW and 7.71 ± 0.005 mW, respectively. The blue laser had an exponential response of the output power and had steady state on 600 s. So, the treatment time was based on this result, the laser irradiation was started on sample after 600 s. This specific amount of time would be used for the treatment. The output power of UV LED had steady response on an earlier time irradiation. The output power was used to determine the dose of light energy. The spectral output test aimed to obtain the diameter of spectral output

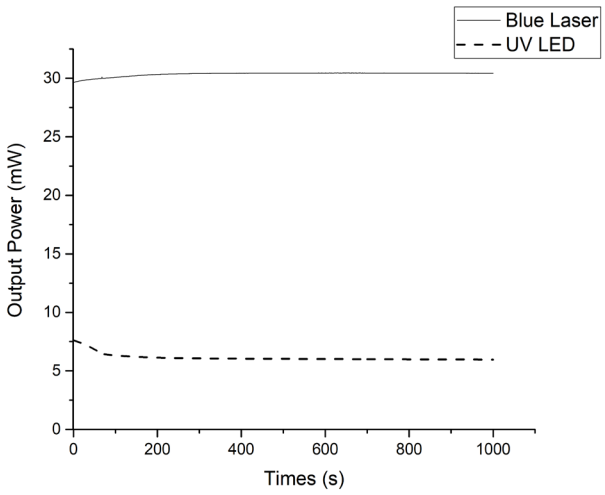

Figure 1: The Output Power of the Light Sources; UV LED and Blue Laser. The blue laser was 34.35 ± 0.004 mW and the UV LED was 7.71 ± 0.005 mW. It was measured with PM100D Powermeter (Thorlab); ----- was UV LED and — was Blue Laser

that is suitable with the bacteria in the microplate. The microplate that was used for bacterial treatment has diameter of 5 mm. The characterization result of the spectral output could be seen in Fig. 2. Based on the fitting result on Fig. 2, the spectral output was (0.1206 ± 0.00871) nm with R = 0.8796. The energy density of light was shown on Table I.

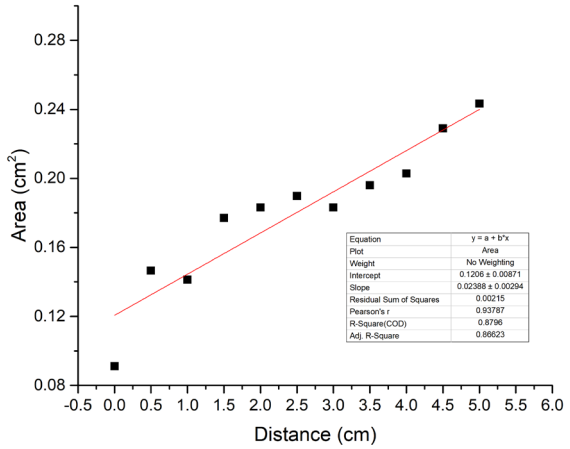

Figure 2: The Spectral Output of UV LED. The spectral output fitting results were (0.1206 ± 0.00871) nm with R 2 = 0.8796; —— was data fitting

The wavelength of light sources was shown on Fig. 3. The peak wavelengths of blue lasers and UV LED were 403.27 nm and 399.79 nm, respectively. The response wavelength of each light source showed similar Full Width at Half Maximum (FWHM) although they had different peak wavelength and power.

Light Treatments

The treatment using UV was performed with two treatment times; 10 s and 20 s to observe the ability of the UV in reducing the biofilm of *Staphylococcus aureus*. The result was shown in Fig. 4.

Table I: The Energy Density for Each Light Sources

Types	Output Power (mW)	Time Irradiation (s)	Area Irradiation (cm^{-2})	Energy Density ($J.cm^{-2}$)
UV LED	7.71	10	0.19	0.41
		20		0.81
Blue Diode Laser	34.35	60	0.036	57.25
		120		114.5
		180		171.75
		240		229

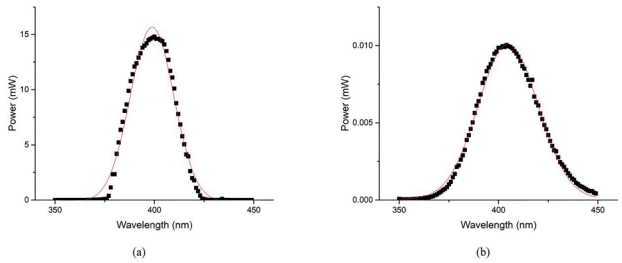

Figure 3: The Wavelength of Light Sources a. Blue Laser b. UV LED. The peak wavelengths of blue lasers and UV LED were 403.27 nm and 399.79 nm, respectively. The response wavelength of each light source showed similar Full Width at Half Maximum (FWHM) although they had different peak wavelength and power.

Based on Fig. 4, it showed that the UV LED could reduce the biofilm for 20 s treatment. The reduction was (74.51±3.40) % for *Staphylococcus aureus*. To explain more about the behavior of UV LED treatment, the treatment was combined with the Blue laser and the result was shown in Fig. 5. The biofilm samples were directly irradiated by the light sources.

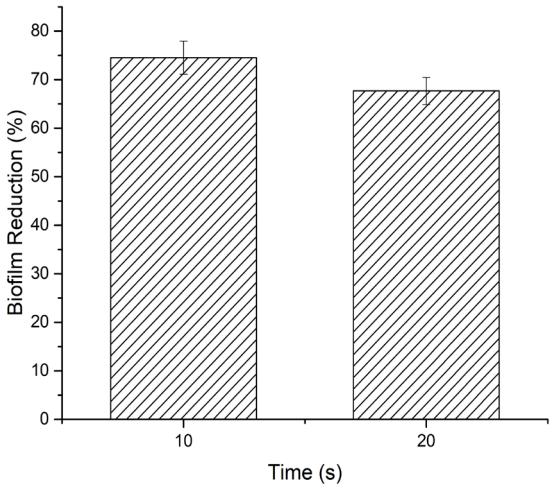

Figure 4: The reduction of Biofilm of *Staphylococcus aureus* due to the treatment using UV LED. The treatment using UV was performed with two treatment times; 10 s and 20 s to observe the ability of the UV in reducing the biofilm of Staphylococcus aureus. It showed that the UV LED 20 s could reduce the biofilm (74.51±3.40) %.

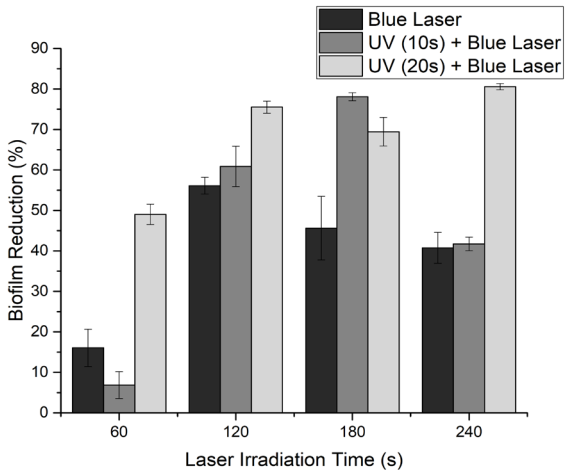

Figure 5: The Biofilm Reduction Due to the Blue Laser and UV-LED. The blue light irradiation for 2 minutes, 3 minutes and 4 minutes indicated no statistically difference in the treatment. The highest biofilm reduction of UVLED - blue laser group was 80.56 ± 0.76 % on combination of 20 s of UV-LED and 5 minutes of blue laser irradiation.

DISCUSSION

The UV irradiation with different time irradiation or dose of light showed a statistically no different response ($p > 0.05$). The UV group showed the response of biofilm reduction of 37.89 ± 11.75 % for 10 s irradiation and 58.07 ± 14.11 % for 20 s irradiation. In the blue laser exposure group, the results of exponential biofilm reduction and the highest reduction at 2 minutes are likely due to this 2 minutes laser at the right energy to kill bacteria, so that at higher exposure times it is in saturated conditions ie many bacteria have died so decreased efficacy.

Statistically, the blue light irradiation for 2 minutes, 3 minutes and 4 minutes indicated no statistically difference in the treatment ($p > 0.05$). However, the highest biofilm reduction was that of 2 minutes or J.cm-2 group. The UV-blue light group implied linier responses for each treatment. Statistically, using different UV time irradiation on combination with UV-blue treatment showed no statistically difference in the treatment ($p > 0.05$) but they were different with blue light group ($p < 0.05$) except 2 minutes of blue light irradiations. The highest biofilm reduction of UVLED - blue laser group was 80.56 ± 0.76 % on combination of 20 s of UV-LED and 5 minutes of blue laser irradiation.

The results of the statistical test (factorial test) of blue laser exposure at various times of exposure show $p = 0.00$ ($p < 0.05$) which indicates that there is at least one pair of different groups. The 4 min group produced the greatest biofilm reduction, differing significantly from the 1 min and 3 min groups, but not significantly different from the 4 min groups.

The exposure of UV A 10 s LEDs has been able to damage the biofilm layer so that the exposure of 20 s becomes smaller because the biofilm layer has been damaged. Likewise, the longer laser exposure shows that there is saturation because many bacteria have died, this is indicated by the ability to reduce smaller.

The selection of light sources especially with specific wavelengths was the first thing to do in the light-based therapy or phototherapy. The wavelength determination was based on the type of molecule that had sensitivity to a certain light source. The light sources in our study had nearly similar wavelength and FWHM. The molecules in the body that could be activated by light in the violet-blue region are porphyrin (Uroporphyrin (89%) and Coproporphyrin III (68.3 – 74.6 %)) Ramberg (24-26). There are other types of molecules could also be activated by these wavelengths, namely riboflavin (335 – 435 nm) (27).

The light effect on the biofilm for the blue laser group showed the reduction forming a Gaussian line. The highest biofilm reduction was obtained in the irradiation treatment for 2 minutes, although 2 min, 3 min and 4 min irradiation treatment were not statistically different. In order to generate ROS, there needs to be adequate energy in the intersystem crossing (ISC) and photochemical process (28). The high energy absorbed by the molecule will be excreted through non-radiation processes, such as fluorescence, phosphorescence, vibration relaxation and internal conversion (29-30).

The UV groups also had the same process but with low energy density that was capable of providing the same biofilm reduction as the blue laser group. It was quite complex because the light source have wavelength and FWHM were in the same region, but in different treatments, they produced similar results. There was a probability that UV (399.79 nm) were capable of activating other molecules than porphyrin and riboflavin so that the low energy density was able to produce a high biofilm reduction. In addition, the UV LED was UVA region which have good penetration to the dermis than UVB or UVC. This allowed UVA to damage the outer layer (EPS) in the biofilm to obtain a high biofilm reduction.

The combination of UV-LED and blue laser afford high reductions (80.56 %) even though the biofilm reduction obtained has no statistical difference with the variation of UV LED and blue laser time irradiation. The UVA damages the biofilm EPS so the bacteria had no protector while blue light irradiation after UV irradiation allows to inactivate bacteria directly. All of that process was depicted in Fig. 6. Overall, the combination UVA and blue light was the best choice than light irradiation standalone (UV treatment or blue laser treatment) for medical application. Some bacteria naturally produce endogenous porphyrins as photosensitivity molecules which are light-sensitive. The ability of porphyrin

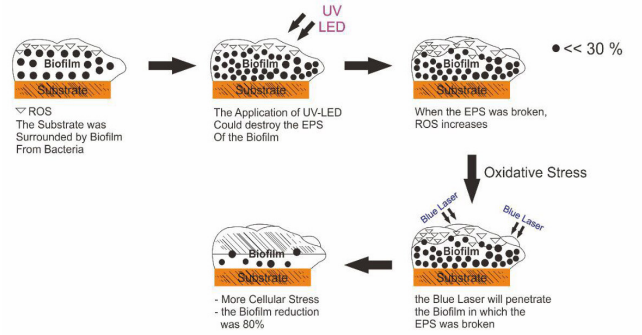

Figure 6: The Mechanism of Biofilm Reduction by Using Blue Laser and UV-LED Light source. The combination of UV-LED and blue laser afford high reductions (80.56 %) even though the biofilm reduction obtained has no statistical difference with the variation of UV LED and blue laser time irradiation. The UVA damages the biofilm EPS so the bacteria had no protector while blue light irradiation after UV irradiation allows to inactivate bacteria directly.

to absorb light is specific (11). The previous research showing that it has 2 specific intensities, i.e. Soret Band located at wavelength of 230-400 nm and Q Band located at wavelength 272 nm, 631 nm, 720 nm, and 930 nm (31). The result show UV (399.79 nm) were capable of activating other molecules than porphyrin and riboflavin so that the low energy density was able to produce a high biofilm reduction. In addition, the UVA region which have good penetration to the dermis than UVB or UVC. This allowed UVA to damage the outer layer (EPS) in the biofilm to obtain a high biofilm reduction.

For future studies, the combination of spectrum and energy density of light sources could be studied with some different photosensitizers to observe the effectiveness of its ability to eradicate the biofilm.

CONCLUSION

Determining the light sources on a therapy for biofilm could be performed by using a violet-blue region light source because they are capable of affording a high biofilm reduction. The combination of 20 seconds UV and 4 minutes blue light provides the highest biofilm reduction which was 80.56%. In conclusion, the use of UV-LED and blue laser had a high performance to reduce the biofilm.

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