Journal of Lasers in Medical Sciences

J Lasers Med Sci 2022;13:e5

Original Article

http://journals.sbmu.ac.ir/jlms

doi 10.34172/jlms.2022.05

Photobiomodulation and Antimicrobial Photodynamic Influence of a 650 nm Wavelength on Staphylocoagulase and Viability of *Staphylococcus aurous*

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Received: December 16, 2020
Accepted: July 28, 2021
Published online February 5, 2022

Abstract

Introduction: *Staphylococcus aureus* is one of the critical pathological bacteria. This bacterium had developed a variety of genetic mutations that made it resistant to drugs and more harmful to humans. In addition, all attempts to design a specific vaccine against *S. aureus* have failed. Therefore, this experiment was designed as a trial for vaccine production, by using a photodynamic treatment (PDT) through partial biological inhibition. The PDT of bacteria mainly focused on reducing the activity of staphylocoagulase (SC), which has a protective feature for bacteria. This study aimed to examine the photodynamic effect of combining a specific wavelength of a laser and a certain dilution photosensitizer, methylene blue (MB) dye. The possible PDT effect on the inhibition of pathogenic enzymatic activity was predicted. This study also aimed to evaluate the inhibitory effect of PDT on the total bacterial account (viability) simultaneously with SC assay.

Methods: A 650nm wavelength diode laser was used with 100 mW output power and 2 minutes of exposure time. Dye dilutions were 50, 100, 150 and 200 µg/mL. The viability of bacteria after and before laser treatment was calculated using single plate-serial dilution spotting methods. The activity of SC was detected by using human plasma for 4 hours incubation of crude-substrate interaction.

Results: The results revealed a significant decrease in enzyme activity and colony-forming units (CFU) after irradiating bacterial suspension with 150 g/mL MB, as well as a decline in CFU. However, irradiation with a laser alone showed a significant increase in SC activity and CFU for the same exposure time.

Conclusion: Besides reducing the production of SC activity, PDT significantly inhibited the viability of *S. aureus*. The application of MB photosensitizer at a concentration of 150 g/mL in combination with a laser wavelength of 650 nm resulted in a complete decrease in the SC activity value as well as the viability of bacteria.

Keywords: Photodynamic Therapy; Staphylocoagulase; Laser; Enzyme activity; Methylene blue.

Introduction

The *Staphylococcus aureus* represents a major causative agent for its exceptional morbidity and mortality rate in human and animal populaces.[1-3] Methicillin-resistant *Staphylococcus aureus* (MRSA) is confused with the anti-toxin opposition marvel.[4] Furthermore, *S. aureus* has a few harmful factors which participate in pathogenicity, such as the coagulase enzyme, which is recognized *S. aureus* from other Staphylococcus species.[5,6] The virulence of staphylocoagulase (SC) is based on supporting bacterial endurance inside phagocytic cells, which is the key to the pathogenic tactic of dodging host immune system reactions.[7] As a result, this enzyme initiates blood coagulation via the prothrombin conformational activation mechanism, in which the N-terminal domain binds with fibrinogen's C-terminal domain and repeats the sequences.[8-11] This activity results in the production of an active proteolytic complex - SC-prothrombin complex - which enhances dividing fibrinogen into fibrin.[12] Here comes the need for new technologies for preventive strategies against vaccination-resistant diseases that can prevent, control, and reduce the risk of the disease process. Different strategies have been used to develop antibodies to forestall *S. aureus* contamination; however, no good outcomes have been acquired.[13] It is known that killed vaccines and live-attenuated vaccines provide protection against varying antigens, but they need more research focus.[3] Thus, antimicrobial photodynamic treatment

Please cite this article as follows: Jadah NA, Shamkhi IA, Shamkhi JA. Photobiomodulation and antimicrobial photodynamic influence of a 650 nm wavelength on staphylocoagulase and viability of *staphylococcus aurous*. *J Lasers Med Sci.* 2022;13:e5. doi:10.34172/jlms.2022.05.

(APDT) can represent a promising modality of *S. aureus* vaccine. APDT has a potential mechanism that can reduce enzyme activity at a certain dose of laser irradiation alone, depending on the production of reactive oxygen species (ROS) from cytological photochromophores of an irradiated cell.[14] Alternatively, a photosensitizer (such as an external photochromophore) can be added to the bacterial solution to act as a catalyst for the production of ROS after laser irradiation. ROS will denature proteins and minerals in the cell membrane and cytoplasm, resulting in cellular apoptosis.[15] In the present study, we aimed to reduce the activity of SC, the most effective weapon of *S. aureus* against the host cells, using APDT on selected pathogenic bacteria (*S. aureus*) without affecting its viability, and evaluating the action of a 650 nm diode laser alone; or with additives of different concentrations of BM as an external photosensitizer to reduce the activity of SC, that may help to develop attenuated Staph-vaccine. i.e. a resulting irradiated bacteria with inhibiting SC activity can be used as attenuated bacteria to construct bacterial vaccine.

Materials and Methods

Laser Irradiation

A diode laser (JD-R303, HUONJE 114 TM/ China) with a 650 nm wavelength and 100 mW power was employed in this experiment, as described in reference 22.[16-22] The following laser parameters were changed to irradiate the bacterial samples, as shown in Table 1.

According to Mahdi and Mohammed,[22] a series of sterile Eppendorf tubes containing 1 mL of 18 hours growth culture (5×10^6 cell/mL) were tightly fastened in a rack tube that was subjected to diode laser exposure to ensure maximum laser distribution evenly in the hole bacterial suspension volume. The setup was designed vertically and was placed in a sterilization hood. Bacteria were prepared for DPT by combining 1ml of the bacterial suspension (18 hours incubation) with 1ml of methylene blue (MB) solution, which was then irradiated and inoculated in 10 mL of Casein Hydrolysate Broth (CHB) (Figure 1).

Table 1. Laser Irradiation Parameters

Laser Parameters	Values
Power density	0.32 W/cm^2
Time of exposure	2 min
Type of laser proliferation	CW

Bacterial Sample

In the present study, *S. aureus* was provided from the central laboratory (Wasit Health Department). These bacterial samples were isolated from infected patients and identified using API test methods. Stock cultures were maintained on Casein Hydrolysate slant agar pH 7.4 (HiMedia) at 4°C and were sub-cultured weekly. Isolates were divided into 55 bacterial groups and labelled according to the type of treatment as follow: five replicates for each treatment: the control group is *S. aureus* without treatment, the IRA group is *S. aureus* with laser irradiation, the 50 PDY group is an *S. aureus* suspension in 50 µg/mL MB and laser irradiation, the 100 PDY group is an *S. aureus* suspension in 100 µg/mL MB and laser irradiation, the 150 PDY group is an *S. aureus* suspension in 150 µg/mL MB and laser irradiation, and the 200 PDY group is an *S. aureus* suspension in 200 µg/mL MB and laser irradiation.

Cultivation Methods

Staphylococcus aureus was cultivated on 20 mL of CHB in100 mL Erlenmeyer flasks at 35°C for 18 hours so that the bacterial growth culture reached the exponential phase, which is optimum for enzyme production. The cultures were shaken in a shaking incubator (LSI-3016R / Labtech Shaking Incubator) at 190 revs. min^{-1}.[23] The bacteria were also cultured in a Casein Hydrolysate Medium (CHM) of pH 7.4 (**HiMedia**) for CFU.

Staphylocoagulase Assay

According to Engels et al,[23], 20 ml of the culture was centrifuged at 12500 g for 2 minutes using a (Beckman

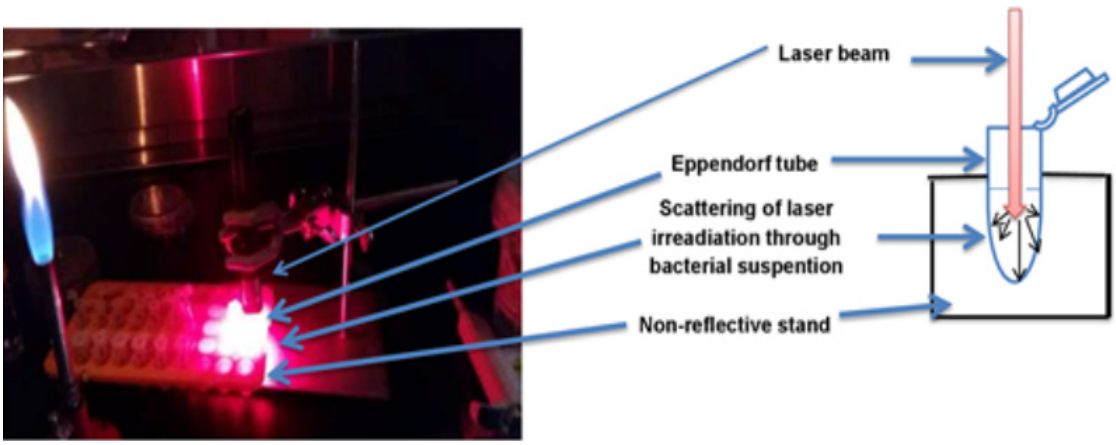

Figure 1. Laser Arrangement for Bacterial Suspension Irradiation.

Coulter/Analytical Ultracentrifuge). Then 0.5 mL of culture supernatant was blended with 0.5 mL of human plasma and this blend was incubated at 35˚C for 4 hours. SC activity was calculated, depending on the clotting time. After SC-plasma reaction, the absorbance at 540 nm was calculated for five replicates of each sample using a UV-VIS spectrophotometer (SP-3000 nano-OPTIMA, Japan). For calculation of the enzyme activity (UmL^{-1}), the following equations were applied:

$$\Delta A = \frac{Absorbance}{time\,of\,incubation\left(minutes\right)} \quad (1)$$

Activity of enzyme in (μ $mol.ml^{-1}.min^{-1}$) or (U/mL) =

$$\frac{\Delta A^{o}}{\varepsilon \times L} \times 10^{6}$$

$\times$ *volume of SC crude in (mL)* (2)

Where ε: extinction coefficient of SC = 10 [24]

L: optical path length

CFU Enumeration

CFU was calculated according to Thomas et al.[25] The single plate-sequential dilution spotting (SP-SDS) approach entails preparing CHM (media) at pH 7.4; dividing 9-cm Petri-dishes into six sectors; and labeling each sector with the bacterial suspension's dilution factor. A stock solution of a growth culture was determined by measuring the optical density at 600 nm utilizing a 1:10 diluted stock in a UV/VIS spectrophotometer (SP-3000 nano-OPTIMA, Japan). A serial dilution of 10^{1}–10^{6} was set up from the 100 stock in 1.5 mL Eppendorf tubes with 3–5 replicates and change the tips. We used sterilized distilled water autoclaved and stored for stock and dilutions preparation. Utilizing an adjusted micropipette, 20 µL of six dilutions were applied as 4–6 miniaturized scale drops in the divided sectors (sample spotting). The inoculated Petri dishes were dried off using the laminar air-flow cabinet and incubated at 37˚C for 18-24 hours. The formula that we used to calculate CFU was (cell/mL)= n × 5× $10^{(d+1)}$, where n = colonies number, and d= dilution level yielding the countable colonies (see Figure 2).

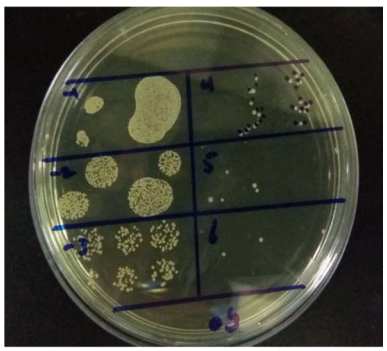

Figure 2. SP-SDS with *S. aureus* Involving 10^{1}–10^{6} Dilutions Showing Acceptable CFU at 10^{4}.

Results

In the present research, we analyzed our results using statistical software, IBM SPSS (version 23.0). A paired samples *t* test was used to analyze the enzyme activity and CFU mean values of five replicates. The comparisons were made before and after DPT with the significance level of 0.05, The dependent variable was made up of two groups: experimental and control Furthermore, we used Excel to present our results in the form of a column chart associated with standard error taps.

In the beginning, the total bacterial numbers were calculated for inoculum and exponential growth culture before and after APDT by using the CFU technique described by Thomas et al.[26] Each experiment was repeated five replicates for each sample. In Figure 3, the mean values of inoculum reveal that the control groups (no treatments), PDY200, PDY100, and PDY50 groups have the lowest values with nearly the same mean value. In contrast, a significant rise in CFU inoculum after irradiation with a 560 nm laser for 2 minutes (IRA group) was seen, and it was immediately inoculated in CHB and broth culture. In addition, a modest rise in the mean values of the total cell number mean values of the PDY15 could be seen.

After 18 hours of shaking incubation, the growth cultures of each group were divided into two parts; the first part was serially diluted for a total bacterial account and then it was re-cultured and incubated for 18-24 hours at 37˚C. The dilution yielding acceptable colonies was selected for CFU enumerations. The results showed a significant decrease in the CFU mean values of IRA groups compared to control groups. At the same time, the mean values of PDY groups produced the lowest bacterial account impact as a result of the highly bacterial dead ratio to inoculum volume after laser-photosensitizer treatment (Figure 4). Generally, there was a noticeable decrease in the viable bacterial number after 18h of the

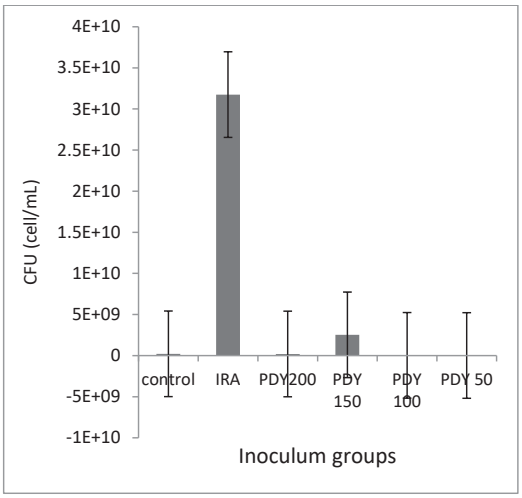

Figure 3. The Mean Values of CFU Inoculum of Control and Irradiated Groups.

incubation period. This is mainly because of the high rate of active nutrition consumption and oxygen content during shaking incubation conditions, shortening the exponential phase compared to the stationary growth phase.

The second part of the growth culture was harvested to separate and extract enzyme crude and then it was followed by SC activity determination steps using a spectrophotometer at 540 nm. The data were analyzed by comparing means using the paired samples *t* test table, which showed a high significance rise and decline in SC activity at IRA and PDY 150 compared to the control respectively (Figure 5). The lowest SC activity mean values were recorded in the 150 PDY group.

Discussion

The goal of this study was to compare the bactericidal effects of laser irradiation alone with laser irradiation combined with the presence of a photosensitizer PS on S. aureus bacteria. There is no consensus about the bactericidal influence of laser treatment. The groups of bacteria were irradiated with a 650 nm diode laser for the same exposure time to clarify the effect of the laser on bacterial growth with respect to the presence or absence of MB.

Our findings approved that irradiation of *S. aureus* with a low power diode laser (650 nm) alone results in a high rise in total cell numbers and enzyme activity after 2 minutes of exposure time. This can be noticed in the IRA groups in Figures 3 and 5, which showed a photo-biostimulation influence response (that appears in the form of increasing in SC activity and bacterial viability values) to 650nm on the inoculum after few minutes of laser irradiation treatment as well as a significant increase in SC activity (*P* value (2-tailed): 0.003 <0.05) that included increases in the cell proliferation rate and biomass of bacteria after overnight culturing. These results are inconsistent with the findings of Chung et al.[26]

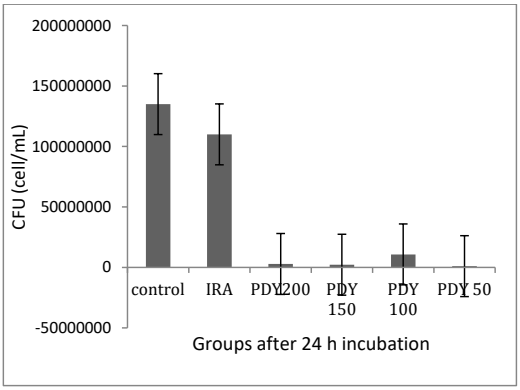

Figure 4. The Mean Values of CFU for Control and Irradiated Groups After 18 hours Incubation.

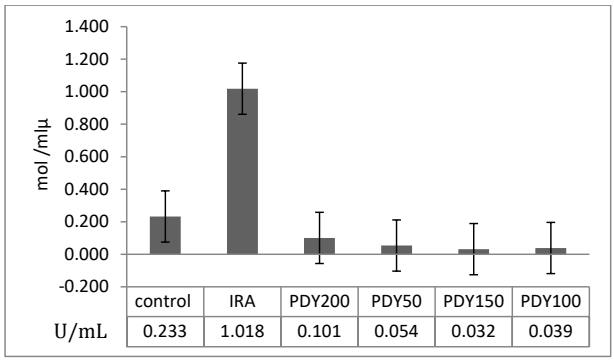

Figure 5. Mean Values of SC Activity as a Function of Non-irradiated and Irradiated Groups.

Three different bacteria species, namely *S. aureus, E. coli, and P. aeruginosa*, were irradiated with nine different frequencies wavelengths of laser beams for 15 minutes, according to Chung et al study. They discovered that none of the laser frequency wavelengths created a significant difference in growth values on any of the three bacterial microscopic models. Along these lines, the importance of their investigation underpins the likelihood that low-level lasers are not adequately restraining or improving the development of microscopic organisms when lighted with the predefined boundaries. We think that exposure of microorganisms to low-level laser treatment (LLLT) for an extended period, similar to the case of Chung's study, leads to a thermal effect instead of photo-biostimulation, and thermal accumulation actively limits the ionization of chromophore in intracellular biomolecules.[27,28]

Another study by Andraus et al revealed that LLLT did not have any bactericidal effect with the light of 660 nm or 808 nm and any hindrance to growth in the illuminated region of plates at different irradiation times (2.15 minutes, 1.7 minutes and 40 seconds).[29] We hypothesize that the form of bacterial biofilm prepared for irradiation represents a critical factor in observing the action of LLLT on bacterial growth and its intracellular biomolecules activities. This idea was based on Chung and Andrau's study, in which they employed an illuminated chamber to irradiate bacterial culture on a medium plate, which provides highly accumulated populations of bacteria and prevents total absorption of the laser wavelength despite using different wavelengths, powers, intensities, and irradiation time. The results of the present experiment contradict the results of Chung and Andraus as we prepared liquid cell suspension to be irradiated and inoculated for subculturing.

Figure 4 shows a slight drop in the CFU mean values of the IRA groups after 24 hours incubation compared to the control group despite a significant increase in the SC activity of the same group (Figure 5). This occurs as a result of acceleration in the cell division rate, nutrition consumption, and shifting in the lag phase after the photo-biostimulation effect. These findings match the results of Jadah et al.[30,31]

APDT has adequacy against a wide spectrum of gram-

positive and gram-negative bacteria and other types of microorganisms. It has a great advantage of the multi-target mechanism,[32-39] autonomously their protection from customary antimicrobial treatment.[40-42] This method requires a close photosensitizer (PS), light, and oxygen. The PS when energized by laser light within the sight of O_2 produces ROS, which are superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) created by type I systems, and singlet oxygen ($^1O_2$).[43-46] Because the latter two are significantly less sensitive and can be detoxified by endogenous antioxidants (both enzymatic and non-enzymatic) incited by oxidative pressure, the effects of O2•- and H2O2 are less severe than those of •OH and $^1O_2$. Examples of that antioxidant system are catalase, superoxide dismutase, peroxidase, regulatory genes RpoE, RpoHII, and RpoHI. Conversely, no enzyme can detoxify $^{\bullet}OH$ or $^1O_2$, making them cytotoxic and intensely deadly.[46] As indicated above, we can use photodynamic conventions where the measure of PS and the presentation time are required to reduce the virulence of *S. aureus*, without affecting total cell viability. As a result, the APDT procedure was used in this trail experiment to combat SC without impacting the total viable count of bacteria.

To provide an optimal cultivation condition for maximum production of the virulence enzyme (SC) from municipal *S. aureus*, which occurred only in the exponential growth phase, we utilized CHB as an optimal production medium and suitable shaking incubation parameters (190 revs. min^1, 35°C for 18 hours).[23] Photosensitizing of MB concentration at 150 µg/mL resulted in a highly significant inhibition influence on CS activity, compared with other groups (200 PDY, 50 PDY, and 100 PDY) which were scaled by CS activity values. It is possible that the photodynamic impact effect is more effective at 150 g/mL MB, the ideal concentration of MB dye for greatest penetration to the inner bacterial structure, resulting in the formation of ROS both inside and outside the bacterial cell's plasma membrane. This can be explained and improved by sudden increases in CFU mean vales in the PDY150 groups in Figure 2. Another APDT effect was the distortion of RNA-related organelles that will be transferred to successive generations; therefore, after 18 hours of cell proliferation, significant inhibition of CS activity was seen (Figure 4) in the 150 PDY group, which means either PDT caused inherited distortion in cytoplasmic organelles responsible for low production of SC or there was a shift in the lag phase of bacterial growth which led to the lowest secretion of SC. For both hypotheses, we produced attenuated bacteria with low potential of CS as a virulence factor. Such findings supported by morphological studies done by Bertoloni et al revealed that the irradiation of the eukaryotic cell with the He-Ne laser at 632 nm increased the packing of the cytoplasmic matrix and number of ribosomes and decreased until almost complete disappearance. Electrophoretic changes improve protein bands to form the cytoplasmic membrane.[46] However, serial studies associated with immune response and another virulence factor of *S. aureus* are needed.

Conclusion

The most effective ADPT parameters which reduced CS activity were the diode laser at a 650 nm wavelength, 100 mW power, 2 minutes irradiation time, liquid bacterial suspension to be exposed directly to laser irradiation, and MB concentration of 150 µg/mL. These conditions cause inhibition in SC activity without affecting bacterial viability after 24 hours of incubation. LLLT can also cause inhibition effects on bacterial cells and their intracellular virulence factors in case of irradiation of liquid bacterial suspension for a long exposure time.

Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Considerations

This article does not contain any studies with human participants or animals performed by any of the authors.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Supplementary files

Supplementary file 1 contains t test analyses.

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