Evaluation of Protoporphyrine IX-Light Emitting Diode Based Antimicrobial Photodynamic Therapy against Staphylococcus Aureus

Fathi Awad, C. Ramprasath, N. Mathivanan, P. Aruna, and S. Ganesan

***Abstract*—The antimicrobial photodynamic activity of protoporphyrine IX (PPIX) in conjunction with red light - emitting diode (LED) and xenon lamp was carried out against *Staphylococcus aureus (S.aureus)*. At 30min illumination using the red LED and 30μM of PPIX 99.8% reduction of *S.aureus* survival was achieved. Based on optofluorometric study, it is further observed that the measurement of steady-state native fluorescence of amino acid, tryptophan provided a method to monitor the Antimicrobial photodynamic therapy (APDT) efficiency by optical means.**

***Index Terms*—Antimicrobial photodynamic therapy, red LED, protoporphyrine IX.**

I. Introduction

Staphylococcus aureus is one of the human pathogens which causes a wide range of diseases such as wound infections, syptic arthritis, osteomyelitis and endocarditis [1,2]. Methicillin-resistant *S.aureus* (MRSA) strains are the most dangerous strain, which are able to cause and develop infections very efficiently and they are resistant to all types of β-lactam antibiotics and other antimicrobials [3]. Further, MRSA infections may be life threatening to immunodeficiency patients such as diabetics' patients.

In this context, photodynamic therapy is used against a wide range of bacteria, viruses and fungi which are extremely harmful [4]. Photodynamic therapy uses light activated chemicals referred as photosensitizer (PS) and visible light of appropriate wavelength. The activation of the PS leads to the generation of Reactive Oxygen Species (ROS) results in cytotoxic effect for the cells mainly in the cytoplasmatic membrane and in DNA [5]. It is reported that many microorganisms are found to be more susceptible to PDT when compared to mammalian cells [6].

As most of the photosensitizers absorb a band of light in the uv-vis regions, even any broad band sources with appropriate filter can be considered as irradiation source for PDT. Recently, Light-emitting diode (LED) has been considered as an alternative novel light source, as they are available in the market in compact size, low price and different wavelengths. Several studies contributed to antimicrobial photodynamic therapy (APDT) development by studying the red LED as a light source for activating different photosensitizers. Peloi et al. found that the exposure of MB to red LED induced more than 93.05% of growth inhibition of *S.aureus*. Furthermore, Umeda et al. found that high power red LED with a low concentration of either methylene blue (MB) or toluidine blue (TBO) showed effective bactericidal effect against two periodontopathic bacteria. However, there is no published study concerning the use of red LED combined to protoporphyrine (PPIX) in APDT against *S.aureus*.

The aim of this study is to evaluate the efficiency of the red light-emitting diode as a light source for photoactivation of protoporphyrine IX in the antimicrobial photodynamic therapy against *S.aureus*. The effect from the red LED was compared to xenon light source. In addition to that, optical technique such as steady state fluorescence spectroscopy was used to characterize the molecular changes in bacteria after APDT.

II. Materials and Methods

A. Bacterial Strain and Growth Conditions

S.aureus (MRSA) was obtained from Centre for Advanced Studies in Botany, University of Madras. The bacteria were kept in tryptic casein agar in stock culture plates till it's used for the experiment. Before the experiments, the microorganisms were grown in Mullen–Hinton broth (MHB)(Himedia,India) at 37℃ for 16 h .They are then harvested by centrifugation and washed three times by normal saline (NS). Subsequently the bacteria were diluted in 0.9% NS at an optical density of 0.05 at 600nm which correspond to approximately $10^7 – 10^8$ colony-forming units (cfu)/mL.

B. Photosensitizer and Light Sources

Protoporphyrine IX (PPIX) was purchased from Sigma Aldrich, India and diluted to a concentration of 30μM in NS. The absorption spectrum of PPIX (figure.1.) was measured using UV/VIS absorption spectrometer (Lambda 35, Perkin Elmer, USA). Two different light sources were used in this study. The red LED (CREE, Germany) which emits red wavelengths (600–700 nm) and Xenon lamp (Mini-Crimescope-MCS400, ISA Jobin Yvon-Spex, Edison,NJ) with a filter wheel and a liquid light guide arrangement. The broad band of the Xenon lamp was filtered using a band pass filter centred at a wavelength of 630nm ± 20nm. The power of the red LED and xenon lamp was found 35 and 20mW respectively.

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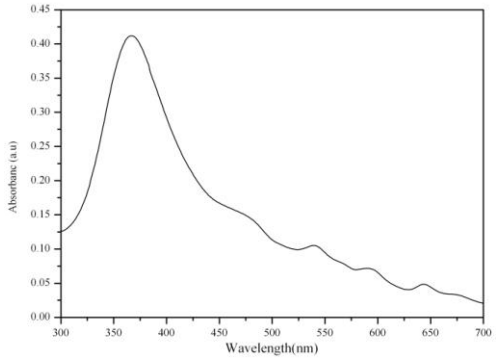

Fig. 1. Absorbance spectrum of PPIX.

C. Lethal Photosensitization

To evaluate the efficiency of the red LED, 100 µL of *S.aureus* was mixed with 50 µL of PPIX in 96 well plates (Tarsons, India) and incubated for 15min. Samples were irradiated in the dark under aseptic conditions in a laminar flow separately with the red LED for 10min, 20min and 30min. One sample was irradiated with Xenon lamp for 10min. Two samples were kept as a control, bacteria alone without PPIX and bacteria mixed with PPIX without light illumination. After treatment, each sample was serially diluted in 0.9%NS solution. The numbers of surviving bacteria (CFU) was determined by viable counting by platting 0.1mL aliquots of each sample in Muller–Hinton Agar and incubated at 37 C° for 48 h.

D. Steady-State Fluorescence Measurements

Steady state fluorescence spectra of tryptophan were recorded using spectrofluorometer (FluoroMax-2, ISA Jobin Yvon-Spex, Edison, NJ*)*. The excitation source (150 W ozone-free xenon arc lamp) coupled to the monochromator delivers light to the sample spot at a desired wavelength, and the fluorescence emission from the samples is collected by another monochromator connected to a photomultiplier tube (R928P,Hamamatzu,Shizuoka-Ken,Japan).The gratings in the excitation and emission monochromators have a groove density of 1200 grooves/mm. During the fluorescence data acquisition, the excitation and the emission slit widths are set at 5nm with an integration time of 0.1 s. The collected signal is transferred to the PC through an RS232 interface. The data were processed by the Windows-based data acquisition program Data Max powered by GRAMS/386t. Two hundred microliter of bacteria was added to one hundred microlitre of PPIX in 96 well plate and incubated for 15min. Samples were irradiated in the dark with the red LED for 10min, 20min and 30min. One sample was irradiated with the Xenon lamp for 10min. One sample was kept without PPIX and light illumination and used as a control. Appropriate volume of NS was added to all samples to bring the total volume of each sample in the quvette to 1.5mL. Samples were excited at 280 nm and emission was collected in the range of 300 nm to 540nm.

III. Results

A. Photodynamic Therapy

Results of *S.aureus* survival reduction after PDT using Xenon lamp for 10min and red LED for 10min, 20min and 30min is presented in figure 2. Protoporphyrine IX produced slight dark toxicity when incubated with bacteria for 15min. It can be observed that when PPIX illuminated with the red LED for 10min, the reduction of survival of bacteria was 99.33% which is higher when compared to the survival reduction when Xenon lamp was used (85%). When the LED illumination time was increased up to 20min, more survival reduction (99.6%) of *S.aureus* was achieved. However, compared to the effect from Xenon lamp and LED for 10min and 20min illumination, the maximum bacteria killing (99.8%) was obtained when the PPIX was irradiated for 30min with the red LED.

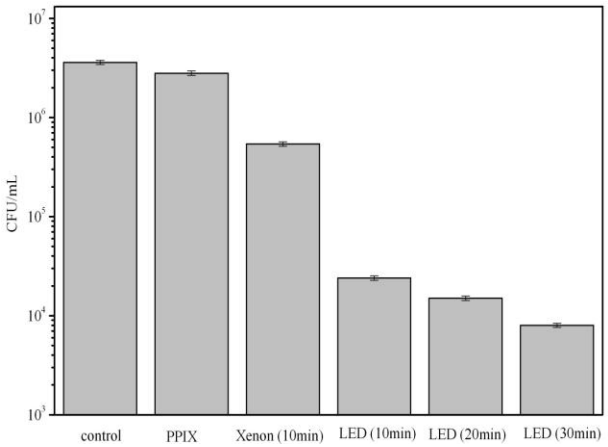

Fig. 2. Effect of PPIX without irradiation and PDT using Xenon lamp and red LED in the reduction of *S.aureus*

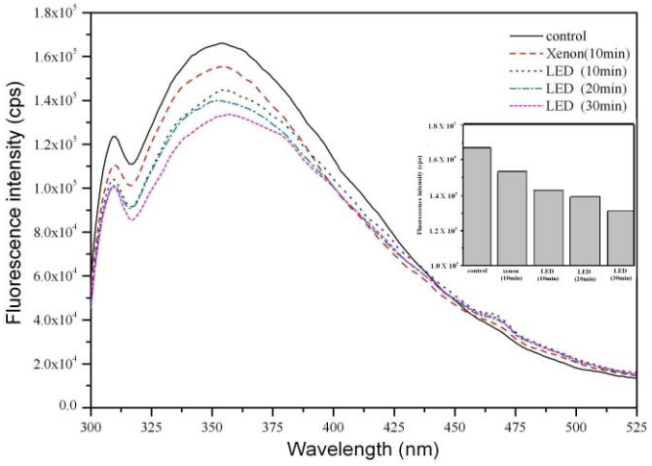

Fig. 3. Steady-state fluorescence spectra of tryptophan and reduction of intensity from *S.aureus* after different treatments

B. Steady- State Fluorescence

Steady- state fluorescence emission spectra of tryptophan from *S.aureus* (maximum emission at 350nm) and reduction of fluorescence intensity are shown in Figure.3. The beak shown at about 310nm is for water Raman. The effect from PPIX irradiation with Xenon lamp for 10min was only 8.1% reduction of tryptophan fluorescence intensity. However, Fluorescence of tryptophan was decreased 14.31% when the bacteria was added with PPIX and illuminated with the red LED for 10min. When the LED irradiation time was increased up to 20min more reduction of fluorescence intensity (16.6%) was observed. The largest reduction of tryptophan fluorescence intensity (21.40%) from *S.aureus* was obtained when the PPIX was irradiated with the red LED for 30min.

IV. Discussions

Although PDT has been considered for various oncological and non oncological applications, still many interested in identifying low cost light sources and photosensitizers for PDT and APDT. In this context, it is worth to note that the development of high power LED has been considered for various industrial and medical applications, as it is less expensive and compact light source. In particular, special attention has been given in the field of PDT and APDT. Further, the field of APDT is still under infant stage as there is lots of controversies with regard to synthesis of Aminolevulinic acid (ALA) induced PPIX and PDT efficacy in different bacterial strains. Protoporphyrine IX, which belongs to the porphyrin group is a well- known, clinically approved PS. Previously reported data showed that PPIX has bactericidal activity against wide range of bacterial species, including MRSA [7]. PPIX absorbs light strongly at about 370nm with several weaker absorption bands including one at 635nm. In PDT, it is favoured to use longer wavelength to activate the photosensitizer to achieve more penetration into tissue with reduced scattering and absorption [8]. The successful of PDT can be achieved by the optimization of large number of parameters [9]. Hence, the selection of an effective light source and PS is essential for the success of the PDT. Based on these, in the present study, an attempt was made to study and compare the antimicrobial effect of LED at 600-700nm and xenon lamp filtered at 630nm in conjunction with PPIX against *S.aureus*.

From figure 2.it is observed that the APDT due to LED is more when compared to xenon lamp. Further there is a decrease in the surviving rate of the bacteria with increase in the exposure time of the LED. Our results agrees with the already reported results of Peloi et al. who found that the exposure of 42.2 μM of MB to the red LED for 20min induced more than 93.05% of growth inhibition for *S.aureus*[10]. Further, Umeda et al found that red LED irradiation for 10s combined with methylene blue at concentrations more than 10 μg/mL, completely killed Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans [11]. In our study when 30 μM PPIX exposed to the red LED for 20min produced 99.6% reduction of *S.aureus*.

It is well known that numerous intrinsic fluorophores such as protein tryptophan is retained in Bacteria and giving intense fluorescence when excited at the UV region [12]. In this study fluorescence spectroscopy has also utilized to get additional information on the molecular mechanism of the antibacterial activity due to APDT. To the best of our knowledge; there is only one study which investigated the changes in steady-state fluorescence of tryptophan from *S.aureus* under different bactericidal agents. Alexandra et al. measured the steady-state fluorescence of tryptophan for five species of bacteria (including *S.aureus*), subjected to three bactericidal agents [13]. They found that the addition of hydrogen peroxide (H_2O_2) to *S.aureus* produced 33% decrease in tryptophan steady- state fluorescence intensity. In this study the largest reduction of tryptophan fluorescence intensity was 21.40% from *S.aureus* is obtained when the PPIX was irradiated for 30min with the red LED. The decreases of tryptophan steady state fluorescence intensity from each treatment indicates that the APDT effect decomposing the tryptophan thought photo oxidation , including destruction of the indole ring [13].

In conclusion, LED at 600-700nm with PPIX of 30 μM concentration may be considered to kill *S.aureus*. Further, it is observed that the APDT efficacy depends on the dose rate. Steady state native fluorescence spectroscopic characterization of amino acid, tryptophan may be used to monitor the post APDT effect.

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