Original Article

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Antibacterial photodynamic therapy on *staphylococcus aureus* and *pseudomonas aeruginosa in-vitro*

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ABSTRACT

Photodynamic therapy (PDT) involves the use of drugs or dyes known as photosensitizers, and light source which induces cell death by the production of cytotoxic reactive oxygen species (ROS). This principle of cell death can be utilized to kill bacteria *in vitro.* We propose the use of blue light emitting diodes (LEDs) and Riboflavin as the light source and photosensitizer for *in vitro* killing of *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Circularly arranged 65-blue LED array was designed as the light source to fit exactly over 7cm culture plate. Riboflavin having non-toxic properties and nucleic acid specificity was used as a photosensitizer. Clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were used in our study. Effect of PDT on viability on these species of bacteria was compared with control samples that included: control untreated, control treated with light only and control treated with riboflavin only. Statistical analysis was done using one-way ANOVA test. PDT against *Pseudomonas aeruginosa* and *Staphylococcus aureus* was significantly ($p<0.05$) effective compared to all control samples. Combination of blue LEDs and Riboflavin in PDT against these bacterial species has been successfully demonstrated *in-vitro*. Therefore, PDT has promising applications in the process of treating superficial wound infections.

Keywords: Photodynamic therapy, photosensitizers, reactive oxygen species *Staphylococcus aureus, Pseudomonas aeruginosa*.

INTRODUCTION

Photodynamic therapy (PDT) is a process which involves bacterial cell death by the use of light energy of appropriate wavelength and photosensitive drugs or dyes. During PDT, a photosensitizer is added to a bacterial sample, which absorbs light energy and causes production of reactive oxygen species (ROS) within or outside microbial cell, depending upon the distribution of PS and penetration of light energy. These ROSs cause bacterial cell death either by oxidative damage to cell membrane or to deoxy-ribonucleic acid (DNA).[1]

Photosensitization of bacteria has shown to be independent of the antibiotic resistance spectrum, non-mutagenic or genotoxic.[2] There appears to be no induction of resistance to PDT even after multiple treatments.[3] Further, microbial selectivity is observed with PDT which can be due to differences in pharmacokinetics of mammalian and bacterial cells.[4] There is potential for the use of PDT in the treatment of dermatological conditions such as infected ulcers, infected burn wounds and skin diseases involving microorganisms. It has the advantage that it may be a cost effective therapeutic option for developing countries as many of the photosensitizer have a minimal cost compared to many of the newer systemic antimicrobials.[5]

For infections of superficial wounds or open surgical wounds, broadband light sources, broad arrays of LEDs or defocused laser beams are considered suitable light source. In our study, blue light emitting diodes are used as a light source, which offers various advantages over other light sources (lasers, UV-lamp) like large output, less thermal destruction, easy fabrication, large area illumination and cost efficiency.[6] Riboflavin, a drug and a food additive, is considered non-toxic and possesses the property of photostimulation. It's been used before as a photosensitizer in combination with UV-A light for decontamination of blood components like platelets, plasma, red cell etc. Its nucleic acid specificity along with its limited tendency towards indiscriminate oxidation makes it a better candidate as photosensitizer for our study.[7-9] Moreover, it can be formulated as a solution to optimize its usefulness for particular application on topical wound infections.

Staphylococcus aureus and *Pseudomonas aeruginosa* have been selected due to their high prevalence in wound infections. These species of bacteria have caused higher rate of morbidity in patients due to the high antibiotic resistance pattern towards the traditional use of antibiotics. Therefore, antimicrobial PDT, which is independent towards the antibiotic resistance pattern of bacteria, could prove beneficial to treat the wound infection.

Our study is focused on *in-vitro* effectiveness of PDT by the use of blue LEDs and riboflavin to kill these bacterial species.

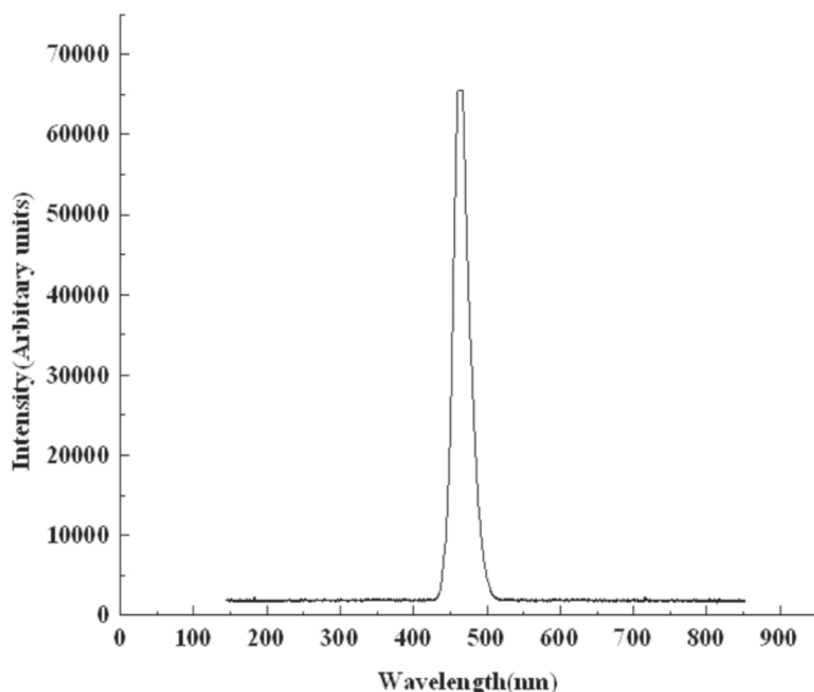

Fig. 1. Emission spectrum of 65-LED array that shows peak spectrum at 470.84nm.The intensity is an arbitary value for the array of blue LEDs

MATERIALS AND METHODS

LED Circuit: Sixty blue LED (RL5-B5515) circular array was used as the light source to uniformly irradiate 7cm culture plate containing bacterial sample. The distance of irradiation was taken 1.5cm.

Bacterial isolates: Twenty clinical isolates of *Staphyloccus aureus* and *Pseudomonas aeruginosa* isolated from different clinical samples were obtained from Nepal Medical College Teaching Hospital (NMCTH) Microbiology Department. These isolates were sub-cultured in Nutrient Agar (NA), Mannitol Salt-base Agar (MSA), and Mackonkey Agar (MA). Confirmatory tests and Gram staining for both of these isolates were performed. Gram positive cocci in clusters observed under miscroscope, yellow fluroscence on MSA and Slide coagulase test (bound coagulase test) positive was used to confirm *Staphylococcus aureus* from other coagulase-negative *staphylococci.* Gram negative bacilli observed under microscope, green fluroscence on NA and MA and Oxidase test positive was used to confirm *Pseudomonas aeruginosa.*

Photosensitizer: Riboflavin 5 phosphate was used as photosensitizer. It was stored in 0- 4^0C in dark for the entire period of experimentation. Spectral analysis of Riboflavin was performed using UV-Visible spectrophotometer (UV-2450 SHIMADZU) to generate absorbance spectrum of Riboflavin at 2mcg concentration.

Antimicrobial Photodynamic therapy on bacteria: The day before performing PDT, pure cultures were inoculated in 100ml Nutrient Broth and incubated (37^0C, 24hrs) to allow complete growth in the liquid growth medium. The overnight incubated broth culture was centrifuged at 3000 rpm for 10 minutes. Supernatant Nutrient Broth was discarded leaving bacterial pellet at the bottom of the centrifuge tube. Phosphate Buffered Saline (PBS) was added to the tube and the bacterial pellet was washed by shaking it gently. The washed pellet was again centrifuged for 10 minutes at 3000rpm. The pellet thus formed was then diluted with PBS. The initial population density of the sample was maintained at 1.5×10^8CFU/ml after comparing with 0.5 McFarland standard solution i.e. Absorbance $_{(630nm)}$ = 0.5 (1.5×10^8CFU/ml). Stock solution of riboflavin i.e.20mg/dl was prepared by dissolving 2mg of riboflavin in 10ml of sterile double distilled water. For the experimentation, three different control samples were prepared which are

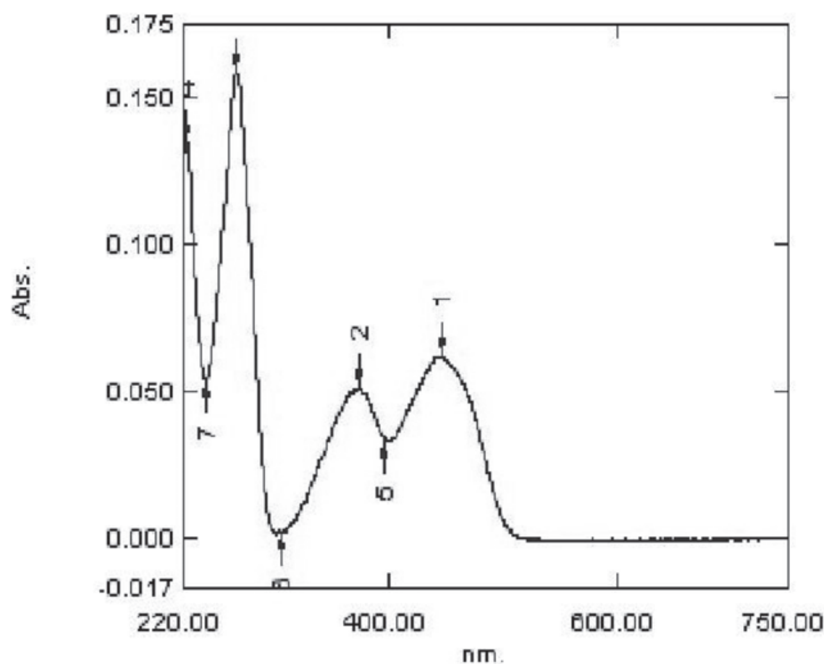

Fig. 2. Absorption spectrum of Riboflavin @ 2mcg.The curve shows peak spectrum at 446.50nm (1), 373.50nm (2), 266.50nm (3), 223nm (4) with absorbance 0.0602, 0.052, 0.159 and 0.135

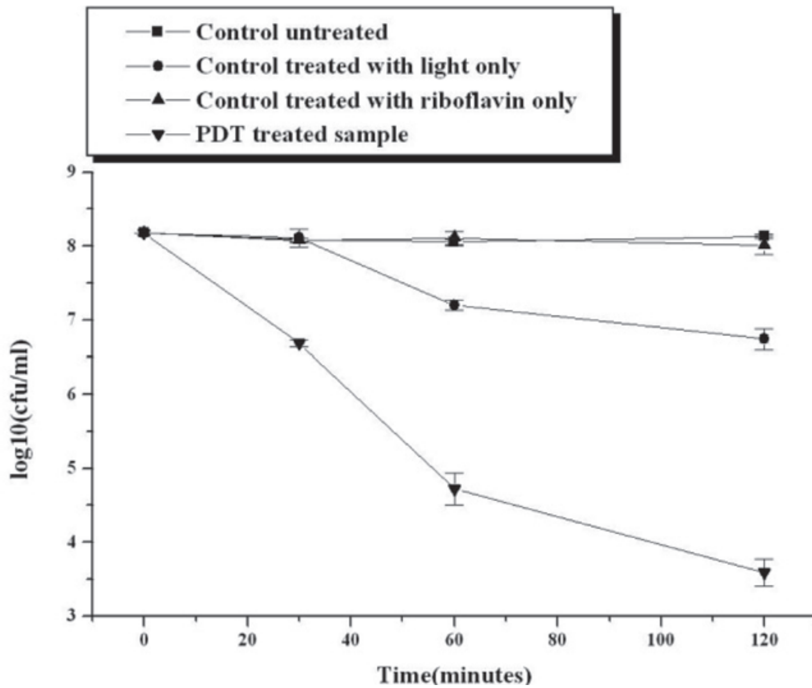

Fig. 3. Result showing PDT on one of the samples of *Staphylococcus aureus.* The experiment was repeated three times and the result shows the mean of data taken at various time intervals. The bar shows the standard deviation from the mean

control untreated, control treated to light only and control treated to riboflavin only.

6ml bacterial sample (with initial population density 1.5×10^8CFU/ml) was aseptically transferred in each of the four 7cm culture plates with properly labeled control samples and PDT treated sample. Stock riboflavin was added in PDT treated sample and riboflavin only treated control sample to make the final concentration of riboflavin of 1mg/dl. Before exposure to light, the samples to be irradiated were incubated in dark for 15 minutes. Two 65 blue led array were used to irradiate PDT treated sample and light only treated sample. These light sources were designed in such a way so as to fit over the 7cm culture plate with a fixed distance of irradiation of 15mm. Irradiation time of 30, 60, 120 minutes were taken for *Staphyloccus aureus* and 45, 90, 180 minutes were taken for *Pseudomonas aeruginosa*. The entire experimentation was carried out in the minimal day light condition and the surface temperature of sample was continuously recorded in order to avoid any significant rise in temperature. After each period of irradiation, serial dilution was performed. The viability count for each sample was done in triplicate plates.

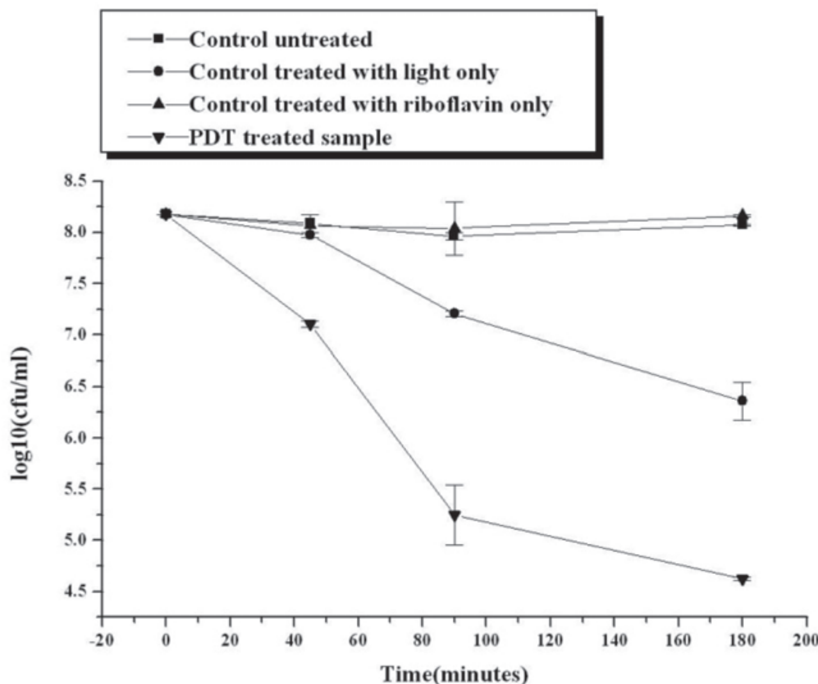

Fig. 4. Result showing PDT on one of the samples of *Pseudomonas aeruginosa.* The experiment was repeated three times and the result shows the mean of data taken at various time intervals. The bar shows the standard deviation from the mean

STATISTICAL ANALYSIS

The results obtained were expressed as means ± standard deviation and were analyzed statistically using one way ANOVA test. Statistical difference were considered significant at $p<0.05$. All the experiments were performed in triplicate and repeated three times. Statistical analysis was performed using 'Origin50' software.

RESULTS

The emission spectrum of the LED array showed the peak spectrum at 470.84nm (Fig 1). Similarly, the spectral analysis of riboflavin showed the peak spectrum at four different regions with major peaks in UV- regions and a peak in the violet (near to blue) region with wavelength 446.50nm (Fig 2).

PDT treated samples in both bacterial species showed significant decrease ($p<0.05$) in the viability when compared to all other control samples. Effect of bacterial killing was more pronounced when time of irradiation was increased in both these bacterial species. Fig. 3 shows the result of experimentation on one of the samples of *staphylococcus aureus*. Experimentation on other nine

samples also showed the significant difference ($p<0.05$) in killing compared to control samples. Fig. 4 shows the experimentation on one of the samples of *Pseudomonas aeruginosa*. Similarly, for rest of the nine samples of *Pseudomonas aeruginosa* a significant difference in killing ($p<0.05$) have also been observed (graph not shown). However, to achieve the same rate of killing, *Pseudomonas aeruginosa* required longer irradiation time than *Staphylococcus aureus.*

DISCUSSION

The present study has successfully demonstrated the efficacy of blue LEDs and riboflavin in the *in-vitro* inactivation of *Staphylococcus aureus* and *Pseudomonas areuginosa*. Various wavelengths of blue light have been successfully used in the inactivation of various range of bacterial pathogens both *in-vivo* and *in-vitro*. 405nm blue led array alone has been successfully used for the *in-vitro* inactivation of wide range of bacterial pathogens. This effect of bacterial inactivation by the use of 405nm blue light can be attributed with the presence of endogenous porphyrins in bacteria.[10] 470nm blue LED alone has also been used to kill methicillin resistant *Staphylococcus aureus in-vitro* [18]. Similarly, PDT using 470 ± 20nm blue led has been found successful in the *in-vivo* inactivation of *Pseudomonas aeruginosa*.[11]

Riboflavin has already been used as photosensitizer in blood decontamination processes as well as in inactivation of bacterial and fungal isolates combined with UV-A light.[12] The cytotoxic effect of riboflavin by light induced photsensitization has been attributed with the production of singlet oxygen, superoxide ions and hydroxyl radicals.[9,13] These cytotoxic reactive oxygen species are primarily involved in cellular death of bacteria, which is either caused due to damage to their DNA or the lysis of their cell wall.

A longer irradiation time was required to kill *Pseudomonas aeruginosa* than *Staphylococcus aureus*. This has been attributed with the complex cell-wall structure of gram-negative species compared to gram-positive ones with lesser photosensitizer and light peneterating the cell-wall structure.[14]

Thus, PDT using blue light emitting diodes and riboflavin has been shown to be effective in killing *Staphyloccus aureus* and *Pseudomonas aeruginosa*. Blue light having soft tissue penetration depth of 2-3mm can be used in treatment of superficial wound infections. Riboflavin can be administered topically on the site of infection and irradiation can be performed for varying time interval to achieve a desired result.

As the use of antibiotics is rendered virtually unsuccessful by the high antibiotic resistance pattern by these bacterial species, a new treatment modality like this can prove to be effective in treating wound infections. A further insight in PDT with *in-vivo* experiment is required to ensure the effectiveness of the antimicrobial PDT.

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