Effectiveness of Antimicrobial Photodynamic Therapy on *Staphylococcus aureus* using Phenothiazinium Dye with Red Laser.

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ABSTRACT

The aim of this study was to evaluate in vitro the bactericidal effect of Antimicrobial Photodynamic Therapy - AmPDT using a phenothiazinium compound (toluidine blue O and methylene blue, 12.5 μg/mL) on *Staphylococcus aureus* (ATCC 23529) irradiated or not with the red laser (λ 660 nm, 12J/cm^2). All tests were performed in triplicate and samples distributed into the following groups: Negative control, Laser, Photosensitizer, and AmPDT. Bactericidal effect of the Antimicrobial Photodynamic Therapy was assessed by counting of colony-forming units and analyzed statistically (ANOVA, Tukey test, $p<0.05$). The results showed, comparing the Laser group with Negative control, a statistically significant increase of counting on the Laser group ($p = 0.003$). The use of the photosensitizer alone reduced the mean number of CFU (64.8%) and its association with the Laser light resulted in 84.2% of inhibition. The results are indicative that the use of Antimicrobial Photodynamic Therapy presented in vitro bactericidal effect on *Staphylococcus aureus.*

Keywords: Photodynamic antimicrobial chemotherapy; *Staphylococcus aureus;* lasers

1. INTRODUCTION

A previous study has shown that methicillin-resistant S. aureus bacteremia is associated with significantly higher mortality rate compared with methicillin-sensitive S. aureus bacteremia[1]. Rates of *Staphylococcus aureus* resistance to methicillin infection is a growing problem: 52.3% of nosocomial infections in patients in the intensive care unit are due to methicillin-resistant S. aureus (MRSA), representing a 37% increase in the incidence of MRSA infections[2].

Bacteria most frequently contaminate chronic wounds, and this contamination normally causes delayed healing and prolonged hospitalization, so innovative and efficacious approaches for combating microbial diseases have been tested[3]. Previous studies support the hypothesis that the use of AmPDT may be a viable alternative to other treatments as its

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effects on microbial cells is markedly different from that observed when using antibiotic. This procedure may be carried out on both sensitive and antibiotic-resistant bacteria causing inactivation of the strains and it does not induce bacterial selection that may cause resistance such as frequently observed during the treatment with antibiotics[4,5].

AmPDT combines the use of a nontoxic photosensitizer associated to a non-ionizing visible light which wavelength has to be effective to excite the photosensitizer to a reactive triplet state. This reaction will generate singlet oxygen and/or superoxide that are highly toxic to the cells[6].

It was hypothesized that the use of an efficacious protocol, in vitro, could be used to treat bacterial infections. Therefore the aim of this study was to evaluate the in vitro bactericidal effect of AmPDT on *Staphylococcus aureus* (ATCC 23529 strain) using 12.5 μg/mL of phenothiazinium dye associated to red laser light using an energy density of 12 J/cm².

2. METHODOLOGY

2.1 Bacterial strain and culture condition

Bacterial strain used in this study was *Staphylococcus aureus* (ATCC 23529 strain) obtained from the Laboratory of Parasite Biology, FIOCRUZ-BA. Cells were aerobically cultured in blood agar (Merck® Darmstadt, Hessen, Germany) at 37°C and grown for 24 hours. For the experiments, colonies were collected with the aid of a calibrated loop of 100 μL and inoculated into 5 mL of tryptic soy broth (Merck®). For the quantification of colony-forming units (CFU), the suspension was standardized by measuring absorbance at ELISA-reader spectrophotometer (Medical Device) to an optical density of 0.5 MacFarland at λ625 nm, corresponding to approximate number 3 x 10^8 CFU. Subsequently, 10 μL of this suspension were inoculated in 1 mL of TSB (Merck®) in a 24-well culture plate (Falcon®, BD Lab., Franklin Lakes, New Jersey, USA). After this dilution, each concentration of the photosensitizer was added and irradiated following experimental protocol.

2.2 Photosensitizer and light source

Toluidine blue O and methylene blue (Fórmula Laboratory, Salvador, BA, Brazil) were used for photosensitization of the *Staphylococcus aureus* strains. The dyes solutions at concentration of 12.5μg/mL were prepared by dissolving in sterile phosphate – buffered saline, pH 7.4 and filtering it through a 0.22 μm membrane (Millipore, São Paulo, SP, Brazil). After filtration, the dye solution was stored in the dark for a maximum of 2 weeks at 4°C before use. A diode laser (λ660

nm, Twin Flex®, MMOptics, São Carlos, SP, Brazil) was used as the light source (Tab.1). The wavelength of the laser corresponded to the maximum absorption of phenothiazinium dyes.[11]

Table 1. Summary of the parameters used on the study

Parameters	LASER
Wavelength (nm)	660
Mode	CW
Spot of the probe (mm²)	4
Power Output (W)	0.04
Exposure Time (s, per session)	300
Energy density (J/cm^2)	12

2.3 Antimicrobial Photodynamic Therapy

Samples were distributed into the following groups: 1. Negative control - untreated by either laser or photosensitizer; 2. Laser group - bacterial suspensions irradiated with laser (12 J/cm^2) in the absence of photosensitizer; 3. Photosensitizer group - bacterial suspensions in the presence of phenothiazinium dye at concentration of 12.5 μg/mL; and 4. Antimicrobial PDT group – bacterial suspensions in the presence of phenothiazinium dye irradiated with laser.

The bacterial suspensions were platted into the 24-well culture plates and the conditions mentioned above and incubated in the dark and at room temperature for 5 minutes. After pre-irradiation time (5 minutes) the bacterial suspensions, with and without photosensitizer, were irradiated according to different energy densities. Immediately after the irradiation, the contents of the wells were mixed before sampling and were seeded in triplicate onto Petri plates divided into four fields containing TSA medium (Merck® Darmstadt, Hessen, Germany) and incubated at 37°C for 24 hours using a calibrated 100 μL loop bacteria. After incubation (24 hours), the number of CFU was determined by counting. Statistical analysis was carried out by One-Way ANOVA and Tukey's Multiple Comparison tests (Graphic Prism Software 4.0) $p < 0.05$ was considered statistically significant.

3. RESULTS

Laser group, the energy density of 12 J/cm², in comparison with the negative control group showed a significant increase in the number of CFU ($p = 0.003$). The photosensitizer group compared to the negative control group showed a statistically significant reduction ($p < 0.001$) in the mean number of CFU. The inhibition percentage was 64.8 using 12.5 mg/ml. The use of the photosensitizer alone reduced the mean number of CFU in 64.8% and its association to the Laser

light increased inhibition to 84.2% (Fig.1). Comparison between AmPDT group and negative control showed a statistically significant reduction of the inhibition ($p < 0.001$) when AmPDT was used.

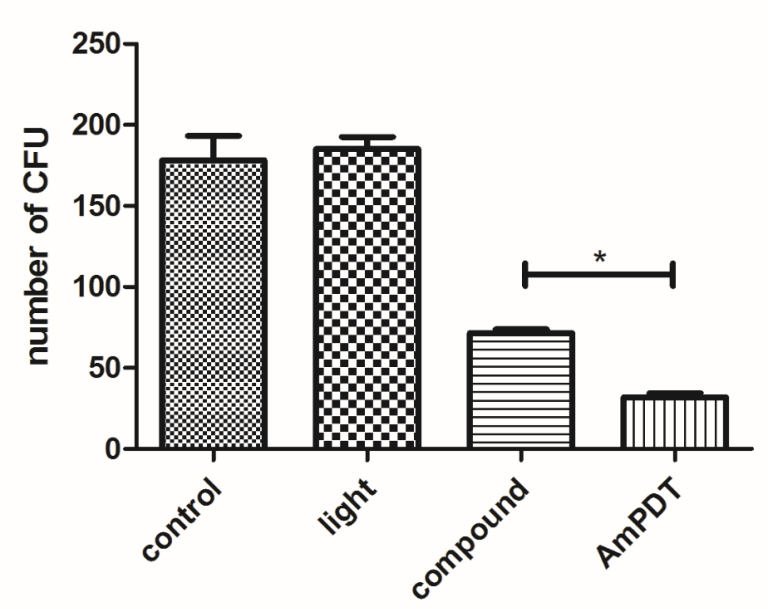

Figure 1: Mean number of CFU obtained for staphylococcus aureus in experimental condition.

4. DISCUSSION

The results of AmPDT may vary according to the cell conditions (density, culture medium, Gram positive or negative bacteria, species, physiological state), photosensitizer (concentration, period of incubation, exposure time) and type and parameters of the light used (energy density, wavelength, power density and other) [7-9]. Despite the present study demonstrated increased proliferation of *S. aureus* ($p = 0.003$) in vitro when using 12 J/cm² of Laser light a previous study[10] also using the red laser, but in wounds and intact skin of rats infected with *Staphylococcus aureus* MRSA, showed reduction on bacterial growth.

The literature is controversial concerning the effects of laser on bacterial growth. Several studies on the effects of laser radiation on bacterial growth demonstrated biostimulatory or proliferative results. It has been advocated that these effects are due to modifications generated by increasing energy intake provided by radiation in the respiratory chain of bacteria and others showed that bactericidal or bacteriostatic effects are related to the absorption of the laser light by chromophores that cause conformational changes in certain molecules generating free radicals and reactive oxygen that

are capable of causing the rupture of the bacterial membranes.[11-13] The irradiation dose and the energy density are the most important parameters in photobiomodulation[14,15].

The result of the present study also demonstrated that the sole use of the photosensitizer (12.5 μg/mL) is capable of a significant inhibition of the growth of *Staphylococcus aureus* in culture when compared to negative controls ($p < 0.001$). On the other hand, another study[9] tested the toxicity of phenotiazinium dyes against methicillin-resistant *Staphylococcus aureus* (ATCC 25923) also using 12.5 μg/mL and found no antimicrobial toxicity when incubated in the dark for 30 min in comparison to control group ($P > 0.05$). It is very important to consider that the sole use of high concentrations of the photosensitizer may cause the killing of bacteria without the use of light due to the high toxicity of the compound. This aspect may have been disregarded in previous studies using the association to different light sources. It is very important to achieve high level of inhibition using much lower concentrations.

The best result of the present study was found using AmPDT that was able to significantly reduce counts up to 84.2% on cultures of *Staphylococcus aureus.* The challenge in AmPDT is to find a therapeutic window, in which hazardous bacteria are efficiently inactivated without harming the surrounding tissue and disturbing the local microenvironment at a given concentration and light dose[7].

5. CONCLUSION

It is concluded that the use of the phenothiazinic compound (12.5 μg/mL) associated to red laser light was able to significantly reduce, in vitro, the proliferation of *Staphylococcus aureus.*

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