Photodynamic Antimicrobial Chemotherapy (PACT) Using Phenothiazines Derivatives Associated with the Red Laser against *Staphylococcus aureus*

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ABSTRACT

The objective of this study was to evaluate the bactericidal effect of photodynamic antimicrobial chemotherapy (PACT) using phenothiazinium dye (Toluidine blue O and methylene blue) at a low concentration of 1µg/mL irradiated with the red laser at doses of 2.4 e 4.8 J/cm² on strain of *Staphylococcus aureus* (ATCC 23529) *in vitro*. For this research, tests were performed in triplicate and the samples were distributed into six test groups: (L_-P_-) Negative control ($L1_+P_-$) and ($L2_+P_-$) bacterial suspensions were irradiated with laser energy 2.4 and 4.8 J/cm^2 respectively in the absence of photosensitizer; ($L1_+P_+$) and ($L2_+P_+$) bacterial suspensions were irradiated with laser in the presence of 1µg/ml of photosensitizer and finally (L_-P_+) bacterial suspensions only in the presence of phenothiazinium dye. Therefore, were analyzed the potential bactericidal PACT by counting of colony-forming units and analyzed statistically (ANOVA, Tukey test, $p<0.05$). The results showed that the negative control group when compared with laser group ($L2_+P_-$) it was observed a statistically significant increase ($p<0.01$) which $L2_+P_-$ showed a higher number of CFU, on the other hand when compared to $L1_+P_-$ no statistically significant difference was found, relation to the groups submitted to PACT, only showed a statistically significant reduction relative to the group irradiated $L2_+P_+$ ($p<0.01$) that showed a decrease in the number of CFU. There was no statistically significant difference between the groups submitted to PDT ($L1_+P_+$ and $L2_+P_+$). Although the results of this study have shown a reduction in average number of colony forming units by the appropriate laser-dye treatment combination, it needs further investigation.

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

1. INTRODUCTION

In recent years, photodynamic inactivation has been proposed as an alternative treatment for localized bacterial infections in response to the problem of antibiotic resistance[1]. Much is already known about the photodynamic inactivation of microorganisms: both antibiotic-sensitive and -resistant strains can be successfully photo inactivated and there is the additional advantage that repeated photosensitization of bacterial cells does not induce a selection of resistant strains[2,3].

Staphylococcus spp. are opportunistic microorganisms known for their capacity to develop resistance against antimicrobial agents. The emergence of resistant strains of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) poses a major challenge to healthcare. MRSA is a major cause of hospital-acquired infection throughout the world and is now also prevalent in the community as well as nursing and residential homes[4,5].

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Staphylococci are important causes of nosocomial and medical-device-related infections. Their virulence is attributed to the elaboration of biofilms that protect the organisms from immune system clearance and to increased resistance to phagocytosis and antibiotics[6].

Photodynamic therapy has emerged as a therapeutic option for the treatment of infectious diseases, combines a nontoxic photosensitizer with harmless visible light of the correct wavelength to excite the photosensitizer to its reactive triplet state, which will then generate reactive oxygen species, such as singlet oxygen and superoxide that are toxic to cells[7]. These reactive oxygen species can damage DNA and the cell membrane, resulting in the leakage of cell components, inactivation of transport systems and cell death[8,9].

At the present time, phenothiazinium salts such as toluidine blue O (TBO) and methylene blue (MB) are used clinically for antimicrobial treatments[10]. The minimal toxicity of these dyes to human cells, plus their ability to produce high quantum yields of singlet oxygen, has produced a great interest in testing the potential of these photosensitizers as photo-activated antimicrobial agents[11].

The objective of this study was to evaluate the bactericidal effect of photodynamic antimicrobial chemotherapy (PACT) using phenothiazine derivatives (methylene blue and toluidine blue O) at a concentration of 1 µg/mL the red laser at doses of 2.4 J/cm² and 4.8 J/cm² strain of *Staphylococcus aureus* (ATCC 23529) *in vitro*.

2. METHODOLOGY

2.1 Bacterial strain and culture condition

Bacterial strain used in this study was *Staphylococcus aureus* (ATCC 23529). This strain was obtained from the Laboratory of Parasitic Biomorphology, Gonçalo Muniz Institute – FIOCRUZ-BA. Cells were cultured in blood agar (Merck®) aerobically at 37°C and were grown for 24 hours.

For the experiments colonies were collected with the aid of a calibrated loop of 100ml and inoculated into 5ml of tryptic soy broth (TSB) (Merck®). For the quantification of colony forming units, the suspension was standardized by measuring absorbance at in an ELISA-reader spectrophotometer to an optical density of of 0.5 Macfarland at a wavelength of 625nm, corresponding to approximate numbers 3 x 10^8 CFU. Subsequently, 10µL of this suspension were inoculated into 1ml of TSB (Merck®). After this dilution the photosensitizer was added to follow experimental protocol.

2.2 Photosensitizer and light source

Phenothiazinium dye (Toluidine blue O and methylene blue) at a concentration of 1000µg/ml was used for photosensitization of the *Staphylococcus aureus* strains. (Fórmula Laboratory, Salvador, BA, Brazil). The dye solution at a concentration of 1µg/ml was prepared by dissolving in sterile PBS (pH = 7.4) and filtering it through a 0.22-µm membrane filter (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 (Twin Flex®, MMOptics, São Carlos, SP, Brazil), emitting light at 660 nm (visible red), was used as the light source. The wavelength of the laser corresponds to the maximum absorption of phenothiazinium dye. The laser settings were as follows on Table 1.

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)	60s/120s
Energy density (J/cm^2)	2.4/4.8

2.3 Photodynamic Antimicrobial Chemotherapy

Samples were distributed into six test groups:
1. L_-P_- : Negative controls untreated by either laser or photosensitizer.
2. $L1_+P_-$: Laser 1 – bacterial suspensions were irradiated with laser energy (2,4J/cm^2) in the absence of photosensitizer.
3. $L2_+P_-$: Laser 2 – bacterial suspensions were irradiated with laser energy (4,8J/cm^2) in the absence of photosensitizer.
4. $L1_+P_+$: Laser 1 + Photosensitizer – bacterial suspensions were irradiated with laser energy (2,4J/cm^2) in the presence of a low concentration of 1µg/ml of photosensitizer.
5. $L2_+P_-$: Laser 2 + Photosensitizer – bacterial suspensions were irradiated with laser energy (2,4J/cm^2) in the presence of a low concentration of 1µg/ml of photosensitizer.
6. L_-P_+: Photosensitizer – bacterial suspensions in the presence of phenothiazinium dye (Toluidine blue O and methylene blue) at a low concentration of 1µg/ml.

The bacterial suspension was put into the 24-well Multiwell Plate (BD Falcon™) to follow the experiments and incubated with TBO/MB at concentration of 1µg/ml in the dark and at room temperature for 5min. The contents of the wells were mixed before sampling. Then with the aid of a handle calibrated 100mL bacteria by depletion were seeded in triplicate onto petri plate divided into four fields containing TSA medium (Merck®) and incubated at 37°C for 24 hours. After incubation the number of colony-forming units (CFU) was determined. Therefore, were analyzed two points in these experiments: the number of colonies per field and reducing the number of colonies per field.

2.4 Statistical analysis

Comparisons between means of groups were analyzed using the One-Way ANOVA and Tukey's Multiple Comparison tests. $P < 0.05$ was considered statistically significant.

3. RESULTS

The reduction of colony forming units in each of the test groups is on Table 2.

Table 2: Total number and average of CFU *Staphylococcus aureus* on different groups

REPLICATE	L-P-	L¹+P-	L²+P-	L-P+	L¹+P+	L²+P+
EXP¹	100	128	136	133	88	62
EXP²	110	130	140	120	79	64
EXP³	105	120	135	115	105	94
AVERAGE	105	126	137	122.6667	90.66667	73.33333

Figure 1 shows the average of CFU obtained for the *Staphylococcus aureus* under each experimental condition.

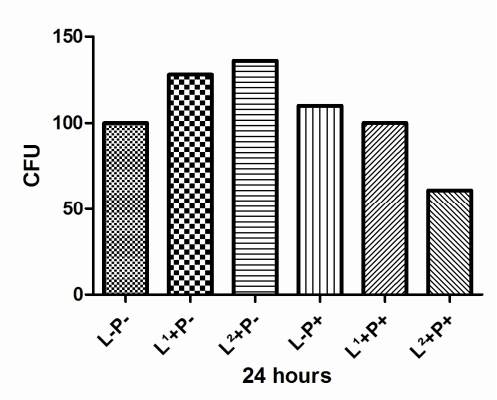

Figure 1. Number of colony-forming units (CFU) on different groups .

Comparing the negative control group (L_-P_-) with laser groups ($L1_+P_-$ and $L2_+P_-$) it was observed a statistically significant increase ($p<0.01$) only relation to $L2_+P_-$ that showed a higher number of CFU.

The negative control group when compared to the groups submitted to PDT ($L1_+P_+$ and $L2_+P_+$), only showed a statistically significant reduction ($p<0.01$) relative to the group irradiated with laser which energy density was 4.8 J/cm^2 ($L2_+P_+$) that showed a decrease in the number of CFU.

A statistically significant reduction in bacterial counts was observed on the group submitted to PDT $L1_+P_+$ which demonstrated a statistically significant reduction in bacterial counts when compared to the other groups $L1_+P_-$, $L2_+P_-$ and L_-P_+ ($p<0.01$, $p<0.001$ and $p<0.01$).

The same was occurred relation to $L2_+P_+$ when compared to the other groups treated only in presence of the light $L1_+P_-$ and $L2_+P_-$ ($p<0.0001$ and $p<0.0001$ respectively), or only in the presence of photosensitizer (L-P+) ($p<0.0001$).

There was no statistically significant difference between the groups submitted to PDT ($L1_+P_+$ and $L2_+P_+$).

4. DISCUSSION

Since the middle of the last century, anti-microbial photodynamic therapy was forgotten because of the discovery of antibiotics. Certainly, in the last decades the total worldwide rise in antibiotic resistance has driven research to the development of new anti-microbial strategies for alternative anti-microbial therapies, like anti-microbial PDT[12].

Successful PDT always involves the optimization of a large number of parameters. Obviously, selection of an effective photosensitizer is essential for the success of the technique. As well as being non-toxic to humans, the ideal photosensitizer needs to absorb a laser beam at the compatible wavelength and has to produce high excitation efficiency[13].

Application of photodynamic inactivation of bacteria by light radiation and photosensitizer has some unknown fields: which are the bacteria with sensibility to light radiation; the direct effect on the microbial population; the type of photosensitizer which is selectively fixed in different bacterial species; the way it should be administered; how to prepare the photosensitizer; what is the therapeutic concentration; how much time must pass from the photosensitizer administration to the exposure to light; which type of source for the radiation is better (continuous or pulse operation); the parameters of the light (wavelength, energy, pulse duration, frequency, time of exposure); how to monitor the biologic response and the treatment[14].

A variety of photosensitizers have been shown to possess antibacterial properties with much recent attention focusing on phenothiazinium dyes and their derivatives[15].

These phenothiazinium based photosensitizers are generally cationic and possess a core structure, which is a planar tricyclic heteroaromatic ring system They are highly effective against a variety of bacterial species but of particular interest is the ability of these dyes to inactivate Gram-positive pathogens[16]. In the present study was used phenothiazinium dyes (Toluidine blue O and methylene blue) at a low concentration of 1μg/ml as photosensitizer, according Sayed, Harris and Phoenix (2005)[17] that realized a study which it has previously been shown that the phenothiazinium dyes and their derivatives are photo-toxic to *S. aureus* and they have considered the possibility that the DNA of the organisms may be a target of these dyes.

Second Chan and Lai (2003)[14] it is obvious that the bactericidal effect is wavelength dependent, since the same power output diode laser with a monochromic infra red light of 830 nm wavelength could not kill the targeted organisms as effectively under the same conditions. Their results suggest that the wavelength of the laser light source used in PDT is a crucial point for optimizing the therapeutic effect and is an important factor in assessing the clinical applicability of this potential therapeutic approach. With respect to phenothiazines (methylene blue, toluidine blue) or porphyrins, there is a still effective absorption of light for wavelengths above 600 nm[12], then for the present study it was used as the light source a diode laser emitting light at 660 nm (visible red), because the wavelength of the laser corresponds to the maximum absorption of phenothiazinium dye.

The results of this study show that exposure of bacterial cultures to laser light in the presence of phenothiazinium dye as a photosensitizer results in a reduction on bacterial growth. The most-effective combination was that of phenothiazinium dye with a 660-diode laser 4.8J/cm^2. This produced a statistically significant reduction compared to all groups that were tested.

The literature is controversial in concerns the effects of laser on bacterial growth. Several researches on the effect of laser radiation on bacterial results indicate biostimulant or proliferative, postulating that these effects are due to modifications generated by increasing energy intake provided by radiation in the respiratory chain of bacteria, others shows that the effect bactericidal or bacteriostatic is related to the absorption of the laser light by chromophores, may cause conformational changes in certain molecules, generating radicals free and reactive oxygen which in promote the rupture of membranes bacterianas[18-20].

In the present study the negative control group (L$_-$P$_-$) when compared with laser group (L2$_+$P$_-$) it was observed a statistically significant increase which L2$_+$P$_-$ showed a higher number of CFU, on the other hand when compared to L1$_+$P$_-$ no statistically significant difference was found. Therefore these different results produced can be assigned to the energy densities used in the same condition. Furthermore it was examined the effects 630, 660, 810 and 905nm of low-intensity laser irradiation delivering radiant exposure of 1-50 J/cm^2 on three species of bacteria in vitro, including *Staphylococcus aureus,* the authors concluded that the response photobiological a microorganism exposure to monochromatic light depends directly on the parameters of irradiation (wavelength, intensity and dose).[21]

5. CONCLUSION

Although the results of this study have shown a reduction in average number of colony forming units by the appropriate laser-dye treatment combination, it needs further investigation.

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