In vitro influence of photodynamic antimicrobial chemotherapy on *staphylococcus aureus* by using phenothiazines derivatives associated with laser/LED Light

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ABSTRACT

The aim of this study was to evaluate the effect of photodynamic antimicrobial chemotherapy (PACT) using phenothiazinium dyes - PTZ irradiated with red laser (ʎ660nm) or red-orange LED (ʎ632±2nm) on *Staphylococcus aureus in vitro*. triplicate tests were performed in 10 groups: control, Laser ($L1_+P_-$ and $L2_+P_-$) bacterial suspensions were irradiated only with laser energy 2.4 and 4.8 J/cm^2 respectively, ($Led1_+P_-$ and $Led2_+P_-$) irradiated only with LED energy 2.4 and 4.8 J/cm^2 respectively, ($L1_+P_+$ and $L2_+P_+$) irradiated with laser in the presence of 1µg/ml of photosensitizer, ($Led1_+P_+$ and $Led2_+P_+$) irradiated with LED in the presence of 1µg/ml of photosensitizer and finally (L_-P_+) only in the presence of PTZ dye. Bactericidal effect of the PACT was assessed by counting colony-forming units. The results showed no significant difference on regards different energy densities on group PACT for both lights. PACT groups ($L2_+P_+$ and $Led2_+P_+$) compared to the Control showed significant reduction of CFUs. LED/Laser groups ($L2_+P_-$ and $Led2_+P_-$) compared to control and PTZ groups showed also significant differences as groups LED/Laser (4.8J/cm^2) increased the average of CFUs. Although the results of this study have shown a reduction in average number of colony-forming units by the appropriate Laser or LED-dye treatment combination, it this topic requires further investigation.

Keywords: Photodynamic antimicrobial chemotherapy; *Staphylococcus aureus;* Light emitting-diodes.

1. INTRODUCTION

In the last decades the total worldwide rise in antibiotic resistance has driven research to the development of new anti-microbial strategies. Much is already known about the photodynamic inactivation of microorganisms: both antibiotic-sensitive and resistant strains can be successfully photoinactivated and there is the additional advantage that repeated photosensitization of bacterial cells does not induce a selection of resistant strains [1,2].

In particular, staphylococcal resistance to methicillin and closely related penicillin was noted since the introduction of penicillinase-stable β-lactam antibiotics like methicillin or cloxacillin[3]. The formation of a biofilm, observed in *Staphylococcus* strains, increases their resistance to antibiotics and protects the cells against the action of the immune system[4].

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A therapeutic option for the treatment of antimicrobial treatments is the photodynamic therapy. It´s consist in visible light to excite the photosensitizer to generate reactive oxygen species, such as singlet oxygen and superoxide that are toxic to cells because can damage DNA and the cell membrane, resulting in the leakage of cell components, inactivation of transport systems and cell death[5,6,7].

Successful PDT always involves the optimisation 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 the light at the compatible wavelength and has to produce high excitation efficiency[8].

Actually, toluidine blue O (TBO) and methylene blue (MB) are photosensitizer used clinically for antimicrobial treatments because the low toxicity of these dyes to human cells, plus their ability to produce high quantum yields of singlet oxygen[9,10].

At the moment, different laser systems and incoherent light sources are used in PDT[11]. In comparison to Lasers, LED technology generates negligible amounts of heat. It is clinically proven to be safe, and has achieved non-significant risk status for human trials by the Food and Drug Administration[12].

The objective of this study was to contribute to PDT development by researching alternative light sources using red laser and red-orange LED light at doses of 2,4 e 4,8 J/cm² to evaluate the bactericidal effect of photodynamic antimicrobial chemotherapy (PACT) using phenothiazinium dye (Toluidine blue O and methylene blue) at a concentration of 1µg/mL on 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 Parasite Biology, 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 100 µL and inoculated into 5 mL of tryptic soy broth (TSB) (Merck®). For the quantification of colony-forming units (CFU), the suspension was standardized by measuring absorbance at in an ELISA-reader spectrophotometer to an optical density of 0.5 Macfarland at a wavelength of 625 nm, corresponding to approximate numbers 1.5 x 10^8 CFU. Subsequently, 10 µL of this suspension were inoculated in 1 mL 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 1000 µg/mL was used for photosensitization of the *Staphylococcus aureus* strains (Fórmula Laboratory, Salvador, BA, Brazil). The dye solution at a concentration of 1 µg/mL was prepared by dissolving in sterile PBS, 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 (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. A red-orange light emitting diode (Prototype, MMOptics, São Carlos, SP, Brazil), emitting light at 632±2 nm, was used as the light source. The Laser and LED settings were as follows on table 1.

Table 1: Summary of the parameters used on the study

Parameters	LASER	Red-orange LED
Wavelength (nm)	660	632±2
Mode	CW	CW
Spot of the probe (mm²)	4	0,5
Power Output (W)	0.04	145±5
Exposure Time (s, per session)	60s/120s	8s/16s
Energy density (J/cm^2)	2.4/4.8	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 photosensitiser.
2. $L1_+P_-$: Laser 1 – bacterial suspensions irradiated with laser energy (2.4 J/cm^2) in the absence of photosensitizer.
3. $L2_+P_-$: Laser 2 – bacterial suspensions irradiated with laser energy (4.8 J/cm^2) in the absence of photosensitizer.
4. $Led1_+P_-$: LED 1 – bacterial suspensions were irradiated with LED energy (2,4J/cm^2) in the absence of photosensitizer.
5. $Led2_+P_-$: LED 2 – bacterial suspensions were irradiated with LED energy (4,8J/cm^2) in the absence of photosensitizer.
6. $L1_+P_+$: Laser 1 + Photosensitizer – bacterial suspensions irradiated with laser energy (2.4 J/cm^2) in the presence of a low concentration of 1 μg/mL of photosensitizer.
7. $L2_+P_-$: Laser 2 + Photosensitizer – bacterial suspensions irradiated with laser energy (2.4 J/cm^2) in the presence of a low concentration of 1 μg/mL of photosensitizer.
8. $Led1_+P_+$: LED 1 + Photosensitizer – bacterial suspensions irradiated with LED energy (2,4J/cm^2) in the presence of a low concentration of 1μg/ml of photosensitizer.
9. $Led2_+P_-$: LED 2 + Photosensitizer – bacterial suspensions irradiated with LED energy (2,4J/cm^2) in the presence of a low concentration of 1μg/ml of photosensitizer.
10. L_-P_+: Photosensitizer – bacterial suspensions in the presence of phenothiazinium dye (Toluidine blue O and methylene blue) at a low concentration of 1 μg/mL.

The bacterial suspension was platted into the 24-well Multiwell Plate (BD Falcon™) to follow the experiments and incubated with TBO/MB at concentration of 1 μg/mL in the dark and at room temperature for 5 min. The contents of the wells were mixed before sampling. Then with the aid of a calibrated 100 μL loop bacteria limiting dil 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 CFU was determined. Therefore, were analyzed two points in these experiments: the number of colonies per field before and after PDT.

2.4 Statistical analysis

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

3. RESULTS

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

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

REPLICATE	L-P-	L^1+P-	L^2+P-	Led^1+P-	Led^2+P-	L^1+P+	L^2+P+	Led^1+P+	Led^2+P+	L-P+
EXP^1	100	128	136	130	160	88	62	78	70	133
EXP^2	110	130	140	140	180	79	64	88	68	120
EXP^3	105	120	135	110	150	105	94	64	60	115
AVERAGE	105	126	137	127	163	91	73	77	66	122

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

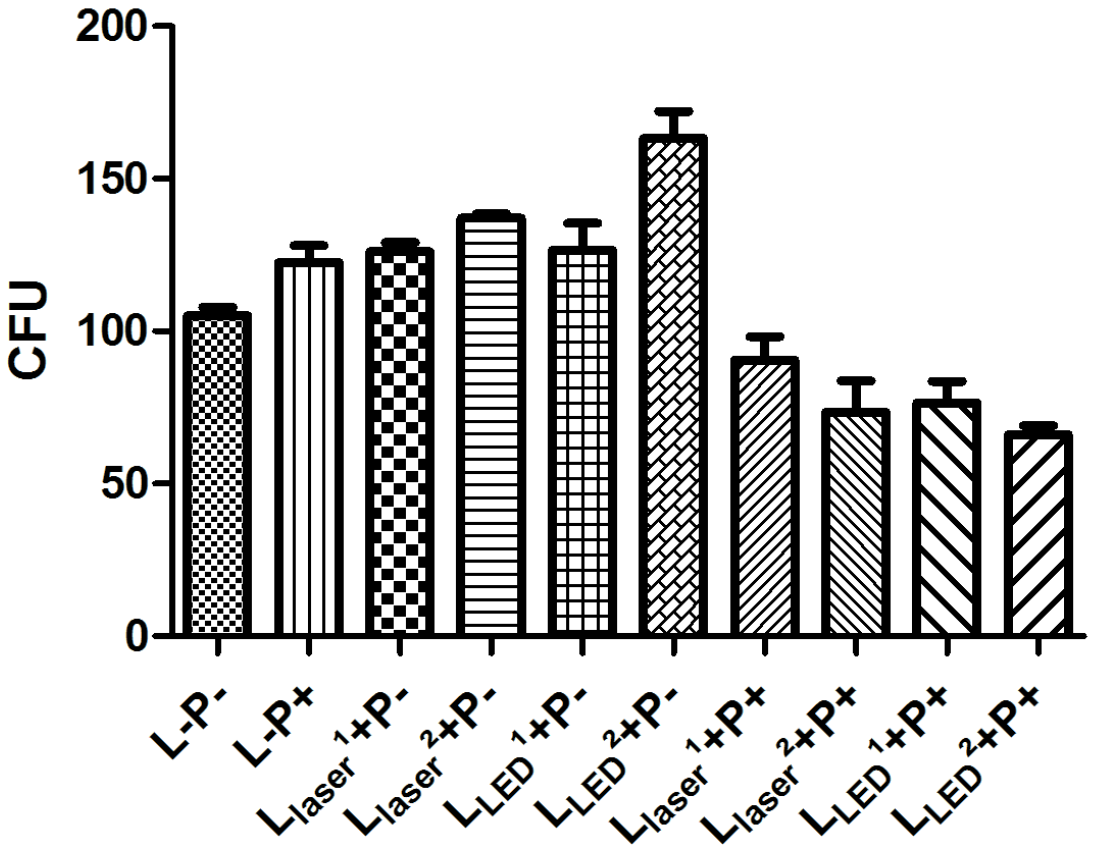

Figure 1**:** Number of colony-forming units (CFU) on different groups

The results demonstrated that the control group when compared to the groups submitted to PACT only showed an increase in the number of CFU on Led2+P- ($P<0,0001$) and a statistically significant reduction relative to the groups Led2+P+ ($P<0,01$).

Relation to the photosensitizer group (L-P+) comparing with control group, light groups and PACT groups there was a statistically significant difference only comparing to PACT groups, which groups showed decrease in the average of CFU counts.

The Light groups (L1+P-, L2+P-, Led1+P- and Led2+P-) showed statistically significant difference relation to PACT groups (L1+P+, L2+P+ Led1+P+ and Led2+P+), which showed a statistically significant reduction on CFU counts.

There was no statistically significant difference between the groups submitted to PACT with different energy densities or sources.

4. DISCUSSION

A large number of PDT parameters may be addressed in detail: bacterial species with sensitivity to light radiation; the selectivity of photosensitizer to different bacterial species; photosensitizer preparation and concentration; type of source for the radiation; the parameters of the light (wavelength, energy, pulse duration, frequency, time of exposure); monitoring the biologic response and the treatment[13].

Phenothiazinium based photosensitizers are highly effective against Gram-positive pathogens[14,15]. In the present study was used phenothiazinium dyes (Toluidine blue O and methylene blue) at a low concentration of 1 μg/mL as photosensitizer, according Sayed, Harris and Phoenix (2005)[16] that realized a study which it has previously been shown that the phenothiazinium dyes 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)[13] it is clear 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 tested organisms as effectively under the same conditions. Their results suggest that the wavelength of the 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[17], then for the present study it was used as the light source a light emitting diode at 632±2nm (red-orange) and a diode laser emitting light at 660 nm (visible red), because this wavelength corresponds to the maximum absorption of phenothiazinium dye.

Studies carried out by Zeina *et al* (2001)[18] suggest that the source of the light is not important for PDT in cases where the wavelength covers the absorption maxima. For instance, they did not observe a difference in photodynamic effect between sunlight and a projector light. Piloi *et al* (2008)[19] using LED light, report that the results of them were comparable with those of studies using lasers, and partially support Zeina´s conclusion. They demonstrated that LED light was as effective against *S. aureus, E. coli* and *C. albicans* as those of experiments using laser.

The literature concerning the effects of laser on bacterial growth is controversial. The effects of laser radiation on bacterial indicate biostimulant or proliferative results, these effects are due to modifications generated by radiation in the respiratory chain of bacteria, others show rupture of bacterial membranes due to conformational changes in certain molecules caused by the absorption of the laser light by chromophores, generating free radicals and ROS.[20-22]

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).[23]

The results of the present study show that exposure of bacterial cultures to Laser and LED light in the presence of phenothiazinium dye as a photosensitiser results in a reduction on bacterial growth. However There was no statistically significant difference between the groups submitted to PACT with different energy densities or sources. By the way, relation to control group a statistically significant reduction on the group Led2+P+ ($P<0.01$), it was observed.

High-potency laser sources are preferable, but their high costs make PDT inaccessible for many institutions and therefore make the dissemination of this technology difficult in most countries. Recently, however, various alternative light sources have been researched to replace the laser in PDT. One of the most interesting of these is the light-emitting diode (LED) because of its low price. It is possible to find different colours of LED light in the market, with radiations covering almost all of the visible electromagnetic spectrum, including red light. This alternative, despite having a lower potency than laser, has several economic advantages and can be used to optimize the investigation of new compounds for PDT[24].

5. CONCLUSION

Although the results of this study have shown a reduction in average number of colony-forming units by the appropriate Laser or LED-dye treatment combination, it this topic requires further investigation.

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REFERENCES

[1] Wainwright, M., Phoenix, D.A., Laycock, S.L., Wareing, D.R., Wright, P.A. "Photobactericidal activity of phenothiazinium dyes against methicillin-resistant strains of Staphylococcus aureus," FEMS Microbiol. Lett. 160 (2), 177–81 (1998).

[2] Lauro, F.M., Pretto, P., Covolo, L., Jori, G., Bertoloni, G. "Photoinactivation of bacterial strains involved in periodontal diseases sensitized by porphycene–polylysine conjugates," Photochem. Photobiol.Sci. 1 (7), 468–470 (2002)

[3] Nishijima, S., Namura, S., Mitsuya, K., Asada, Y. "The incidence of isolation of methicillin-resistant Staphylococcus aureus (MRSA) strains from skin infections during the past three years (1989–1991)," J. Dermatol. 20 (4), 193-197 (1993).

[4] Lynch, A.S., Robertson, G.T. "Bacterial and fungal biofilm infections," Annu Rev Med. 15(59), 415-428 (2008).

[5] Castano, A.P., Demidova, T.N., Hamblin, M.R. "Mechanisms in photodynamic therapy. Part one. Photosensitizers, photochemistry and cellular localization," Photodiag. Photodyn. Ther. 1, 79-93 (2004).

[6] Wainwright, M. "Photodynamic antimicrobial chemotherapy (PACT)," J. Antimicrob. Chemother. 42(1), 13-28 (1998).

[7] You, C.,Chern-Hsiung, L. "Bactericidal effects of different laser wavelengths on periodontopathic germs in photodynamic therapy" Lasers Med. Sci. 18(1), 51–55 (2003).

[8] Hamblin, M.R., Hasan, T. "Photodynamic therapy: a new antimicrobial approach to infectious disease?," Photochem. Photobiol. Sci. 3 (5), 436-450 (2004).

[9] Wainwright, M. "The use of dyes in modern biomedicine," Biotech. Histochem. 78(3-4), 147-155 (2003).

[10] Wainwright, M. "The development of phenothiazinium photosensitisers," Photodiagn. Photodyn. Ther. 2, 263-272 (2005).

[11] Baeumler, W. "Light sources for photodynamic therapy and fluorescence diagnosis in dermatology," Comprehensive series in photoscience, Elsevier, 1(2), 83–98 (2001).

[12] Whelan, H.T., Smits, R.L., Buchmann, E.V., Whelan, N.T., Turner, S.G., Margolis, D.A., Cevenini, V., Stinson, H., Ignatius, R., Martin, T., Cwiklinski J., Philippi, A.F., Graf, W.R., Hodgson, B., Gould, L., Kane, M., Chen, G., Caviness, J. "Effect of NASA light-emmiting diode irradiation on wound healing," J. Clin. Laser Med. Surg. 19(1), 305–314 (2001)

[13] Chan, Y., Lai, C. "Bactericidal effects of different laser wavelengths on periodontopathic germs in photodynamic therapy." Lasers Med. Sci. 18(1), 51-55 (2003).

[14] Phoenix, D.A. and Harris, F. "Phenothazinium based photosensitisers: antibacterials of the future?," Trends Mol. Med. 9(1), 283-285 (2003).
[15]Wainwright, M. and Giddens, R.M. "Phenothiazinium photosensitisers: choices in synthesis and application," Dyes Pigments 57(3), 245-257 (2003).
[16]Sayed, Z., Harris, F., Phoenix, D.A. "A study on the bacterial photo-toxicity of phenothiazinium based photosensitisers," FEMS Immunol Med Microbiol 43(3), 367–372 (2005).
[17]Maisch, T. "Anti-microbial photodynamic therapy: useful in the future?," Lasers Med Sci 22(2), 83-91 (2007).
[18]Zeina, B., Greenman, J., Purcell, W. M. and Das, B. "Killing of cutaneous microbial species by photodynamic therapy," Br. J. Dermatol. 144(2), 274–278 (2001).
[19] Peloi, L.S.,Soares, R.R.S.,Biondo, C.E.G., Souza, V.R., Hioka, N., Kimura, E. "Photodynamic effect of light-emitting diode light on cell growth inhibition induced by methylene blue," J. Biosci. 33(2), 231–237 (2008).
[20]Kawamoto, K., Senda, N., Shimada, K., Itol, K., Hirano, Y., Murai, S. "Antibacterial effect of yellow He-Ne laser irradiation with crystal violet solution on *Porphyromonas gingivalis*: an evaluation using experimental rat model involving subcutaneous abscess," Lasers Med. Sci. 15 (4), 257-262 (2000).
[21]Karu, T.I. "Primay and secondary mechanisms of action of visible to near-IR radiation on cells," J Photochem Photobiol. Part B. (49), 1-17 (1999).
[22]DeSimone, N.A., Hristiansen, C., Dore, D. "Bactericidal effect of 0.95-mw helium-neon and 5-mw indiumgallium-aluminum-phosphate laser irradiation at exposure times of 30, 60, and 120 seconds on photosensitized *Staphylococcus aureus* and *Pseudomonas aeruginosa in vitro,"* Phys. Ther. 79 (9), 839-846 (1999).
[23]Nussbaum, E.L., Lilge, L., Mazzulli, T. "Effects of 630-, 660-, 810-, and 905- nm laser irradiation delivering radiant exposure of 1-50 J/cm^2 on three species of bacteria in vitro," J Clin. Laser Med. Surg. 20 (6), 325-33 (2002).
[24]Mang, T. S. "Lasers and light sources for PDT: past, present and future," Photodiag. Photodyn. Ther. 1, 43-48 (2004).