Association phenothiazine and laser on growth of *C. tropicalis* fluconazole-resistant

Rafael Araújo Gomes Júnior[c], Susana C. P. S. de Oliveira[a,b], , Juliana S. C. Monteiro[a,b,e], Gustavo M. P. Santos[c], Fernando J. P. Sampaio[a,b], Maria F. M. Gesteira[a,b], Fátima A. A. Zanin[a,b,d], Aldo Brugnera[a,b,d], Antônio L. B. Pinheiro* [a,b,d] and Marcos A. Vannier-Santos[c]

[a]Center of Biophotonic, School of Dentistry, Federal University of Bahia, Salvador, BA, Brazil, 40110-150; [b]National Institute of Optics and Photonics, São Carlos, SP, Brazil, 13560-970; [c]Laboratory of Parasite Biology, Centro de Pesquisas Gonçalo Muniz, Fundação Oswaldo Cruz FIOCRUZ, Salvador, BA, INCT-INPeTAM, Brazil. [d]Brugnera & Zanin Institute, São Paulo, SP, Brazil 01434-000; [e]Department of Biology, Estate University of Feira de Santana, Feira de Santana, BA, Brazil, 44036-900.

ABSTRACT

Candidiasis is caused by Candida species found on the skin, gastrointestinal tract and mucous cavities of the humans and may be acute, chronic, localized or systemic. Alhough *C. albicans* is the species most often identified as responsible for this type of infection *C. Tropicalis* has been considered an emerging cause. The effect of the association of phenothiazine - PTZ and laser on fluconazole-resistant C.tropicalis growth was tested. 2.5 x 10^6 CFU/mL 100mg/mL of phenothiazine with the pre-irradiation time of 10 min were irradiated with laser light (660 nm; 4.8 and 12 J/cm^2 (L1 and L2 respectively) 40 mW) followed by incubation in RPMI for 24h. The following conditions were tested: control (control), laser (L1 and L2), phenothiazine (F1 and F2), and PACT (F1L1 and F2L2). Statistically significant diferences were seen between groups (L-F +) and (F + L +) for both conditions of the laser, with a growth inhibition of the yeast around 67 and 51%, respectively, however, when using only the laser there was an increase of 18% in the survival of these cells. PACT´s efficacy on fluconazole-resistant *C. tropicalis* depended on both the time of pre-irradiation and concentration of the PTZ.

1. INTRODUCTION

Candidiasis is a fungal infection that usually occurs in situations where there is a reduction of the immune response, and therefore this condition is considered an opportunistic infection. This condition can be a problem for immuno-incompetent host, which may be caused by various Candida species such as *C. albicans, C. glabrata, C. tropicalis*, among others[1,2]. Candida species can be found on the skin, gastrointestinal tract and mucous cavities of the humans and may be acute, chronic, localized or systemic.

The levels of incidence in Brazil can be up to 15 times more than in developed countries. Moreover, currently there are reports of resistance among different species, moreover different resistance mechanisms have been observed, including mechanisms of action that reduce the effectiveness of Amphotericin B, an antifungal of choice for the treatment of ringworm. In this case there is a modulation in ergosterol biosynthesis. Other resistance mechanisms such as antioxidant enzymes and increase in efflux of compounds are examples of other pathways of cell adaptation by this fungus[3].

Recently, antimicrobial resistance has lished a problem, however the photodynamic therapies are showing great promise in combating localized infections. Currently cases of microbial control and microbial inactivation have been described in different strains, resistant or not[4,5].

* albp@ufba.br; phone 55 71 3283-9010; fax 55 71 3283-9010; www.laser.odontologia.ufba.br

Mechanisms for Low-Light Therapy IX, edited by Michael R. Hamblin, James D. Carroll, Praveen Arany,
Proc. of SPIE Vol. 8932, 89320G · © 2014 SPIE · CCC code: 1605-7422/14/$18 · doi: 10.1117/12.2038566

The effectiveness of this treatment is due to the combination of a non- toxic compound and a length of wave harmless, but able to activate the photosensitizer. This activation allows the generation of oxygen 'singlet' and / or superoxide that can act on several cellular targets and change organelles such as the nucleus, membrane, mitochondria[6,7,8].

The objective of this study was to evaluate in vitro the efficacy of photodynamic therapy Antifungal associating a phenothiazine compound at concentrations of 50μg/mL and 100μg/mL the Red Laser (λ660nm) at doses of 4.8 and 12 J/cm² on populations of fluconazole resistant *Candida tropicalis*.

2.METHODOLOGY

Cultivation and experimental conditions

The culture was maintained on potato agar at 37 ° C for 24 hours.

For the experiments cells were counted $2.5x10^5$ cells / ml and incubated under different conditions for 24 hours at 37°C and then recounted. In these tests four groups [(G1= 50μg/mL phenothiazine and 5 minutes pre-irradiation) (G2= 50μg/mL phenothiazine and 10 min pre-irradiation) (G3= 100μg/mL and phenothiazine were assessed 5 minutes pre-irradiation) (G4= 100μg/mL phenothiazine and 10 minute pre-irradiation), and each seven conditions [control, L1 (4.8 J/cm²), L2 (12 J/cm²), F1 (phenothiazine)*; F2 (phenothiazine)*; F1L1 and F2L2].
*** phenothiazine: F1 = F2 (this new group was created just to respect the pre irradiation time)**

Photosensitizer and light source

Phenothiazinium dye at a concentration of 1000μg/ml was used for photosensitization of the *C. tropicalis* fluconazole resistant 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)	40
Exposure Time (s, per session)	120 / 360
Energy density (J/cm2)	4,8 /12

Statistical analysis

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

3. RESULTS

The application of laser energy at different doses, 4.8 and 12 J/cm², were unable to inhibit the growth of yeasts, as was expected (Fig. 1). The different groups were efficiencies that varied with the concentration of the photosensitizer and the time of pre-irradiation.

There was a statistically significant inhibition in proliferation between the control and treatment conditions, however between treatments there were no statistically significant differences.

The lethal effect of the photo activation of phenothiazine may be in group 4 where the pre-irradiation time was 10 minutes and the concentration of the compound was 100 µg/mL. In this group, L1F1 condition showed a percentage inhibition of about 70% in the control grating and approximately 40% compared to the condition F1 (phenothiazine) (Figure 1D).

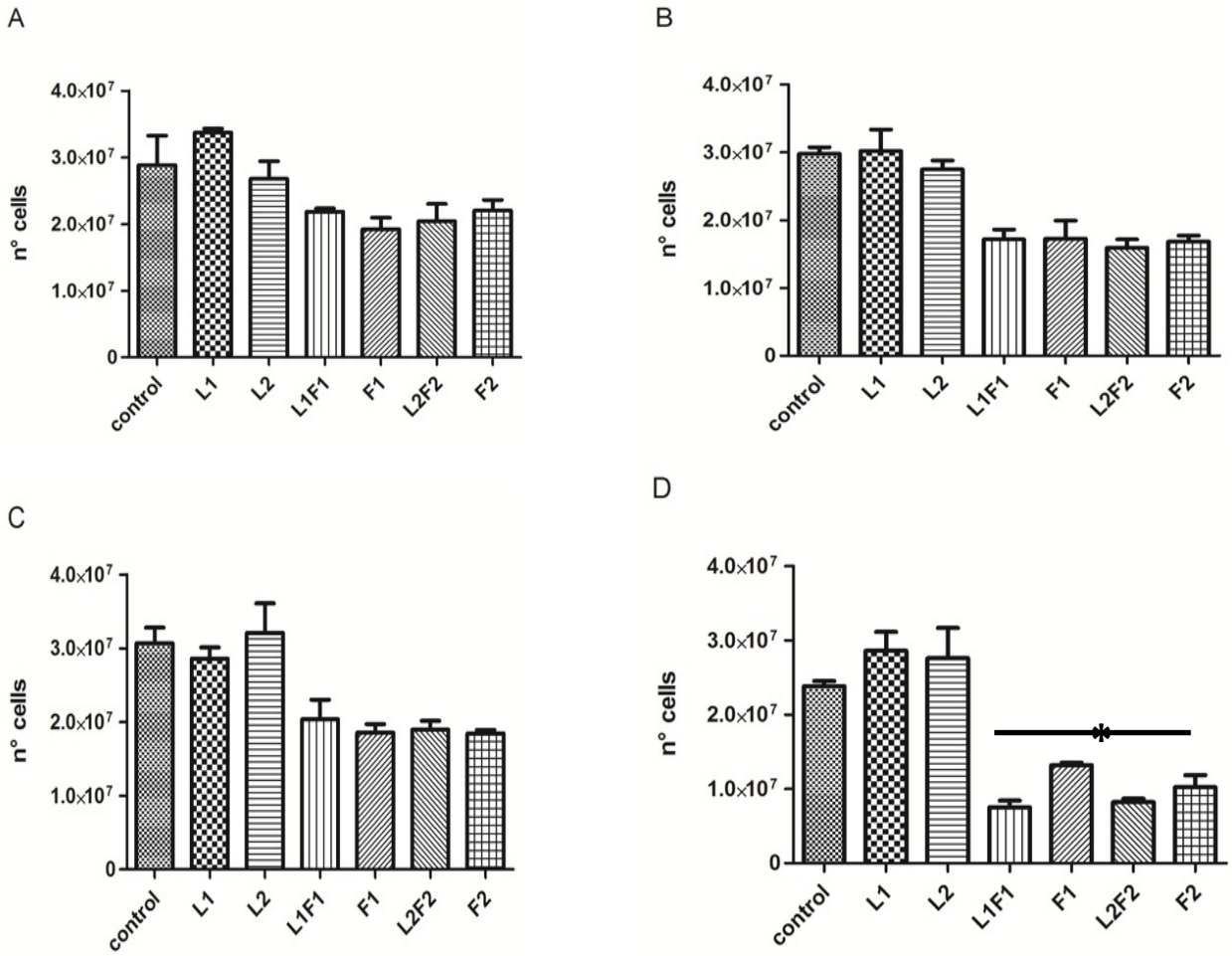

fig. 1: Microbial growth. A) Yeast growth in the group phenothiazine 50 µg/mL and the time to 10 minutes as pre-irradiation. B) Growth of yeast in phenothiazine group 100 µg/mL and the time to 5 minutes as pre-irradiation C) Growth of yeast in phenothiazine group 50 µg/mL and the time to 5 minutes as pre-irradiation D) Growth of yeast in phenothiazine group 100 µg/mL and the time to 10 minutes as pre-irradiation

4. DISCUSSION

Laser therapy is still controversial about the results on microorganisms. there are articles showing that laser radiation as biostimulator or promoter of proliferation, probably supplying power to the biological system, where the target molecules, the chromophores are part of respiratory chain. However, there are descriptions of microbicides static or from microbial growth in the generation of structural and conformational changes in molecules some effects and production of ROS, which are connected to cell death. However, studies indicate that cultures of microorganisms as laser irradiated in the presence of phenothiazine compounds result in reducing microbial growth[9-12].

The PDT is used as an alternative for the inhibition of proliferation of microorganisms and depends upon photoactivation of the compounds for the therapeutic success. Yet these mechanisms of resistance to therapeutic protocols have been described, however there is a possibility that organisms overexpressing antioxidants may be less responsive to contain higher concentrations of superoxide dismutase, for example[5,13,14].

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[15].

For a photosensitizer act optimally in yeast cells it needs, before crossing the membrane, pass through a cell wall. Consequently, fungi are organisms that may require a longer period of pre-irradiation. Therefore, it is believed that the protocols presented in this study may be improved with the increase of time of pre-irradiation.

Phenothiazine when photoactivated can induce cell death by different pathways, because it can interact with the plasma membrane and nucleus. This may be related to the ability of the fungicide compound tested the same microorganism in question presents a production of efflux proteins or even greater resistance to ROS.

As mentioned above the action on two important targets for the yeast may be the key to successful treatment. It is worth remembering that the release of ROS in the nucleus can induce programmed cell death similar to apoptosis, whereas membrane lesions may be linked to necrotic events. Therefore the activation of different cell death pathways may enhance the effectiveness of the model[16].

It is important to remember that the polymerization occurs in the compounds inside and outside the cell (data don't show). Therefore, when photoactivated outside the yeast phenothiazine compounds can cross biological membranes and therefore can trigger more toxic response of yeast. This response is secondary to PDT. This secondary pathway may act without the release of singlet oxygen and other ROS by photoactivation (fig 1).

Adaptation of the protocols in order to increase the fungicidal effect is important. However, it is noteworthy that the above protocol was interesting since it was able to inhibit about 70%, compared to the negative control, and about 40%, compared to the phenothiazine, of the fungal growth of a resistant strain, which has potential mechanisms as redox defense (fig. 1)[1]. In this sense, studies esclarençam and elucidate the mechanisms of cell death are themselves important.

5. CONCLUSION

The study showed that there is the prospect of using PDT as a treatment alternative to candidiasis, however increase the potential fungicides is required, as well as the elucidation of mechanisms of cell death should be performed

ACKNOWLEDGMENTS

Work at the author´s laboratories was supported by grants from Centro de Pesquisas Gonçalo Muniz, Fundação Oswaldo Cruz (FIOCRUZ).

.

REFERENCES

[1] Mascelino, M. T., Raponi, G., Oliva, A., Mastroanni, C. M., Vullo, V. "Candidaemia in immune-compromised hosts: incidence and drug susceptibility, " J. Clin. Exp. Pathol. 2, 131. 2:131. doi: 10.4172/2161-0681.1000131 (2012).

[2] Pfaller, M. A. "Epidemiology and control of fungal infections," Clin. Infect. Dis. 19(1), 8-13 (1994).

[3] Nucci, M., Queiroz-Telles, F., Tobón, A. M., Restrepo, A., Colombo, A.L. "Epidemiology and control of fungal infections in Latin America" Clin. Infect. Dis. 23(1), 317-322 (2010).

[4] 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)

[5] 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 7(1), 468–470 (2002).

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

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

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

[9] 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).

[10] 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).

[11] DeSimone, N.A., Hristiansen, C., Dore, D. "Bactericidal effect of 0.95-mw helium-neon and 5-mw indiumgalliumaluminum- 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).

[12] Oliveira, S. C. P. S., Pires-Santos, G. M., Monteiro, J. S. C., Miranda, A. F. S., Sampaio, F. J. P., Gesteira, M. F. M., Zanin, F. A. A., Vannier-Santos, M. A., Pinheiro, A. L. B. "Photodynamic Antimicrobial Chemotherapy (PACT) Using Phenothiazines Derivatives Associated with the Red Laser against *Staphylococcus aureus"* Proc. SPIE 8569, 1-6 (2013).

[13] Tegos, G.P., Masago, K., Aziz, F., Andrew Higginbotham, A, Stermitz, F.R., Hamblin, M.R. "Inhibitors of bacterial multidrug efflux pumps potentiate antimicrobial photoinactivation," Antimicrob. Agents Chemother. 52(9), 3202-3209 (2008).

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

[15] Calin, M.A., Parasca, S.V. "Light sources for photodynamic inactivation of bacteria," Lasers Med. Sci. 24(3), 453-460 (2009).

[16] Ramos, E.; Olivos-García, A.; Nequiz, M.; Saavedra, E.; Tello, E.; Saralegui, A.; Montfort, I.; Tamayo, R. P. "*Entamoeba histolytica*: Apoptosis induced in vitro by nitric oxide species" Exp. Parasitol. 116(3), 257-65 (2007).