Photodynamic therapy can kill *Cryptococcus neoformans* in *in vitro* and *in vivo* models

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ABSTRACT

Cryptococcosis is an infection caused by the encapsulated yeast *Cryptococcus neoformans* and the most afflicted sites are lung, skin and central nervous system. A range of studies had reported that photodynamic therapy (PDT) can inactivate yeast cells; however, the *in vivo* experimental models of cryptococcosis photoinactivation are not commonly reported. The aim of this study was to investigate the ability of methylene blue (MB) combined with a low-power red laser to inactivate *Cryptococcus neoformans* in *in vitro* and *in vivo* experimental models. To perform the *in vitro* study, suspension of *Cryptococcus neoformans* ATCC-90112 (10^6cfu/mL) was used. The light source was a laser (Photon Lase III, DMC, São Carlos, Brazil) emitting at λ=660nm with output power of 90mW for 6 and 9min of irradiation, resulting fluences at 108 and 162J/cm². As photosensitizer, 100μM MB was used. For the *in vivo* study, 10 BALB/c mice had the left paw inoculated with *C. neoformans* ATCC-90112 (10^7cfu). Twenty-four hours after inoculation, PDT was performed using 150μM MB and 100mW red laser with fluence at 180J/cm^2. PDT was efficient *in vitro* against *C. neoformans* in both parameters used: 3 log reduction with 108J/cm² and 6 log reduction with 162J/cm². In the *in vivo* experiment, PDT was also effective; however, its effect was less expressive than in the *in vitro* study (about 1 log reduction). In conclusion, PDT seems to be a helpful alternative to treat dermal cryptococcosis; however, more effective parameters must be found in *in vivo* studies.

Keywords: Antifungal; Diode laser; Methylene Blue; Photodynamic antimicrobial chemotherapy; Yeast photoinactivation; Photosensitizers.

1. INTRODUCTION

Photodynamic therapy (PDT) is a phototherapy based on the utilization of substances that can photosensitize biological tissues and are capable of being activated in the presence of light. The cells that are considered therapeutical targets are stained with the photosensitizing agent and irradiated with light [1-6]. The photodynamic process rapidly generating reactive oxygen species (ROS) as for instance peroxides, hydroxyl radicals, superoxide ions and singlet oxygen, the last being implicated as the major causative agent of cellular damage in photodynamic process [7]. In addition, this technique has been shown to have effects against a range of pathogens and also against drug-resistant microorganisms [8-10]. Yeast photoinactivation is a challenge on antimicrobial PDT due to the relatively lower susceptibility of these cells to oxidant agents. By the other hand, this phototherapy is a substantial hope against antifungal resistant species.

Cryptococcosis is an infection caused by the encapsulated yeasts *Cryptococcus neoformans.* Human infection is acquired by inhalation of airborne propagules from an environmental source, which can colonize the host respiratory tract without producing any disease [11,12]. When the host immunity is compromised (i.e., HIV infection, transplantation, corticosteroid therapy and lymphoma) the latent form can be reactivated and disseminate hematogenously to cause systemic infection. It can disseminate to cause localized infections involving the skin, eyes, myocardium, bones, joints and central nervous system (CNS). Recently, an immunocompetent cryptococosis case was reported [13].

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Brain infections and meningitis are the major causes of death for this yeast infections [14]. *C. neoformans* infection requires prolonged antifungal therapy and is associated with neurological sequelae and neurosurgical interventions [15,16]. Paralysis may persist as permanent sequelae of the disease and involves one or more cranial nerves [17,18]. Furthermore, often cryptococcosi meningoencephalitis can be a fatal disease [19,20].

The aim of this study was to investigate the ability of methylene blue (MB) combined with a low-power red laser to inactivate *Cryptococcus neoformans* in *in vitro* and *in vivo* experimental models.

2. MATERIALS AND METHODS

2.1 In vitro study

2.1.1 Inoculum preparation

Cryptococcus neoformans ATCC 90112 was sub-cultured from vial stock onto Sabouraud dextrose agar in air atmosphere for 48h at 30°C. The estimation of the cell concentration was made by a Neubauer camera with exclusion criterion to identify unviable cell. The inoculum initial quantification was confirmed by cells growing in the control group.

2.1.2 Irradiation source and photosensitizer

An InGaAlP diode laser (Photon Lase III, DMC, São Carlos, Brazil) with wavelength of 660 nm was used in this study. Yeast strain was irradiated from the top of a well microtitulation plaque and the laser beam passed through all the suspension at 0.3cm^2 spot size with output power of 90mW at 6min. and 9min. of irradiation.

The solution of methylene blue (MB) was prepared by the dissolution of the powder (Sigma Ltd, Poole, UK) in distillated water in a concentration of 10mM, which was filtered through a sterile filter membrane (0.22 μm, Millipore, São Paulo, Brazil). The photosensitizer was added to the yeast suspension in proportion of 1/100 that resulted in a final concentration of 100μM [4,21,22].

2.1.3 Photodynamic therapy *in vitro* and colony-forming units (cfu) determination

Yeasts were harvested onto Sabouraud dextrose agar and then suspended in phosphatase buffer saline (PBS) at a concentration of approximately 10^6 cfu/mL. This cell density was chosen because higher yeast cells concentration may inhibit photodynamic effect [23]. The suspension strain was divided into four groups.

The control group (L-PS-) was untreated by either laser or photosensitizer (PS). In the laser groups (L+PS-), the yeast suspensions were irradiated for 9 min. with an energy dose of 162 J/cm^2 in the absence of the photosensitizer. In the PS group (L-PS+), the yeast suspension stained by 100μM MB was maintained for 10 min. in cell contact to evaluate its toxicity in dark condition. In the PDT groups (L+PS+), aliquots of yeast suspensions with PS were putted in a 96-wells flat-bottomed microtiter plaque, and then irradiated with laser, in fluence rate of 300mW/cm². Groups were also carried out to observe each fluence-rate at 6 and 9 min. of laser illumination; thereafter, they were serially diluted in PBS to generate dilutions of 10^{-1} to 10^{-4} times the original concentration [4]. Ten-μL aliquots of each dilution were streaked onto a Sabouraud agar plate in triplicate and incubated to allow colony formation [24].

2.1.4 *In vitro* statistics

The yeast colonies were counted and converted into cfu for analysis. All samples were submitted to this process and statistical analysis of the experimental data was performed using one-way analysis of variance (ANOVA). Mean comparisons were carried out with the Tukey's test, which retains the overall significance level at 5% ($P<0.05$) [25].

2.1.5 Electron microscopy

Scanning electronic microscopy (SEM) was carry out to observe possible changes on yeast morphology following PDT. Yeast cells were harvested from the well microtitulation plaque and incubated in 2.5% glutaraldehyde for 1h. at room temperature. The samples were then serially dehydrated in alcohol, applied on a glass slide, coated with gold-palladium,

and viewed with scanning electron microscope (Phillips XI, Eindhover, Holland). Sets of cultures from control group and PDT group were prepared.

2.2 *In vivo* study

2.2.1 Animals

Ten female BALB/c mice, body mass of 24-26g, were used in this study. The experiments were performed according to COBEA (Brazilian College of Animal Experience), an institute associated with ICLAS (International Council of Laboratory Animal Science) and it was approved by Ethical Committee for Animal Research of IPEN-CNEN/SP. All mice were housed under the same conditions and maintained on food and water *ad libitum*.

2.2.2 Cryptococosis induction and Photodynamic therapy *in vivo*

Cryptococcus neoformans ATCC-90112 were harvested from Sabouraud dextrose agar and then suspended in PBS at a concentration of approximately 10^8cfu/mL, which 0.1mL of the suspension was slowly inoculated in mice left paw.

Following tree days of the inoculation, the mice were divided into two groups: In PDT group, 0.05mL of 150μM MB was injected in the afflicted paw with a needle and a pre irradiation time of 30min. was set. In the control group, 0.05mL of PBS solution was injected to allow discrepancies in fungal recuperations.

Irradiation was performed with a diode laser (Photon Lase III, DMC, São Carlos, Brazil) emitting wavelength λ=660nm and output power of 100mW. Energy of 54J for 9min. of irradiation was delivered in contact with the paw, performing a dose of 180J/cm².

The animals were euthanized in CO_2 camber following the treatment. A sample of the tissue from each paw was removed aseptically, weighed, macerated in 1mL PBS, and then serially diluted in PBS to generate dilutions of 10^{-1} to 10^{-5} times the original concentration [4]. Ten-μL aliquots of each dilution were streaked onto a Sabouraud plus cloranphenicol agar plate in triplicate and incubated to allow colony formation [24].

2.2.3 *In vivo* statistics

The yeast colonies were counted and converted into cfu/g for analysis. All samples were submitted to this process and statistical analysis of the experimental data was performed using independent t-test, which retained the overall significance level at 5% ($p<0.05$).

3. RESULTS AND DISCUSSION

Neither light nor photosensitizer alone presented any effect on yeast cells inactivation. No statistically significant differences were observed among (L-PS-), (L+PS-), (L-PS+). One-hundred μL of methylene blue at 10 min. of cell contact had no toxicity effect on the samples, as well as the laser alone did not alter the number of viable yeast cells.

The overall finding was that yeast cells MB-stained are killed *in vitro* after red illumination (fig. 2).

The reduction of yeast cells viability in the tested PDT groups was effective in the used parameters. Note that at 6 min. of irradiation (108J/cm²), a statistically significant decrease on yeast viability was found (3 log reductions). A higher photofungicidal activity against *C. neoformans* (6 log reduction) was observed at 9min. of irradiation (162J/cm²).

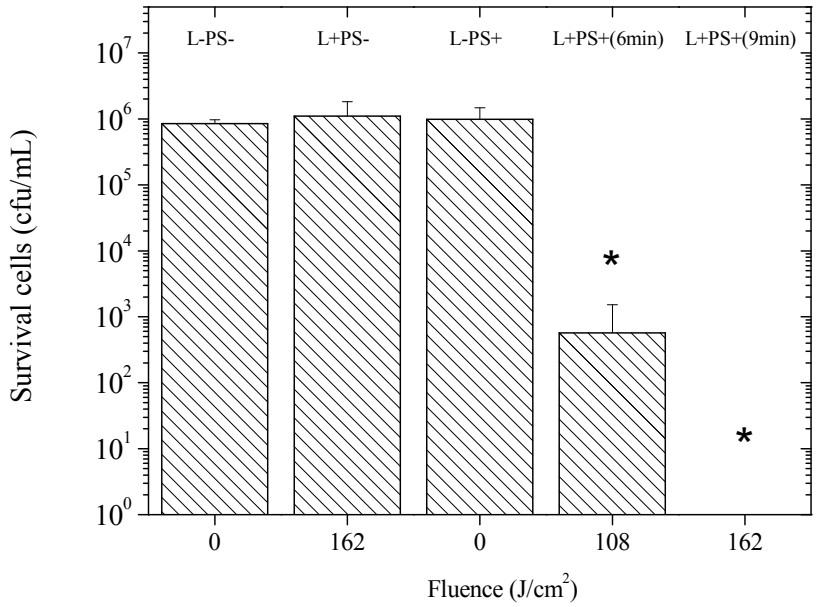

Fig. 2 - Means values +SD of *Cryptococcus neoformans* survival cells comportment. Neither light nor photosensitizer alone presented any effect on yeast cell inactivation. The samples which were treated by PDT (L+PS+) presented an irradiation time-dependent inactivation following laser irradiation at 6min and 9 min. Bars are standard deviations (SD) and (*) are statistical differences regarding to the control group (L-PS-).

Examination of cells in control group (L-PS-) revealed typical globular cells, which were observed on electron microscopy. Furthermore, some of them had lost their integrity due to the sample preparation (e.g. dehydrate process) (fig. 3A). On the other hand, the yeast cells harvested from PDT group (L+PS+9min) presented structural morphological changes compared to the control group. Note in fig. 3B that the yeast cells languish and their capsule are not so smooth, comparing to control group (compare fig. 3B to 3A).

The alterations on cell wall following PDT do not suggest a breakdown of the membrane (fig. 3B). In fact, it seems to be an apoptosis-like mechanism of death. Apoptosis mediated by methylene blue-PDT was previously described by Ball *et al.* [26]. Signals for apoptosis should be produced by an intracellular ROS disequilibrium, which triggers a mechanism of organelle autophagocytosis, and leads to cell destruction by one or more programmed cell death pathways. The mitochondria are particularly involved in the initiation of damage. The incomplete reduction of oxygen during the phosphorylase oxidation process may cause damage, including lipid and DNA peroxidation. Injure at the external mitochondrial membrane leads to further damage in the electron transfer chain and it may releases cytochrome C molecules in the fungal cytoplasm, which accelerates the metabolic cell failure [27,28].

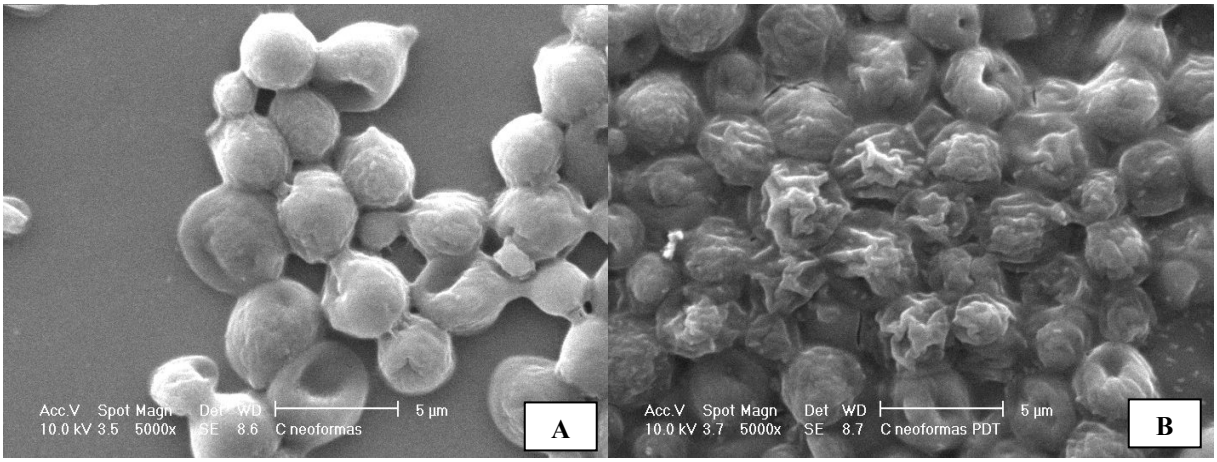

Fig. 3 – Scanning electron microscopy of *Cryptococcus neoformans* yeast cells are present in the figure (A) without treatment, and (B) following PDT. The yeast cells shown are representative of those seen for each condition. Scale bars represent 5µm.

Animals treated by PDT presented reduction of *C. neoformans* viability. The overall finding was that MB-stained infected tissue was able to kill yeast cells in the mice afflicted paw following red laser irradiation. The parameters used *in vivo* presented a moderated fungicidal effect. In addition, *C. neoformans* recover showed a statistically reduction after 9 min. of illumination, and the microbial reduction was about 1 log (fig. 4).

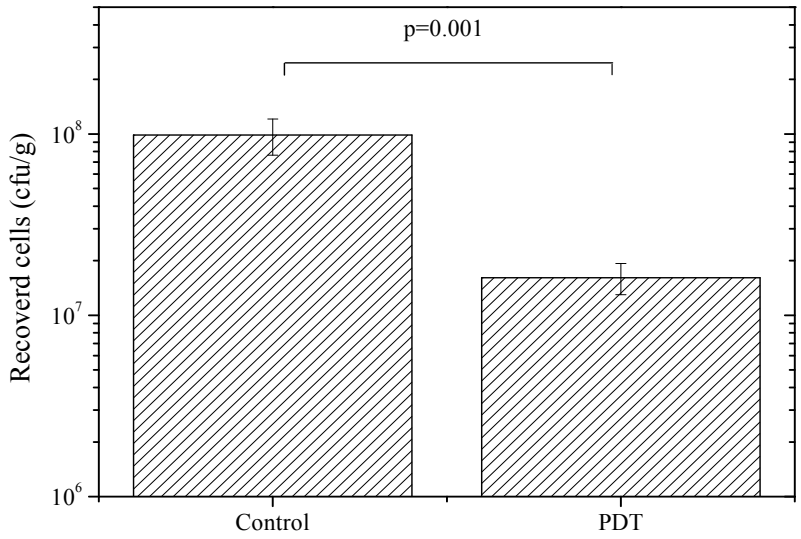

Fig. 4 - Means values ±SD of *Cryptococcus neoformans* survival cells (cfu/g). The animal treated by PDT presented a lower recuperation of yeast cells following 9 min. of laser irradiation on the stained tissue. Bars are standard deviations (SD).

Fuchs *et al*. [29] showed that *C. neoformans* is susceptible to photoinactivation and their results showed until 6 logs reduction of this yeast. Furthermore, cell wall structure of their samples had injuries caused by the lack of *ROM2* locus. In our work, the inactivation degree was changed by the illumination parameters. At 300mW/cm², it was found about 6 logs reduction, which shows that this capsulate yeast can be damaged by PDT. Although phototoxic effect on *C. neoformans* following PDT has being demonstrated in the literature, to the best of our knowledge it is the first application in animal model to treat dermal cryptococosis.

4. CONCLUSION

In conclusion, PDT seems to be an alternative to treat dermal cryptococcosis; however, more effective parameters must be found for *in vivo* studies.

5. ACKNOWLEDGMENTS

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