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In vitro reduction of pathogenic *sporothrix schenckii* fungus by photodynamic therapy

Terapia fotodinâmica na redução in vitro do fungo patogênico sporothrix schenckii

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Abstract

Sporotrichosis is a disease that affects the lymph vessels, skin and some internal organs. Most cases are presented as a subacute chronic mycosis caused by the *Sporothrix schenckii* fungus; fairly common in tropical regions. The aim of this study was to evaluate the susceptibility of *Sporothrix schenckii* yeast cells to the effects of photodynamic inactivation. For this, the viable cells were separated into four groups: irradiated with photosensitizer group (L+F+); irradiated without photosensitizer group (L+F-), without irradiation and with photosensitizer group (L-F+); and without irradiation and without photosensitizer group (L-F-). The methylene blue photosensitizer concentration used was 0.1 mg/mL, and the Aluminum Gallium Arsenide laser dose was 26.3 J/cm2. Then, counting of colony forming units (CFUs) was performed in each group. The main result was that the irradiated group with photosensitizer (L+F+) was the one that showed no growth of CFUs. Thus, it was concluded that *Sporothrix schenckii* can be inactivated by use of photodynamic therapy

Keywords: Sporotrichosis. *Sporothrix schenckii*. Methylene blue. Laser.

Resumo

A esporotricose é uma doença que afeta os vasos linfáticos, pele e alguns órgãos internos. A maioria dos casos se apresenta como uma micose subaguda à crônica, provocada por fungos do complexo Sporothrix schenckii, bastante comuns em regiões tropicais. O objetivo deste estudo foi avaliar a suscetibilidade aos efeitos da inativação fotodinâmica em células leveduriformes de fungos do complexo Sporothrix schenckii. Para tal, as células viáveis foram separadas em quatro grupos, sendo estes: grupo irradiado com fotossensibilizador (L+F+); grupo irradiado sem fotossensilizador (L+F-), grupo não irradiado com fotossensibilizador (L-F+); e grupo não irradiado sem fotossensibilizador (L-F-). A concentração do fotossensibilizador azul de metileno utilizada foi de 0,1 mg/mL, e a dosagem do laser de Arseneto de Gálio Alumínio foi de 26,3 J/cm2. Em seguida, foi realizada a contagem das unidades formadoras de colônias (UFCs) em cada grupo. Como principal resultado, verificou-se que o grupo irradiado com fotossensibilizador (L+F+) foi o único que não apresentou crescimento de UFCs. Dessa forma, concluiu-se que fungos do complexo Sporothrix schenckii podem ser inativados com o uso da terapia fotodinâmica.

Palavras-chave: Esporotricose. Sporothrix. Azul de Metileno. Lasers.

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INTRODUCTION

Sporotrichosis is a subcutaneous mycosis of subacute to chronic evolution, caused by the thermo-dimorphic fungi of the *Sporothrix schenckii* complex, which are known as mycelial (23 to 28°C) or yeast (35 to 37°C)[1]. These organisms are more prevalent in tropical regions, the soil being one of their main reservoirs, and they are a major source of contamination along with a number of plant organisms, which are also recognized as important reservoirs[2].

The cutaneous and lymphobuccal forms of sporotrichosis are the most common, and result from inoculation of the fungus by small lesions or traumas. Other forms, such as pulmonary, osteoarticular, meningeal and their dissemination, usually occur after inhalation of spores[3]. The treatment of fungal infection occurs by the administration of oral antifungal agents[4,5]. However, due to the indiscriminate use of these drugs, drug-resistant isolates can be identified, which consequently leads to therapeutic failures and remission of mycosis in humans[6-9]. The diagnosis consists of the microscopic identification of yeast structures of the *Sporothrix schenckii* complex fungi by the material collected from the lesions[10,11].

Cutaneous sporotrichosis affects people of any age, both sexes, and in various locations favorable to the fungus. The affected individuals hardly adhere adequately to the treatment, since this is, in most cases, of long duration (3 to 6 months). In addition, costs also negatively interfere with treatments, especially due to the need to use relatively new antifungal drugs[12].

There are innumerable microorganisms that can be inactivated by visible light after treatment with an appropriate photosensitizer (PS), which, in addition to reducing the number of microorganisms in vitro[10], has demonstrated local and non-systemic therapeutic action, thus, avoiding the side effects of a conventional treatment; especially when administered in immunocompromised patients[10].

The process of photodynamic therapy is based on the topical or systemic administration of a non-toxic photosensitizer, followed by low-dose irradiation with visible light and an adequate wavelength[13]. When in contact with oxygen found in cells, the activated PS can react with molecules and, by transfer of electrons or hydrogen, can lead to the production of free radicals (called the type I reaction), or by transferring energy to the oxygen (type II reaction), which leads to the production of singlet oxygen ($^1O2$). Both situations can lead to cell death and destruction of the diseased tissue. The $^1O2$ reacts with cellular components, since the unsaturated organic compounds are generally susceptible to the action of $^1O2$. The first barrier to $^1O2$ is the cell membrane, and, because it has unsaturated lipids in its composition, this can be damaged during the described process, causing cellular inviability[14,15].

The interest in efficient fungicide treatments has been increasing due to the increasing number of fungal pathogens responsible for nosocomial infections or opportunistic mycoses in immunocompromised patients. Safe and specific agents are scarce, and most of them are just fungistatic. In addition, the routine use of antifungals can progressively lead to the appearance of resistant strains[16]. Yeasts, such as *Saccharomyces marxianus*, have been used as model organisms to elucidate the types of damage caused by photodynamic therapy (PDT) in eukaryotic cells[14]. This fact has aroused interest in PDT in fungi, and the target has mainly been its use in pathogenic or potentially pathogenic fungi[17].

Considering the potential of this technique in the treatment of fungal infections and the importance of developing new antifungal strategies, PDT can be considered a promising alternative for the treatment of patients with sporotrichosis[18,19]. Therefore, the objective of this study was to evaluate the susceptibility of *Sporothrix schenckii* complex fungal yeast cells to the effects of photodynamic inactivation.

METODOLOGY

Cultivation of *Sporothrix schenckii* fungi

The fungal mycelial form of the *Sporothrix schenckii* complex (Ss40 isolates) was grown on Sabouraud dextrose agar (Difco, Detroit, USA), and incubated at 37°C for 7 days. Subsequently,

yeast cells were transferred to an Erlenmeyer flask containing 200 mL of brain and heart infusion (BHI, Acumedia), supplemented with dextrose (18 g/L), pH 8.0, and incubated at 37°C for 10 days under slow shaking. Fungus yeast cells were used in all experiments.

Sample Preparation

Yeast cells were transferred to 50 mL falcon tubes and washed in autoclaved deionized water, centrifuged at 1300 rpm, 4°C, for 10 minutes. This procedure was repeated 3 times, and the precipitate was resuspended in 5 mL of sterile physiological solution (0.85% NaCl). After washing, the counting of viable cell counts was performed in a spectrophotometer (B582, Micronal, Brazil) with a wavelength of 530 nm and an optical density of 0.284. From the 106 suspension of *Sporothrix schenckii* complex fungi, 20 trials were performed, 5 of which were for each experimental group, which were as follows: Group L+F+ (irradiated with laser in the presence of the photosensitizer); Group L+F- (irradiated only with laser); Group L-F+ (treated only with the photosensitizer); Group L-F- (not irradiated by laser and without photosensitizer).

Photosensitizer and Laser

The methylene blue photosensitizer (Sigma-Aldrich, St. Louis, MO, USA) was used at the concentration of 0.1 mg/mL for each sample and dissolved in sterile double-distilled water, filtered in a sterile membrane with pores 0.22 µm in diameter (Millipore, São Paulo, Brazil). After filtration, the solution with the photosensitizer was kept in the dark.

The light source used was the Aluminum Gallium Arsenide (Easy Laser, Clean Line, Taubaté, Brazil) laser, with a wavelength of 660 nm, an energy density of 26.31 J/cm², 10 J of energy, 35 mW of power, and 285s of irradiation in an area of 0.38 cm².

In vitro photosensitization

In all experimental groups, 0.1 mL of S. Schenckii suspension was added to each well of the sterile, capped, flat bottom 96-well microtiter plates (Costar Corning, New York, USA). Subsequently, cells from the L+F+ and L-F+ groups received 0.1 mL of photosensitizer. In the L+F- and L-F- groups 0.1 mL of physiological solution was added. Then all plates were shaken for 5 minutes on an orbital shaker (Solab, Piracicaba, Brazil). After this period, the contents of the wells of the plates belonging to the L+F+ and L+F- groups were irradiated according to the above protocol. The irradiation was carried out under aseptic conditions, in a laminar flow chamber in the absence of light, where a black matte screen with a hole diameter coinciding with the well inlet was used, thus avoiding light scattering.

After irradiation, serial dilutions were performed, and 0.1 mL aliquots of each dilution were seeded in duplicate on Sabouraud dextrose agar plates (Difco, Detroit, USA). After incubation, counts of colony forming units per milliliter (CFU / mL) were performed, and the numbers obtained were transformed into logaritham (Log). The results were submitted to analysis of variance ANOVA and to Tukey's test, considering statistical difference when $p \leq 0.05$, with the aid of Minitab software (Inc. PA, USA).

RESULTS

The use of photodynamic therapy for inactivation of fungi of the Sporothrix schenckii complex, according to the conditions described in Table 1, promoted a significant reduction in the number of CFUs. The concentration of the methylene blue photosensitizer did not vary between groups. Evaluating the four groups, we observed a reduction of 100% of the CFU / mL in the L+F+ group in relation to the control group (L-F-). The mean values of the CFU / mL for the L-F-, L+F- and L-F+ groups were similar, with no statistically significant difference between these groups, although colony growth was observed.

The *Sporothrix schenckii* complex fungi were sensitive neither to the photosensitizer used alone (L-F+), nor to the application of laser alone (L+F-). In all experimental conditions, these results indicate that the group undergoing photodynamic therapy achieved microbial growth inhibition of the *Sporothrix schenckii* complex fungi (Figures 1A, B, C and D, and Figure 2).

Table 1 – Description of the concentrations (mg/mL) and the density of laser energies (J/cm2) used in the PDI (Taubaté – SP, March 2016).

Experimental Groups	(A) L-F-	(A) L-F+	(A) L+F-	(B) L+F+
Methylene blue dye (mg/mL)	0.1	0.1	0.1	0.1
Aluminum Gallium Arsenide Laser (J/cm²)	26.31	26.31	26.31	26.31
Sporothrix schenckii	3.23	3.21	3.20	0

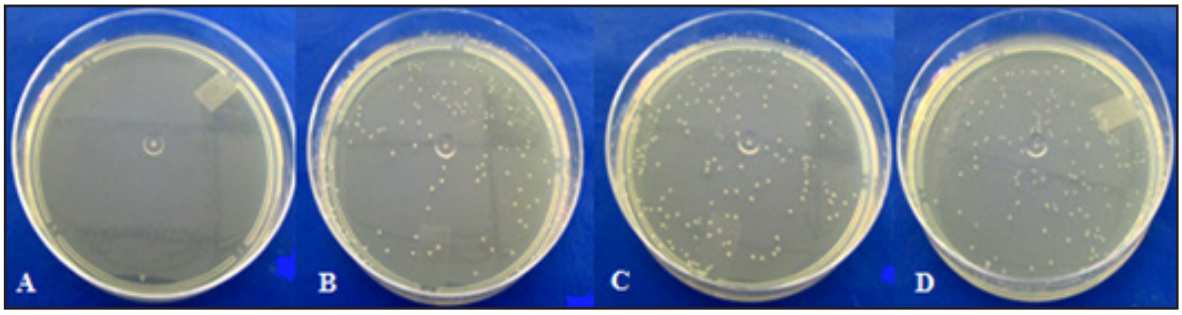

Figure 1 – Growth of fungi of the *Sporothrix schenckii* complex in Petri dishes containing Sabouraud-dextrose agar, according to experimental groups. (A) Group L+F+; (B) L+ F- group; (C) L-F+ Group; (D) L-F- (control) group. (Taubaté - SP, March 2016).

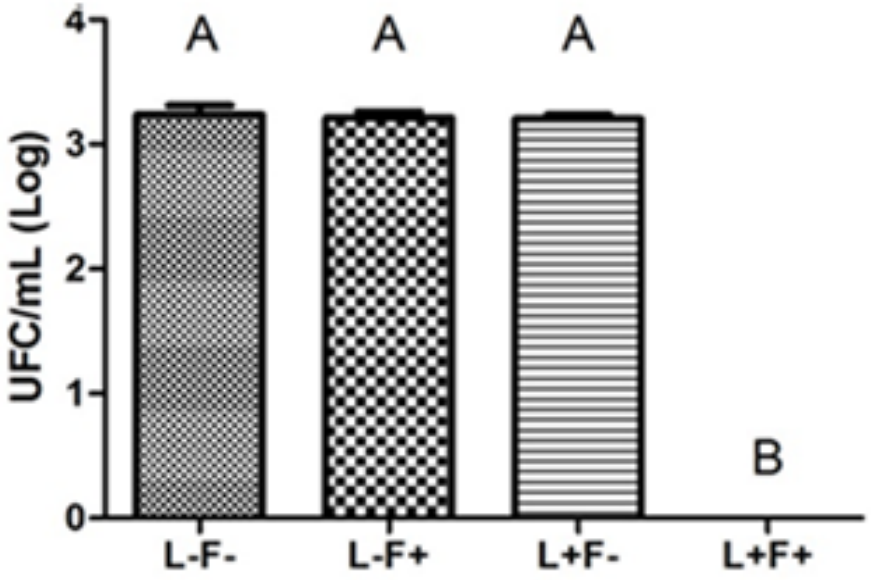

Figure 2 – CFU count after PDT with Aluminum Gallium Arsenide laser. (A) Groups L+F-; L-F+; L-F-. (B) Group L+F+ ($p < 0.0001$). (Taubaté - SP, March 2016).

DISCUSSION

Advances in sporotrichosis treatments have always been concentrated on the use of antifungal agents of various classes. However, with the appearance of resistance to these therapies, the need for exploration of new technologies for the treatment of this infection has appeared, thus justifying advances in *in vitro* research lines[10].

Although there are studies that demonstrate the use of dye-associated light as an effective procedure for microbiological inactivation, some variables still generate considerable influence in this treatment, such as: the type and concentration of the dye, the species of the microorganism, the pre-irradiation period, the source of light, and the dose used[10,13]. Tests with laser irradiation, or even non-irradiated dye in the presence of yeast suspension, did not show any deleterious effects on the growth of the *Sporothrix schenckii* complex fungi[13,15-19]. However, the concentration of 0.1 mg/ml, followed by low power GaAlAs laser irradiation at energy densities of 26.31 J/cm², reduced the number of log CFU mL of the fungus.

According to Souza et al.[20], the activation of methylene blue photosensitizers at a concentration of 0.1 mg/mL at 685 nm of laser light (28 J/cm2), reduced the number of CFU/mL by 88.6% of C. *albicans*. In the present study, using a light source with an energy density dose of 26.31 J / cm², 10 J of energy, 35 mW of power, and 285 seconds of irradiation in an area of 0.38 cm², a reduction of the log number of microbial CFU/mL was obtained. This shows similar susceptibility of the *Sporothrix schenckii* complex fungi compared to the susceptibility of the studies developed with Candida albicans[20].

Also in this study, photoactivation using methylene blue (0.1 mg/mL), followed by low power GaAlAs laser irradiation at energy densities of 26.31 J/cm², reduced the number of log CFU / ml *Sporothrix schenckii* complex fungi. In a similar way, it has been shown that the combination of methylene blue and aluminum gallium arsenide (685 nm and 28 J/cm2) caused a reduction of 88.6% of C. *albicans*, 84.8% of C. *dubliniensis*, 91.6% of C. *krusei*, and 82.3% of C. *tropicalis*[20,21]. In the present experiment, the same laser energy density of Gallium Aluminum Arsenide in J/cm² was used, in addition to the same concentration of methylene blue dye in the samples, in order to evaluate its effect on S. *schenckii*[2,5,8-10], because the reduction of *Ckefyrse* results from the existence of a membrane efflux pump[22,23].

The number of colony forming units per milliliter (CFU/mL) and the numbers obtained were transformed into logarithem (log), submitted to analysis of variance ANOVA and Tukey's test, and considering the existence of statistical difference when $p < 0.05$, this in vitro study demonstrated that there was no fungal growth under the experimental conditions already described. For the experimental condition of PDT with the *Sporothrix schenckii* complex fungi, L+F+ was the group in which the best results were obtained. In this case, it can be assumed that there was a greater production of singlet oxygen, causing a greater chance of destruction of intracellular organelles, which effectively inhibited the growth of the *Sporothrix schenckii* complex fungi.

In the group irradiated with the laser only (L+F-, log of 3.20), there was fungal growth. Similarly, in the groups treated with the photosensitizer in the absence of laser (L-F+, log of 3.21), and in the group not irradiated by the laser and without photosensitizer L-F-, log of 3.23) there was considerable growth. Based on these data, it can be inferred that only in the presence of the photosensitizer and the low-power laser is it possible to reduce microbial growth. Thus, the limited effect of the therapy imposed on these groups is observed, since the growth of the microorganism was observed. What can be expected is that the isolate used, even if it consists of a wild culture with all its mechanisms of defense and exchange of genetic material, can present a greater sensitivity to the therapy since there are different types of *Sporothrix schenckii* cataloged, with several genotypes[10,11].

Thus, this study indicated the efficiency of methylene blue as a photosensitizer associated with a low power laser in inhibiting the growth of *Sporothrix schenckii* complex fungi. Thus, this research opens new perspectives for *in vivo* studies in order to explore the potential application of the protocol described for the treatment of sporotrichosis.

CONCLUSIONS

Based on the experimental conditions described in this work, the photodynamic therapy associated with the methylene blue dye was effective in the inactivation of the fungal suspension of the *Sporothrix schenckii* complex. The viability and / or inviability of the microorganisms in the photodynamic inactivation was independent of the concentration of the dye and the energy density of the laser, since these were the same in the different groups tested. The combination of irradiation with laser in the presence of the photosensitizer was effective in the in vitro control of the species studied.

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