Antimicrobial photodynamic therapy in rat experimental candidiasis: evaluation of pathogenicity factors of Candida albicans

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Objective. This study evaluated the effects of photodynamic therapy on pathogenicity of Candida albicans.

Study design. Fifty-six rats were submitted to development of candidiasis on the tongue dorsum by C. albicans inoculations. After 5 days, different treatments were administered: laser and photosynthesizer methylene blue (L+P+); laser only (L+P−); photosensitizer only (L−P+); and physiologic solution only (L−P−). Samples of the oral cavity were collected for a count of colony-forming units per ml. Colonies were isolated for evaluation of proteinase and phospholipase activities. The rats were killed for microscopic analysis of the tongue dorsum. The data were analyzed by analysis of variance, Kruskal-Wallis, and Bonferroni tests.

Results. The number of C. albicans recovered from the oral cavity of the rats was similar between the groups (P = .106). The L+P+ group showed fewer microscopic lesions of candidiasis than the L−P− group (P = .001). The L+P+ group presented lower proteinase activity compared with the other groups, with significant difference between the groups L+P+ and L−P− (P = .018).


Fungi are important agents of human disease. Among the most important fungal pathogens are yeast species belonging to the genus Candida. These species can cause a wide range of human diseases, ranging from superficial mucosal infections, such as vulvovaginal and oropharyngeal candidiasis, to life-threatening invasive infections.1-4 Several virulence factors contribute to the pathogenicity of C. albicans, including the ability to adhere to epithelial cells, the ability to form hyphae, and secretion of extracellular enzymes.5-7 During the initial stages of superficial mucosal infection, C. albicans forms filamentous hyphae, which show thigmotropism, a phenomenon also known as contact guidance, in addition to releasing various hydrolytic enzymes, such as extracellular phospholipases and secretory aspartyl proteinases.5

The widespread use of topical and systemic antifungal agents as the conventional treatment for oral candidiasis has resulted in the development of resistance in C. albicans.8 Therefore, the study of additional methods for the control of C. albicans, such as photodynamic antimicrobial chemotherapy (PACT), has become essential. PACT is a process that combines light and a photosensitizing drug, promoting a phototoxic effect on the treated cells, in general via oxidative damage. The potential of PACT to promote microbial eradication is becoming progressively more accepted. The technique involves the production of highly cytotoxic singlet oxygen and other reactive oxygen species, promoting photodynamic microbial damage.9,10

As an antifungal therapy, PACT is very much a developing science, and the vast majority of published work has understandably centered on in vitro laboratory investigations. Various Candida species, photosensitizers, and irradiation protocols have been used. In most cases, complete killing of the yeast has been readily achieved. Critically, no reports on development of resistance to antifungal PACT currently exist, and the treatment has not been associated with mutagenic effects or genotoxicity in either fungi or cultured human cells.2 The most extensively investigated photosensitizer classes investigated in in vitro antifungal PACT studies have been the phenothiaziniums,11-14 the porphyrins,15,16 and the phthalocyanines.17

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The efficacy of PACT on yeasts of the Candida genus has also been demonstrated by some in vivo studies. Teichert et al. tested several concentrations of methylene blue associated with a diode laser on buccal candidiasis in immunosuppressed mice. Analysis of the results indicated the efficacy of PACT in reducing yeast, an effect that was directly proportional to the photosensitizer concentration. Junqueira et al. evaluated the effects of photodynamic therapy (PDT) on buccal candidiasis in rats and verified that the rats treated with a laser and with methylene blue developed more discrete candidiasis lesions compared with the control groups. Mima et al. observed that PACT with porphyrin and red light–emitting diode (LED) promoted significant reduction in the viability of C. albicans in buccal candidiasis in immunosuppressed mice.

Though many in vitro and in vivo studies have shown inactivation of C. albicans by PACT, few studies have focused on the effects of PACT on the virulence factors of C. albicans. Munin et al. demonstrated that PDT using methylene blue as a photosensitizing drug inhibits both the growth and the germ tube formation of C. albicans. Soares et al. verified that toluidine blue with LED inhibits in vitro growth and adhesion of different Candida isolates to buccal epithelial cells. Regarding the effects of PACT on secretion of extracellular enzymes, which is an important virulence factor of C. albicans, there are no studies in the literature. Therefore, the objective of the present study was to evaluate the effects of PDT on secretion of the extracellular enzyme by C. albicans using a murine model of buccal candidiasis.

**MATERIAL AND METHODS**

**Experimental animals**

This study was approved by the Research Ethics Committee of the São José dos Campos School of Dentistry under protocol no. 035/2007-PA/CEP. Fifty-six male rats (Rattus norvegicus, Albinus, Wistar) negative for the Candida genus in the buccal cavity, weighing ~250 g (120 days old), were included in the study. Each group was composed of 14 rats: 10 with experimental candidiasis and 4 without experimental candidiasis (Fig. 1).

**Induction of experimental candidiasis**

The rats were given a solution of 0.1% tetracycline hydrochloride (Terramycin; Pfizer, São Paulo, Brazil) in their drinking water. This treatment was initiated 7 days before the inoculation of C. albicans suspension and was maintained throughout the experiment. A suspension of C. albicans containing $5 \times 10^8$ viable cells/mL was prepared according to Reed et al. A reference strain (ATCC 18,804) of C. albicans was
used. For the inoculation of this suspension, the rats were sedated via intramuscular injection of xylazine chloride solution (Vetbrands, São Paulo, Brazil) and ketamine (Vetbrands) in the proportion 1:0.5 mL at a dose of 0.05 mL/100 g of body weight. The _C. albicans_ suspension (0.2 mL) was dropped into the mouths of the rats with the aid of a 1 mL syringe. The material was spread on the tongue dorsum with a swab that had been previously soaked in the suspension. This procedure was repeated for 3 consecutive days.

**Photosensitizer and laser**

A 0.1 mg/mL solution of methylene blue was used as the photosensitizer and was prepared by dissolving the powder (Sigma, São Paulo, Brazil) in a physiologic solution of 0.85% sodium chloride (NaCl). The solution was filtered through a sterile membrane filter with 0.22-μm-diameter pores (TPP, Trasadingen, Switzerland) and stored in the dark.

The light source used was a gallium-aluminum-arsenide (GaAlAs) laser (Photon laser III; DMC, São Carlos, Brazil) with a wavelength of 660 nm, output power of 100 mW, energy density of 245 J/cm², and 57.3 g NaCl, 0.55 g CaCl₂, and 8% sterile egg yolk emulsion were used. Test strains were spot inoculated (6 mm) and plates were incubated at 37°C for up to 5 days. Each isolate was tested in duplicate. The diameter of each colony and the total diameter of the colony and precipitation zone (Pz) was measured, and phospholipase activity was scored according to the method described by Price et al.²⁶ The Pz value, representing the ratio of the colony alone to the diameter of the colony plus the Pz, was determined. The results were classified as negative (Pz = 1 cm), positive (0.64 cm ≤ Pz < 1 cm), and strongly positive (Pz < 0.64 cm). Thus, a high Pz value means low enzymatic activity.

The isolates were tested for proteinase secretion in bovine serum albumin (BSA) agar that contained yeast carbon base (1.17%), yeast extract (0.01%), and BSA (0.2%), according to Ruchel et al.²⁷ The medium was adjusted to pH 5.0, sterilized by filtration, and added to autoclaved 2% agar. Test strains were spot inoculated (6 mm), and plates were incubated at 37°C for up to 5 days. Each isolate was tested in duplicate. After incubation, plates were stained with 0.5% amido black and the zone of clearance around the colony was recorded. Scoring was carried out by determination of the Pz value, as for phospholipase activity.

**Killing the rats**

In each experimental group, the rats were killed 1 day after the respective treatments, corresponding to 7 days after experimental candidiasis induction. The tongues were removed and analyzed by stereomicroscopy (Carl Zeiss, Jena, Germany).

**Microscopic analysis of the tongue dorsum**

For light microscopy analysis, the tongues were fixed in 10% formalin for 24 hours and hemisected in the
The histologic analyses of the tongue dorsum verified that most of the rats showed few yeast and Candida hyphae in the keratin layer. However, many tissue lesions were observed, characterized by epithelial lesions and inflammatory infiltrate in the lamina propria. The epithelial lesions included epithelial hyperplasia, disorganization of the basal layer, exocytosis, spongiosis, loss of filiform papillae, hyperparakeratosis, and the formation of intraepithelial microabscesses. Regarding chronic inflammatory infiltrate in the conjunctive tissue, the following scores were attributed: 0 (absence of inflammatory cells), 1 (discrete inflammatory infiltrate), 2 (moderate inflammatory infiltrate), and 3 (accentuated inflammatory infiltrate).

Statistical analysis

Statistical analysis was performed using the Minitab Program, using a 5% level of significance (\( P < .05 \)). The CFU/mL (log) results and Pz values of enzymatic activities were analyzed by analysis of variance (ANOVA) and the Tukey test. For evaluation of the results obtained from the histologic analysis, the Kruskal-Wallis and Bonferroni tests were used.

RESULTS

The number of C. albicans (CFU/mL) recovered from the oral cavity after the experimental treatment presented reduced numbers of C. albicans compared with samples collected before the treatment for all groups studied. ANOVA was applied to the difference value between the CFU/mL obtained before and after the experimental treatment. There was no significant difference between the groups L+P+, L+P–, L−P+, and L−P− (\( P = .106 \)).

Phospholipase activity was positive or strongly positive for all C. albicans isolates. No isolate presented negative activity. The phospholipase activity was strongly positive for 40% of the isolates of the L+P+, 60% of the L+P–, 80% of the L−P+, and 60% of the L−P− group. Regarding the Pz value, there was no significant difference between the groups (\( P = .298 \)). Proteinase activity was strongly positive for all C. albicans isolates. The L+P+ group presented lower proteinase activity compared with the other groups, with significant difference between the groups L+P+ and L−P+ (\( P = .018 \)). The data of phospholipase and proteinase activities are shown in Fig. 3.

The histologic analyses of the tongue dorsum verified that most of the rats showed few yeast and Candida hyphae in the keratin layer. However, many tissue lesions were observed, characterized by epithelial lesions and inflammatory infiltrate in the lamina propria. The epithelial lesions included epithelial hyperplasia, disorganization of the basal layer, exocytosis, spongiosis, increased mitosis numbers in the basal layer, loss of filiform papillae, microabscesses and hyperparakeratosis. In these areas of tissue lesion, the lamina propria exhibited predominately mononuclear inflammatory infiltrate and, occasionally, congested vessels. These tissue lesions were located at the transition between simple conical and giant papillae (Fig. 4).

The epithelial lesions were quantified and showed that the L+P+ group presented fewer candidiasis lesions than the other groups, with significant difference between the L+P+ group and the L+P–, L−P+, L−P− groups (Fig. 5). In the semiquantitative analyses of inflammatory infiltrate, the median scores were similar in all the groups studied (data not shown).
DISCUSSION

Many oral *C. albicans* infection models in nonhuman animals have been described, with persistent infections usually requiring some form of immunosuppression or other manipulation, oral fungal burdens in mice and rats are variable and often decline rapidly.28

In this study, experimental candidiasis was induced by tetracycline administration and by *C. albicans* inoculations. Five and 6 days after the *Candida* inoculation, the rats were subjected to the following experimental treatments: laser and methylene blue (L+P+); laser only (L+P−); photosensitizer only (L−P+); and physiologic solution (L−P−). Before and 1 day after the experimental treatment, samples of the oral cavity of the rats were collected to determine the CFU/mL count; these corresponded to 5 and 7 days after the *Candida* inoculation. The number of *C. albicans* (CFU/mL) recovered from the oral cavity of the rats before and after experimental treatment presented log reductions of 0.35 (L+P+), 0.88 (L+P−), 0.53 (L−P+), and 0.56 (L−P−). These reductions did not present significant differences between the groups, demonstrating that it was not related to the experimental treatment but to the experimental candidiasis model studied. According to Samaranayake and Samaranayake,29 during the development of experimental candidiasis, the yeasts and hyphae are eliminated from the organism by the host’s immune system.

The experimental candidiasis model used in this study made possible the recovery of 4-5 CFU/ml(log) of *C. albicans* after 5 and 7 days after yeast inoculation in all of the groups studied. Mima et al.8 also recovered 4-5 CFU/mL(log) from mouse tongues after 5 days of *C. albicans* inoculation for the control group (L−P−). However, the recovery of *C. albicans* in their group treated with PDT (L+P+) was 3-4 CFU/mL(log). Those authors did not count the number of CFU/mL before the experimental treatment, to avoid removing *Candida* cells from the tissue with the swab, which could potentially interfere with the results, decreasing the CFU/mL values.

In the histologic analyses, candidiasis lesions were observed on the tongue dorsum of the rats. These lesions were characterized by epithelial hyperplasia, disorganization of the basal layer, exocytosis, spongiosis, increased mitosis numbers in the basal layer, loss of filiform papillae, hyperparakeratosis, and inflammatory infiltrate in the lamina propria. These lesions are all characteristic of the development of experimental candidiasis on rat tongues and have been described by several authors, including Allen et al.,30 Jorge et al.,31 and Junqueira et al.19,32 Samaranayake and Samaranayake29 reported that although infection by *Candida* is restricted to the keratin layer in the epithelial surface, tissue changes occur in the deepest layers of the epithelium and can probably be attributed to extracellular

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**Fig. 4.** Sagittal section of the tongue dorsum of a rat from the L−P− group. Tissue lesion characterized by loss of filiform papillae, epithelial hyperplasia (diamonds) and hyperparakeratosis (arrow). Hematoxylin-eosin; original magnification ×200.

**Fig. 5.** Numbers and medians of epithelial lesions observed in the histologic analyses of the tongue dorsum. Different letters represent significant difference between the groups: L+P+ and L+P− (P = .007), L+P+ and L−P+ (P = .001), and L+P+ and L−P− (P = .001).
enzyme production, such as proteinase and phospholipase.

The \( L+P^+ \) group presented fewer candidiasis microscopic lesions than the other groups (\( L+P^-, L^-P^+, L^-P^- \)), suggesting that PDT showed some effect on experimental candidiasis in rats. According to Maisch,\(^3\) for PDT to be toxic for the fungus, it must induce the inflammatory pathway that activates immune cells, such as macrophages and neutrophils, which kill Candida.

Junqueira et al.\(^19\) using methodology similar to that used in the present study, also verified that the group treated with 1 session of laser and methylene blue exhibited fewer candidiasis microscopic lesions on the tongue dorsum compared with the control group (\( L^-P^- \)). However, in most of the rats, the infection process was not completely terminated, and those authors reported that more in vivo work should be performed and should include more PDT sessions. In the present study, PDT was used on the tongue dorsum for 2 consecutive days and candidiasis microscopic lesions were not observed in 6 of 10 rats analyzed in the \( L+P^+ \) group. Most likely, for clinical success of the treatment of buccal candidiasis, many PDT sessions should be requested. However, the application of many PDT sessions is difficult to perform in rats, because these animals cannot bear many anesthetic treatments.

In the present study, the effects of PDT on healthy oral tissue were also evaluated in 16 rats that did not receive \( C. albicans \) inoculations. Histologic alterations after applications of photosensitizer or laser treatments were not observed on the healthy tongue dorsum. Luan et al.\(^34\) also reported that no necrotic or inflammatory changes were found in the gingival, dentin, dental pulp, or alveolar bone of mice after application of toluidine blue photosensitizer and laser irradiation. Thus, the results of this study suggest that PDT with methylene blue is a safe antimicrobial treatment for oral candidiasis without damaging effects on normal tissues.

To evaluate the effects of PDT on pathogenicity factors of \( C. albicans \), the yeast recovered from the oral cavity of the rats was submitted to tests of phospholipase and proteinase activity. The group that received PDT (\( L+P^+ \)) showed higher \( P_z \) values for phospholipase and proteinase and thus lower enzymatic activity compared with the other groups. However, significant differences were observed only for proteinase activity.

It is probable that the yeast that were not killed by PDT exhibited lower proteinase activity owing to interference of PDT on the cell secretion pathway. According to Naglik et al.,\(^35\) the pathway of proteinase synthesis starts in the rough endoplasmic reticulum where the N-terminal signal peptide is removed by the signal peptidase complex. Later, in the trans-Golgi network (TGN), the propeptide is cleaved after a conserved Lys-Arg site by the subtilisin-like endoproteinase Kex2. Leaving the TGN, the proteinases are packaged into secretory vesicles, are transported to the plasma membrane, and either are released to the extracellular space (sap 1-8) or remain attached to the cell wall (sap 9 and 10).

Although there was no significant difference among the groups for phospholipase \( P_z \) value, the phospholipase activity was strongly positive for only 40% of the isolates of the \( L+P^+ \) group, whereas it was strongly positive for 60% of the \( L+P^- \), 80% of the \( L^-P^+ \), and 60% of the \( L^-P^- \) group. These results suggest that more studies should be developed to investigate the role of PDT on the phospholipases and secretory aspartyl proteinases, and consideration should be given to providing comparisons with antifungal drug therapies.

We conclude that PDT reduced the candidiasis microscopic lesions and the proteinase activity of \( C. albicans \) in a murine model of oral candidiasis, without damaging effects to normal tissues.

**REFERENCES**


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