

Effect of erythrosine- and LED-mediated photodynamic therapy on buccal candidiasis infection of immunosuppressed mice and *Candida albicans* adherence to buccal epithelial cells

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Objective. This study evaluated the effects of photodynamic therapy (PDT) on buccal candidiasis in mice and on the adherence of yeast to buccal epithelial cells (BECs) in vitro.

Study Design. A total of 56 immunosuppressed mice with buccal candidiasis were subjected to PDT, consisting of treatment with erythrosine (400 $\mu\text{mol/L}$) followed by exposure to a green LED (14.34 J cm^{-2}). After treatment, the yeasts recovered from the mice were quantified (CFU/mL) and analyzed for the effects of PDT on their adherence to BECs. The data were analyzed using ANOVA, the Tukey test, Kruskal-Wallis test and Student *t* test.

Results. PDT significantly reduced the amount of yeast present in the lesions by 0.73 \log_{10} ($P = .018$) and reduced *C. albicans* adherence to BECs by 35% without damaging adjacent tissues ($P = .045$).

Conclusions. Photodynamic therapy exhibited antifungal effects against *C. albicans* biofilms formed in vivo and reduced the capacity of *C. albicans* to adhere to BECs in vitro. (Oral Surg Oral Med Oral Pathol Oral Radiol 2012;114:67-74)

Buccal candidiasis is the most common fungal infection in immunocompromised patients, such as individuals with acquired immunodeficiency syndrome (AIDS) and is often an indicator of the progression of human immunodeficiency virus (HIV) infection.¹ Buccal candidiasis is an opportunistic infection that affects individuals who use broad-spectrum antibiotics and immunosuppressive drugs, individuals with a prosthesis, and those with xerostomia or poor buccal hygiene.²⁻⁴ The most common acute manifestation of buccal candidiasis is pseudomembranous candidiasis, which begins with the adherence of yeast to

buccal epithelial cells (BECs) and subsequent colonization, biofilm formation, and damage to host tissues. Biofilms formed by *Candida albicans* are composed of blastoconidia, pseudohyphae, and hyphae embedded in extracellular polymeric substances that form channels and pores.^{5,6} This structure constitutes an important virulence factor that confers protection from the immune system and resistance to chemical and antifungal agents.^{5,7,8}

The antifungals fluconazole and amphotericin B are primary treatments for buccal candidiasis, and they have satisfactory performance against *Candida*; however, some strains have reduced susceptibility to antifungals because of recurrent episodes of candidiasis and selection for resistant strains.^{6,9} Schelenz et al.⁹ isolated 269 strains of *Candida* spp. from the buccal cavity of patients with hematological malignancies or head and neck solid tumors. The authors evaluated the sensitivity profile of these strains to antifungal agents and found resistance to fluconazole (4.5%), itraconazole (11.7%), ketoconazole (11.3%), voriconazole (0.75%), and caspofungin (41.1%); however, all strains were susceptible to amphotericin B. Thus, amphotericin B has been used in treatment of *Candida* infections caused by fluconazole-resistant strains; however, amphotericin B can have substantial side effects, and a few cases of resistance have been reported.⁶

As an alternative to conventional antibiotic therapy, photodynamic therapy (PDT) has demonstrated potent antifungal activity against *Candida* spp., both in vitro and in vivo.¹⁰⁻¹⁴ In this therapy, a photosen-

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sitive agent, which is activated by a light source at a specific wavelength in the presence of oxygen, resulting in the production of reactive oxygen species and free radicals, is administered. These compounds disrupt the *Candida* cytoplasmic membrane and cause an increase in cellular permeability with subsequent damage to intracellular targets. It has been suggested that this intracellular damage might compromise virulence factors of the microorganism, such as the capacity to adhere to host epithelial cells, production of proteinases, reduction of biofilms and formation of germ tubes.^{10,14-19}

Methylene blue- and laser-mediated PDT has been shown to reduce the epithelial alterations and chronic inflammatory responses related to buccal candidiasis in rats, without observed reductions in the number of yeasts.^{12,14} In contrast, other studies have demonstrated a reduction of 1.0 to 2.7 log₁₀ of *C. albicans* cells in immunosuppressed mice with buccal candidiasis following treatments with high concentrations of methylene blue or Photogen photosensitizers with long periods of irradiation under laser or blue/red LEDs.^{13,20} Mice have also been used to study *C. albicans* and offer a few advantages over a rat model in that mice do not harbor *Candida* spp. in their buccal cavity and therefore do not elicit an adaptive immune response against this yeast^{3,21}; moreover, immunosuppression is the basis for inducing candidiasis in the mouse model, which reflects the oral ecology of a patient with AIDS.²²

At present, the effects of PDT on *C. albicans* growth, adherence, and virulence factors are not well defined. Thus, the aim of this study was to evaluate the action of PDT mediated by erythrosine, a dye commonly used in dentistry for detecting dental biofilms, in the treatment of buccal candidiasis in an immunosuppressed mouse model. The yeasts (*C. albicans*) that survived PDT were subsequently evaluated for their capacity to adhere to BECs in vitro.

MATERIAL AND METHODS

Experimental animals

The Animals Research Ethics Committee of the School of Dentistry of São José dos Campos (UNESP) approved this study under protocol number 022/2009-PA/CEP. Fifty-six adult male mice (*Mus musculus*, Albinus, Swiss), weighing 30 to 60 g and with no *Candida* in their buccal cavities, were included in the study. Animals were divided into 2 groups: 48 animals were used to study the effect of PDT on experimental candidiasis induced by *C. albicans*, and 8 animals were used to study the effect of PDT on the tongue dorsum without inoculation with *C. albicans*.

Photosensitizer and light source

Erythrosine (Aldrich Chemical Co., Milwaukee, WI, USA) was used for the sensitization of *C. albicans* in the buccal cavity. A 400- μ mol/L solution of erythrosine was prepared by dissolving the powdered dye in phosphate-buffered saline (PBS, pH 7.4). The solution was filter sterilized through 0.22- μ m pore-size membranes (MFS, Dublin, CA, USA). After filtration, the dye solution was stored in the dark. As light source, a green light-emitting diode (LED) (MMOptics, São Carlos, SP, Brazil) with a wavelength of 532 ± 10 nm at an output power of 90 mW and 16.2 J of total energy for 3 minutes was used. The fluency rate was 80 mW cm⁻² and the fluence was 14.34 J cm⁻². The irradiated area was 1.13 cm². The parameters used in this study were determined from previous in vitro studies involving *C. albicans* biofilms according to Costa et al.¹⁹

Induction of experimental candidiasis

The methodology described by Takakura et al.²³ was used to induce experimental candidiasis with some modifications. Briefly, the animals were immunosuppressed with 2 subcutaneous injections of prednisolone (Depo-Medrol, Laboratórios Pfizer Ltda., Guarulhos, SP, Brazil) at a dose of 100 mg/kg of body weight 1 day before and 3 days after infection with *Candida*. Tetracycline chloride (Terramicina, Laboratórios Pfizer Ltda., Guarulhos, SP, Brazil) was administered in the drinking water at a concentration of 0.83 mg/mL beginning 1 day before infection and maintained throughout the experiment. A 50- μ L intramuscular injection of chlorpromazine chloride (10 mg/kg of body weight; Amplictil, Sanofi Aventis, Suzano, SP, Brazil) in each thigh was used to sedate the animals.

C. albicans (ATCC 18804), grown for 24 hours at 37°C on Sabouraud dextrose agar (Himedia, Mumbai, Maharashtra, India), were resuspended in 10 mL of PBS and centrifuged at 358g for 10 minutes. The pellet was resuspended in 10 mL of PBS and adjusted to 10⁸ viable cells/mL after counting in a Neubauer chamber (Laboroptik GMBH, Bad Homburg, Germany). A sterile swab (Absorve, Cral, São Paulo, SP, Brazil) soaked in the *C. albicans* suspension was used to inoculate the sedated mice by rubbing the swab for 1 minute on the tongue dorsum. The group of mice that were not inoculated with *C. albicans* received tetracycline and prednisolone.

Photodynamic therapy

Twenty-four hours after the second injection of prednisolone, PDT was conducted on the animals under anesthesia through an intramuscular injection of xylazine (Anazedan, Vetbrands, Jacaré, SP, Brazil) and ketamine (Dopalen, Vetbrands, Jacaré, SP, Brazil) at a

ratio of 0.8:0.5 mL, respectively, and a dose of 1.6 mL/100 g of body weight.

A 50- μ L volume of erythrosine solution was pipetted onto the tongue dorsum. After 1 minute (preirradiation time), the LED was applied for 3 minutes (P+L+, n = 14). The effects of the photosensitizer itself (P+L-, n = 14) and the light source alone with PBS (P-L+, n = 14) were evaluated. The control group received only PBS with no LED irradiation (P-L-, n = 14).

Microbiological analysis

Samples from the same animal were collected before and after each experimental treatment because the animals could present different susceptibilities to infection that could interfere with the interpretation of the results. Samples from the tongue dorsum were collected with a mini-swab, which was immediately placed in a test tube containing 0.99 mL of PBS and shaken for 1 minute. This solution was estimated to be a 10^{-2} starting dilution of *Candida* from the soaked swab. Subsequently, serial dilutions were made, and 0.1 mL of each dilution was plated onto the surface of Sabouraud dextrose agar (Himedia) containing chloramphenicol (Vixmicina, São Paulo, SP, Brazil). Dilutions were plated in duplicate and incubated at 37°C for 48 hours. *Candida* colonies were counted on plates exhibiting 30 to 300 colonies for the determination of colony-forming units (CFU)/mL. Plates with fewer than 30 colonies from the initial 10^{-2} dilution were estimated to contain 10^{-1} *Candida* cells.²

Yeasts recovered from the animals of the groups P+L+ (n = 10) and P-L- (n = 10) were transferred to Yeast Peptone Dextrose broth (YPD, Himedia) supplemented with 20% glycerol in Eppendorf tubes and stored at -80°C.

One day after the experimental treatments, an excessive dose of anesthetic was administered to kill the mice. The tongues were then removed for macroscopic and microscopic (optical and scanning electron microscopy [SEM]) analyses.

Macroscopic analysis of candidiasis on the tongue dorsum

After the animals were killed, characteristic lesions of candidiasis on the tongue dorsum were observed using a stereomicroscope (Zeiss, Göttingen, Germany). For quantification of lesions on the tongue dorsum, scores were assigned from 0 to 4: 0, normal; 1, white patches on less than 20% of the surface; 2, white patches covering between 21% and 90% of the surface; 3, white patches on more than 91% of the surface; and 4, thick white patchy pseudomembranes covering more than 91% of the surface.²³

Optical microscopy of the tongue dorsum of mice

For microscopic analysis of the lesions, the tongues were fixed in 10% formalin for 24 hours. After embedding in paraffin, 5- μ m-thick tissue slices were cut and stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS). The presence of candidiasis was analyzed using optical microscopy (Olympus, CX41, Toquio, Japan) at $\times 400$ magnification, and histologic sections were examined for the presence of yeasts and hyphae, the location and extent of the lesions, and the alteration of the involved tissues. The presence of yeasts and hyphae was quantified in accordance with the methodology of Junqueira et al.,²⁴ attributing the following scores to 21 histologic fields in the anteroposterior direction: 1, 1 to 5 yeasts/hyphae; 2, 6 to 15 yeasts/hyphae; 3, 16 to 50 yeasts/hyphae; and 4, more than 50 yeasts/hyphae.

The intensity of the tissue lesions was evaluated, and the inflammatory response of the conjunctive tissue was scored according to the methodology of Junqueira et al.¹² For the epithelial lesions, 7 tissue alterations were analyzed: epithelial hyperplasia, disorganization of the basal cell layer, exocytosis, spongiosis, loss of filiform papillae, hyperkeratosis, and development of intraepithelial microabscesses. The inflammatory infiltrate was scored using the following scale: 0, absence of inflammatory cells; 1, mild inflammatory infiltrate; 2, moderate inflammatory infiltrate; and 3, intense inflammatory infiltrate.

SEM of the tongue dorsum

For the SEM analysis, 2 tongues from each experimental group were inoculated with *C. albicans* and fixed in 2.5% glutaraldehyde in phosphate buffer (0.1 mol/L and pH 7.3) for 24 hours at 4°C. The tongues were washed with a physiological salt solution (0.85% NaCl) for 30 minutes. The specimens were subsequently dehydrated in a series of ethanol solutions (50%, 70%, and 90% for 20 minutes each and 100% for 20 minutes 3 times). After dehydration, the tongues were dried to the critical point using CO₂ (Denton Vacuum DCP 1, Moorestown, NJ). The tongues were fixed on aluminum stubs and coated with gold for 120 seconds at 40 mA (BAL-TEC 50 D 050 Sputter Coater, Liechtenstein) and evaluated using SEM (JEOL JSM 5600, Tóquio, Japan), at 15 kV.

Optical microscopy and SEM analyses of the in vitro adherence of *C. albicans* to human BECs

Yeasts recovered from the animals in the groups P+L+ (n = 10) and P-L- (n = 10) after experimental treatments were stored at -80°C and then tested for their ability to adhere to BECs in vitro. Use of human BECs was approved by the Human Research Ethics Commit-

tee of the School of Dentistry of São José dos Campos (UNESP) under protocol number 008/2011-PH/CEP.

Adherence tests were performed according to Soares et al.¹⁸ with modifications. BECs from 4 young healthy adults were collected by rubbing the buccal mucosa with a sterile swab (Absorve), which was transferred to a Falcon tube containing 10 mL of PBS and vortexed for 3 minutes. Subsequently, the swab was removed, and the pooled BECs were centrifuged at 1096g for 10 minutes and washed 4 times with PBS to remove the attached yeasts. The pooled BECs were suspended in PBS at 10^5 cells/mL after counting in a Neubauer chamber (Laboroptik GMBH).

Yeasts were plated onto Sabouraud dextrose agar (Himedia) and incubated at 37°C for 24 hours. Subsequently, the yeasts were suspended in PBS at an appropriate volume to achieve an optical density of 1.5 at 520 nm on a spectrophotometer (B582, Micronal, São Paulo, SP, Brazil). One milliliter of yeast inoculum was added to tubes containing 4 mL of Sabouraud dextrose broth (Himedia) to achieve 10^6 to 10^7 cells/mL. In a fresh tube, 500 μ L of this suspension was added to 500 μ L of the BEC suspension and incubated at 37°C for 1 hour in a shaker at 75 rpm (Quimis, Diadema, SP, Brazil).

The yeast and BEC suspension was subsequently filtered through a 12- μ m polycarbonate filter and washed twice with 50 mL of PBS to remove the unattached fungi. Then, the filter was placed firmly on a glass slide to allow BEC adherence to occur. For the optical microscopy analysis, the slide was air dried, fixed with heat, and Gram-stained. One hundred BECs were analyzed, and the adherent yeasts were quantified at $\times 400$ magnification. The assays were performed in duplicate, and the results are expressed as the mean number of adhered yeasts per 100 BECs.

For the SEM analysis, 1 slice from each group (P+L+ and P-L-) was analyzed. The slices were fixed in Petri plates containing 10 mL of 2.5% glutaraldehyde for 1 hour. After fixation, the slices were immersed in 10 mL of ethanol solution at concentrations of 10%, 25%, 50%, 75%, and 90% for 20 minutes each, followed by 100% ethanol for 1 hour. The slices were dried in a bacteriologic incubator at 37°C for 24 hours. The slices were mounted on aluminum stubs and prepared for SEM analysis as described previously.

Statistical analysis

The data for the amount of recovered yeast (CFU/mL) and the epithelial lesions were analyzed using ANOVA and the Tukey test. The scores from the macroscopic analysis of yeasts and hyphae counts and the inflammatory infiltrate were evaluated using the Kruskal-Wallis test. The results obtained for the adherence of *C.*

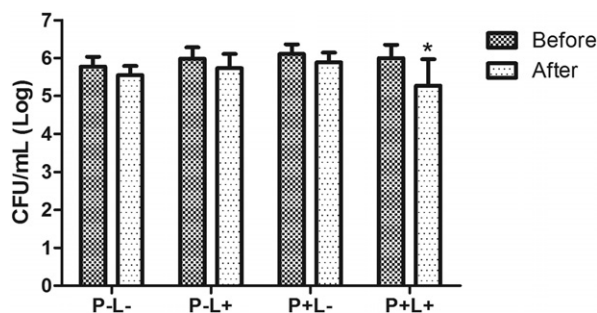


Fig. 1. Mean values of *C. albicans* (log CFU/mL) recovered before and after treatment in each of the experimental groups (n = 10 per group): P-L-, control group; P+L-, photosensitization with erythrosine; P-L+, irradiation with LED; P+L+, photosensitization with erythrosine and irradiation with LED. *Significant difference between the P+L+ group and the remaining groups, P-L-, P-L+, and P+L- (Tukey test, $P = .018, .030$ and $.018$, respectively).

albicans to BECs were analyzed using Student *t* test. A *P* value less than .05 was considered statistically significant.

RESULTS

Effect of PDT on viability

The amounts of *C. albicans* recovered from the buccal cavity of the mice before and after the experimental treatments in the P-L-, P-L+, P+L-, and P+L+ groups are shown in Figure 1. All experimental groups showed a reduction in the number of viable cells after treatment with mean reductions of 0.22 log₁₀ (P-L-), 0.25 log₁₀ (P-L+), 0.22 log₁₀ (P+L-) and 0.73 log₁₀ (P+L+). The reduction in the number of viable cells in the PDT group (P+L+) was significantly greater than that in the other groups (P-L-, P-L+, and P+L-).

Macroscopic analysis

Macroscopic analysis revealed the presence of candidiasis lesions on the tongue dorsum of all the tongues from groups P-L-, P-L+, and P+L- with a median score of 2. For the group treated with PDT (P+L+), 1 animal presented a tongue dorsum with a normal appearance (0 score), and 9 animals presented candidiasis lesions with median score of 1. The differences between the PDT group and the other groups did not achieve statistical significance ($P = .098$), however. The candidiasis lesions were characterized by the presence of whitish regions with areas of papillary atrophy localized primarily in regions of simple conic papillae (Figure 2).

Microscopic analysis

In the optical microscopic analysis, the candidiasis lesions were characterized by the presence of yeasts and

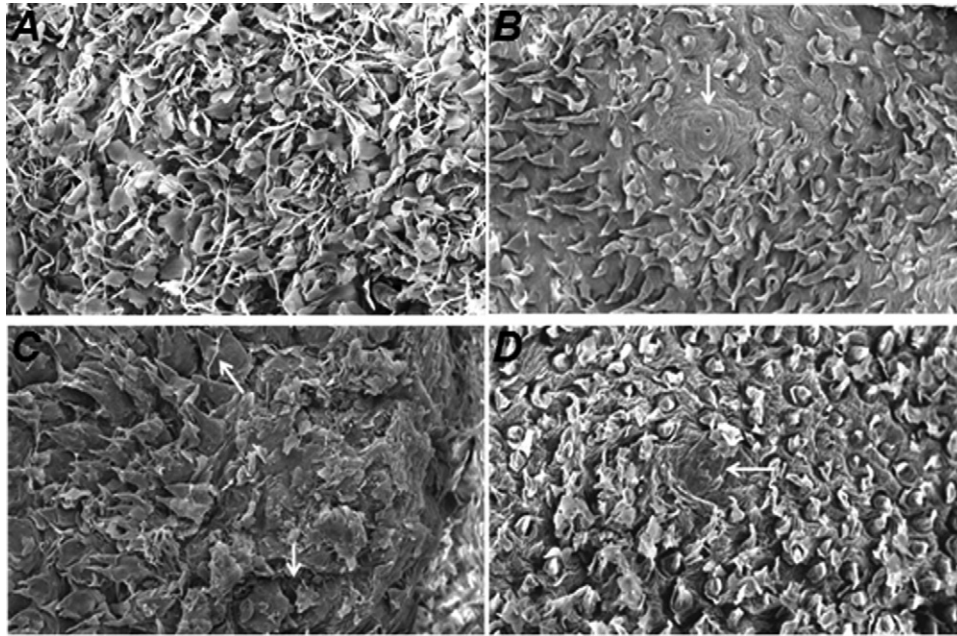


Fig. 5. SEM of the tongue dorsum of mice. P-L- (A): numerous hyphae among the destroyed simple conic papillae are observed (original magnification, $\times 250$). P-L+ (B) and P+L+ (D): atrophy and destruction of the simple conic papillae with increased interpapillar surface and presence of the fungiform papillae (arrow) are shown (original magnification, $\times 160$). P+L- (C): destruction of the giant papillae and presence of hyphae (arrow) are observed (original magnification, $\times 150$).

(P-L-, P-L+, P+L-, and P+L+) presented normal macroscopic and microscopic characteristics.

Effect of PDT on adherence of *C. albicans* to BEC

An analysis of the yeasts recovered from the P-L- and P+L+ groups following the experimental treatments revealed that *C. albicans* obtained from mice subjected to PDT (P+L+) demonstrated a statistically significant reduction (35%) in their capacity to adhere to BECs compared with the control group (P-L-) ($P = .045$). Optical microscopy revealed budding blastoconidia and germ tubes adhered to BECs, and budding blastoconidia were also observed using SEM (Figure 6).

DISCUSSION

In this study, we used the experimental candidiasis model described by Takakura et al.,²³ which was deemed suitable for the induction of buccal candidiasis because large numbers of viable *Candida* (5 and 6 \log_{10}) were recovered 4 days after inoculation. The large quantity of yeasts obtained could be attributed to the sedation of animals during inoculation with *C. albicans*, which maintained the microbial suspension in the buccal cavity during the approximately 3 hours of sedation time. The large numbers of *Candida* cells could also be attributed to the state of immunosuppression of the animal conferred by the potency of the immunosuppressive administered (prednisolone).

PDT treatment (P+L+) with 400 $\mu\text{mol/L}$ erythrosine and 14.34 J cm^{-2} fluence LED irradiation reached reduction of 0.73 \log_{10} of *C. albicans* biofilm in vivo that was significantly different compared with the other groups (P-L-, P+L-, and P-L+). Mima et al.¹³ used the same methodology for inducing buccal candidiasis as the present work. The animals treated with a Photogen at concentrations from 400 to 1000 mg/mL and irradiated with a blue LED and red LED showed reduction of 1.04 to 1.59 \log_{10} of *C. albicans* cells. In this study, the reduction could have been greater if the number of PDT sessions was increased; however, multiple treatments were not feasible because 10 of 66 animals died, which could be attributed to the state of immunosuppression of the animals and the virulence of the *C. albicans* strain, which is correlated with rapid mortality in mice.^{25,26}

Under optical microscopy, many yeasts and hyphae, numerous epithelial lesions, and mild inflammatory infiltrate were observed on the tongue dorsum of the animals resembling the characteristic lesions of buccal candidiasis that have been reported by others.^{13,23,27} The quantifications of the yeasts/hyphae and the tissue lesions were similar among the groups studied, and no significant differences was found. Similarly, Mima et al.¹³ did not observe differences in the histologic lesions of candidiasis between the groups treated with Photogen- and LED-mediated PDT and the control

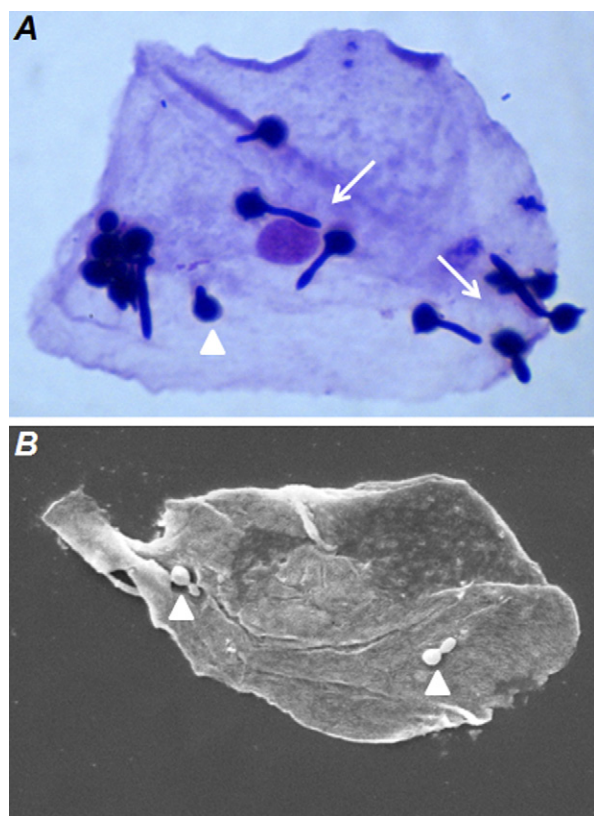


Fig. 6. Optical microscopy (A) and SEM (B) of in vitro adherence of *C. albicans* to BECs. Presence of adherent budding blastoconidia (▲) and germ tubes (arrows) are shown (original magnification, $\times 1000$ [A] and $\times 1100$ [B]).

group in an experimental candidiasis model in immunosuppressed mice. In a buccal candidiasis model in immunocompetent rats, however, Junqueira et al.¹² and Martins et al.¹⁴ observed fewer epithelial lesions and a decrease in inflammatory infiltrate for the P+L+ group treated with methylene blue ($312.6 \mu\text{mol/L}$) at the same concentration and an AsGaAl laser with a fluence of 26 J cm^{-2} and 245 J cm^{-2} compared with the P-L-, P-L+, and P+L- groups, respectively.

In this article, and in the study by Mima et al.,¹³ a statistically significant reduction in the number of CFU/mL was observed, with similar microscopic lesions among the experimental groups. Conversely, Junqueira et al.¹² and Martins et al.¹⁴ observed a similar recovery of *C. albicans* among the groups and found that the microscopic lesions were reduced in the group treated with PDT. These differences might be attributed to the experimental model or light source used. In this work and in the study of Mima et al.,¹³ an LED was used for the PDT of buccal candidiasis in immunosuppressed mice, whereas Junqueira et al.¹² and Martins et al.¹⁴ used a laser for the PDT of buccal candidiasis in immunocompetent rats. The immunosuppression in-

creased *C. albicans* colonization in the buccal cavity, which facilitated the counting the CFU/mL in the recovery of *C. albicans*, enabling better comparative analyses between the experimental groups in the present study and in the study of Mima et al.¹³ Furthermore, the inflammatory response between rats and mice is naturally different, with rats responding more quickly to infection because of an innate immune response that is more potent than that of mice. Concerning the light source, the laser presents modulatory effects on inflammation and tissue responses, which might explain the small number of intense histologic lesions observed in the group treated with PDT in the studies by Junqueira et al.¹² and Martins et al.¹⁴

The tongues analyzed by SEM after 5 days of inoculation showed alterations on the surface tongues. The SEM analysis showed fewer hyphae on the tongue dorsum of the group subjected to PDT (P+L+) compared with the control group (P-L-). Although a lower recovery of *C. albicans* was observed with PDT, there was no difference between the groups in the quantification of yeasts and hyphae from the histologic analysis. The data showed that PDT treatment affected the fungal structures exposed on the tongue surface without reaching the inner layers of keratin. Increasing the efficacy of PDT against buccal candidiasis by combining sessions of PDT with systemic antifungal treatments might help prevent *C. albicans* invasion and limit episodes of relapse that are common in immunosuppressed individuals. The development of resistance to PDT is unlikely because antioxidant enzymes, such as superoxide dismutase and catalase, could protect against some free radicals. These enzymes do not offer protection against singlet oxygen generated in the reaction, however, which is a great advantage of combining sessions of PDT.²⁸

Adherence is an important step in *C. albicans* colonization and subsequent biofilm development, which could lead to host tissue damage. The yeasts recovered after the PDT (P+L+) treatments of the biofilms formed in vivo were impaired in their ability to adhere to BECs by 35% compared with the yeasts obtained from the control group (P-L-). Nevertheless, this statistically significant reduction in the adherence observed in the present study was lower than the 55% reduction in adherence to BECs of fluconazole-sensitive and fluconazole-resistant *C. albicans* achieved by Soares et al.¹⁸ after in vitro PDT mediated by toluidine blue ($25 \mu\text{mol/L}$) and a red LED (180 J cm^{-2}). An important difference between these 2 studies is that Soares et al.¹⁸ applied PDT on *Candida* spp. planktonic cultures, whereas *Candida* in suspension is more susceptible to PDT than sessile cells. This difference might contribute to the increased reduction in adherence to BECs. These

authors hypothesized that PDT caused a reduction in adherence by disrupting the cytoplasmic membrane.

In summary, the results of the present study demonstrate that erythrosine- and LED-mediated PDT showed antifungal activity in buccal candidiasis in an immunosuppressed murine model without damaging the adjacent tissue and significantly reducing the adherence of *C. albicans* to BECs.

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