



Universidade de São Paulo

Biblioteca Digital da Produção Intelectual - BDPI

Departamento de Física e Ciências Materiais - IFSC/FCM

Artigos e Materiais de Revistas Científicas - IFSC/FCM

2010-03

Susceptibility of *Candida albicans* to photodynamic therapy in a murine model of oral candidosis

Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontology, New York : Mosby, v. 109, n. 3, p. 392-401, Mar. 2010

<http://www.producao.usp.br/handle/BDPI/50181>

Downloaded from: Biblioteca Digital da Produção Intelectual - BDPI, Universidade de São Paulo

Susceptibility of *Candida albicans* to photodynamic therapy in a murine model of oral candidosis

Ewerton Garcia de Oliveira Mima, DDS, MSc, PhD,^a Ana Cláudia Pavarina, DDS, MSc, PhD,^b Lívia Nordi Dovigo, DDS, MSc,^c Carlos Eduardo Vergani, DDS, MSc, PhD,^b Carlos Alberto de Souza Costa, DDS, MSc, PhD,^d Cristina Kurachi, DDS, MSc, PhD,^e and Vanderlei Salvador Bagnato, MSc, PhD,^e São Paulo, Brazil
SÃO FRANCISCO UNIVERSITY, SÃO PAULO STATE UNIVERSITY, AND UNIVERSITY OF SÃO PAULO

Objective. In vivo studies of antimicrobial PDT in animal models of oral candidosis are scarce and the association of porphyrin and LED light has not been evaluated for in vivo photoinactivation of *Candida*. In this study the effectiveness of photodynamic therapy (PDT) on the inactivation of *Candida albicans* in vivo was evaluated.

Study design. Seventy-one 6-week-old female Swiss mice were immunosuppressed, provided tetracycline to their drinking water, then orally swabbed with a suspension of *C. albicans* (10^7 CFU/mL). Four days after oral inoculation, PDT was performed on the dorsum of the tongue after topical administration of Photogem at 400, 500, or 1000 mg/L and followed 30 minutes later by illumination with LED light (305 J/cm^2) at 455 or 630 nm ($n = 5$ each). After swabbing to recover yeast from the tongue, the number of surviving yeast cells was determined (CFU/mL) and analyzed by ANOVA and Holm-Sidak tests ($P < .05$). Animals were humanely killed, and the tongues surgically removed and processed for histological evaluation of presence of yeast and inflammatory reaction.

Results. PDT resulted in a significant reduction in *C. albicans* recovered from the tongue ($P < .001$) when compared with mice from the positive control group. There was no difference between the concentrations of Photogem and LED light wavelengths used. Histological evaluation of the tongue revealed that PDT causes no significant adverse effects to the local mucosa.

Conclusion. PDT promoted significant reduction in the viability of *C. albicans* biofilm without harming the tongue tissue. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;109:392-401)

Oral candidosis is the most common infection of the oral cavity and is caused by *Candida* species, the commonest being *Candida albicans*.^{1,2} The predisposing factors of oral candidosis include immunocompromised states, diabetes mellitus, dental prostheses, xerostomia,^{3,4} and prolonged use of antibiotics or immunosup-

pressive drugs.^{5,6} With the advent of the human immunodeficiency virus (HIV) infection, increased attention has been paid to oral candidosis, because up to 90% of HIV-infected individuals suffer from oral *Candida* infection.³

The widespread use of topical and systemic antifungal agents as conventional treatment for oral candidosis has resulted in the development of resistance in *C. albicans*.⁷ Although resistance of *C. albicans* to polyenes is rare, several mechanisms of azole resistance⁸ have been reported, including changes in the cell wall or plasma membrane, which lead to impaired azole uptake; overexpression of or mutations in the target enzyme of azoles; and the efflux of drugs mediated by membrane transport proteins.⁹ Resistance appears to increase proportionally with the extend of previous exposure to the antifungal drugs.¹⁰ Moreover, because of the fungistatic rather than fungicidal effect of azoles,⁷ the host defenses are essential for eradicating the infection.¹¹ Therefore, in immunosuppressed patients, the use of azole agents to treat oral candidosis can be ineffective.

Thus, it is necessary to develop alternative therapies for the treatment of oral candidosis. A promising modality is photodynamic therapy (PDT), which uses a

This research was supported by São Paulo Council of Research (FAPESP – Grant No. 2005/02193-4 and 2005/03226-3, and CePOF – CEPID Program).

^aAssistant Professor, Dentistry School, São Francisco University–USF, Bragança Paulista, SP, Brazil.

^bAdjunct Professor, Department of Dental Materials and Prosthodontics, Araraquara Dental School, São Paulo State University–UNESP, Araraquara, SP, Brazil.

^cPostgraduate Student, Department of Dental Materials and Prosthodontics, Araraquara Dental School, São Paulo State University–UNESP, Araraquara, SP, Brazil.

^dAdjunct Professor, Department of Physiology and Pathology, Araraquara Dental School, São Paulo State University–UNESP, Araraquara, SP, Brazil.

^eProfessor, Physics Institute of São Carlos, University of São Paulo–USP, São Carlos, SP, Brazil.

Received for publication Jul 1, 2009; returned for revision Sep 25, 2009; accepted for publication Oct 4, 2009.

1079-2104/\$ - see front matter

© 2010 Mosby, Inc. All rights reserved.

doi:10.1016/j.tripleo.2009.10.006

photosensitizing agent and an appropriate wavelength of light. The interaction between the photosensitizer (PS) and light in the presence of oxygen produces reactive species, such as singlet oxygen and free radicals, which causes cell damage and death. In this sense, the mechanism of PDT for inactivating fungi differs completely from that of antifungal agents. Owing to the nonspecific oxidizing agents, organisms resistant to conventional antifungal agents could be successfully killed by PDT and it seems unlikely that resistance to such therapy will be developed. Although PDT is more usually applied for treating cancer, several studies have reported that microorganisms, such as bacteria, viruses, and fungi, can be killed by PDT.^{12,13} It has been demonstrated that PDT is effective against oral species and may not promote damage to host cells and tissues.^{14,15} However, most studies on cell damage are short-term investigations and safety studies have been performed over the short term. Kömerik et al.¹⁵ observed complete inactivation of *Porphyromonas gingivalis* in the maxillary molar region of rats after PDT, with no adverse effects on the periodontal structures after 3 days and significant reductions in bone loss after 90 days. Moreover, Zeina et al.¹⁶ detected no genotoxicity (immediate and delayed effects) on skin cells after a PDT protocol that was effective for killing microbial species. These authors concluded that there is a wide safety margin for PDT between microbial elimination and damage to host cells.

In vitro investigations have shown that *Candida* spp. are susceptible to photoinactivation.¹⁷⁻²⁴ Usually, dyes (toluidine blue and methylene blue) and porphyrins are used as PS combined with red laser light. However, light sources with simpler technology and lower cost than lasers, such as light-emitting diodes (LED), have been successfully applied in PDT.^{25,26} In addition, there are different colors of LED light, with radiations covering almost the entire visible electromagnetic spectrum. Investigations with the aim of confirming the effectiveness of antimicrobial PDT in animal models are scarce. Only one in vivo study reported complete inactivation of *C. albicans* with topical methylene blue and red laser light in a murine model of oral candidosis.²⁷ Nevertheless, dyes have the undesirable effect of staining teeth, lips, tongue, buccal mucosa, esthetic restorations, and prosthetic surfaces. Therefore, a non-dye sensitizer, such as the porphyrins, would be preferable. A previous in vitro study showed that PDT mediated by Photogem and blue LED light resulted in complete inactivation of planktonic suspensions and significant reduction in biofilm viability.²⁸ However, in vivo application of antimicrobial PDT using porphyrins and LED light has not yet been well established and animal models may provide outcomes more closely

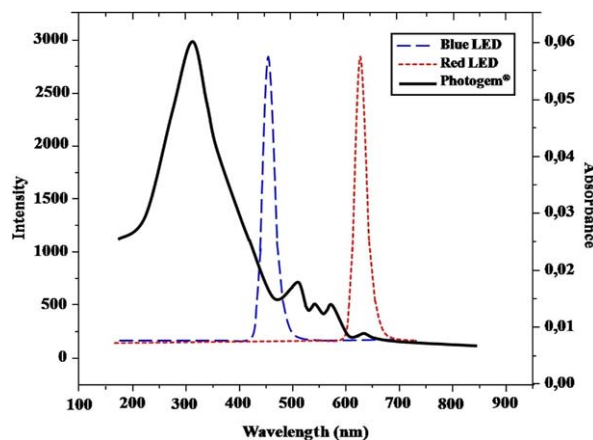


Fig. 1. Absorption bands of Photogem and intensity of blue (455 nm) and red (630 nm) LED light.

correlated to clinical situations. Thus, the aim of the present investigation was to contribute to in vivo antimicrobial PDT development by reporting on the photoinactivation of *C. albicans* in a murine model of oral candidosis using a porphyrin in association with LED light sources of different wavelengths.

MATERIALS AND METHODS

Photosensitizer and light sources

The PS used in this study was a hematoporphyrin derivative produced in Moscow, Russia (Photogem; Limited Liability Company Photogem, Moscow, Russia). Stock solutions of Photogem (pH 6.6) were prepared by dissolving the powder in sterile saline and kept in the dark instantly before use. The absorption bands of Photogem are shown in Fig. 1.

Two handpieces with a blue (455 nm) or red (630 nm) light-emitting diode (LED, LXHL-PR09, Luxeon III Emitter, Lumileds Lighting, San Jose, CA) were designed by the “Instituto de Física de São Carlos” (University of São Paulo, São Carlos, SP, Brazil). The output power of light delivered at the end of each handpiece (5 mm in diameter) was 200 mW. The wavelengths of 455 nm (blue) and 630 nm (red) were chosen because the absorption bands of Photogem match these wavelengths. Although the highest absorption of Photogem is close to 455 nm (see Fig. 1), the wavelength of 630 nm achieves a higher penetration into the tissue.

Microorganisms and culture condition

A reference strain (ATCC 90028) of *C. albicans* (ATCC, Rockville, MD) was evaluated. This strain was maintained in yeast-peptone-glucose (YEPD, 1.0% yeast extract, 2.0% peptone, 2.0% glucose, 0.1 M cit-

rate-phosphate buffer pH 5.0) and glycerol medium at -70°C . The yeast was reactivated by cultivation in Sabouraud Dextrose Agar (SDA, Acumedia Manufactures Inc., Baltimore, MD) containing $5\ \mu\text{g}/\text{mL}$ gentamicin at 37°C for 48 hours before each experiment. The yeast suspended in sterile saline (pH 5.3) was inoculated in 5 mL of Tryptic Soy Broth (TSB, pH 7.2, Acumedia Manufactures Inc., Baltimore, MD) and grown aerobically at 37°C for 24 hours. Each culture was harvested after centrifugation at 2000 rpm for 10 minutes, washed twice with sterile distilled water, and resuspended in sterile saline (4.5×10^7 colony-forming units [CFU]/mL).

Preparation of animals and oral inoculation

The research protocols for using mice and all the animal experiments were approved by the Ethics Committee for Animal Investigations (Araraquara Dental School, São Paulo State University). Seventy-one 6-week-old female Swiss mice were used for all animal experiments. The mice were kept in cages housing 5 animals in a temperature-controlled room ($23 \pm 2^{\circ}\text{C}$) with a 12:12-hour light/dark cycle. Standard mouse chow and tap water were given ad libitum.

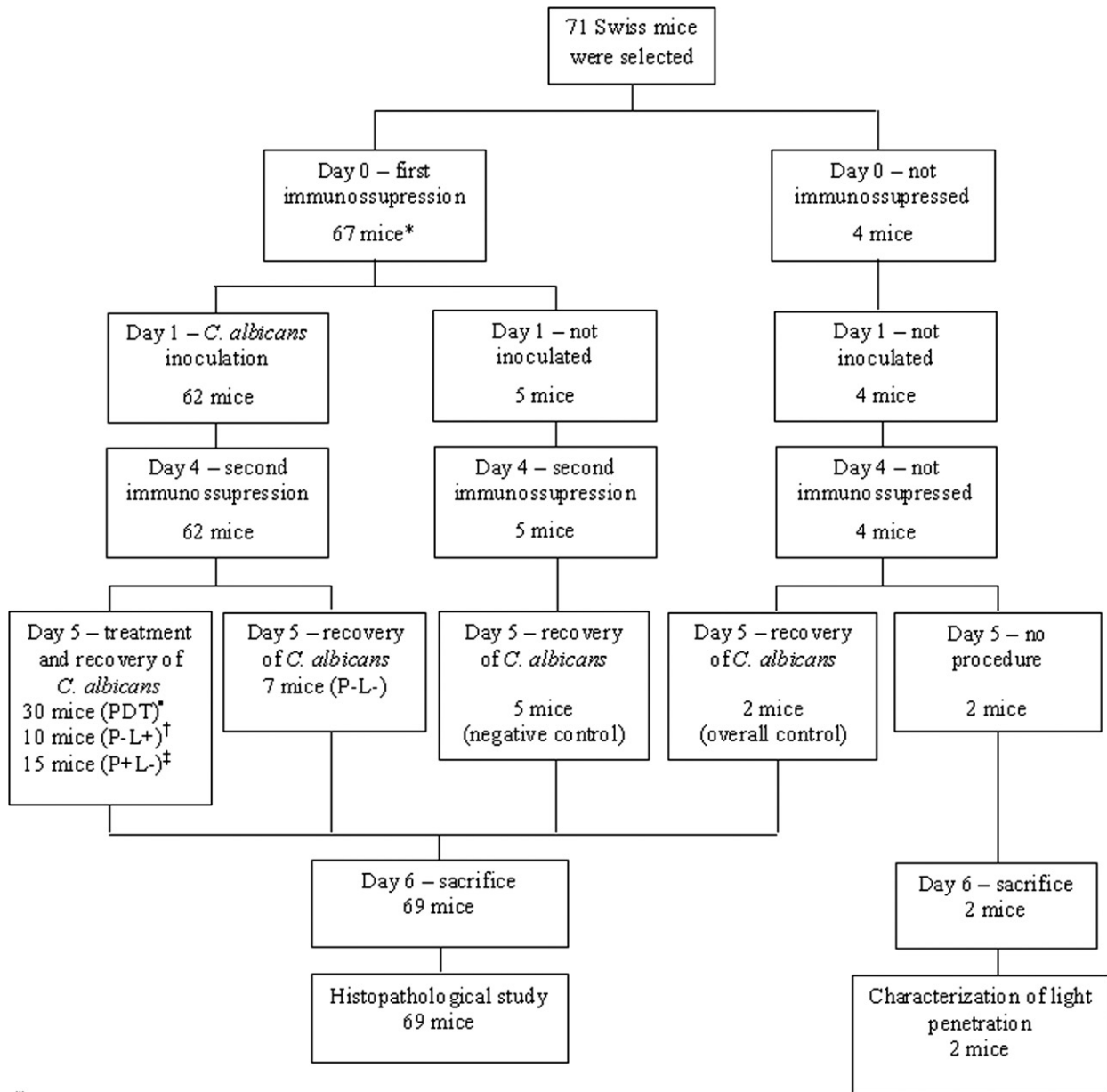
The methodology described by Takakura et al.²⁹ was used to induce oral candidosis in mice. The timeline of events used in this study can be seen in Fig. 2. The animals were immunosuppressed with 2 subcutaneous injections of prednisolone (Depo-Medrol, Laboratórios Pfizer Ltda., Guarulhos, SP, Brazil) at a dose of 100 mg/kg body weight 1 day before and 3 days after the infection with *Candida* (days 0 and 4 in Fig. 2). The mice were given tetracycline hydrochloride (Farmácia Santa Paula, Araraquara, SP, Brazil) in their drinking water at the concentration of 0.83 mg/mL beginning on day 0. On day 1, animals were anesthetized by an intramuscular injection with $50\ \mu\text{L}$ of 2 mg/mL chlorpromazine chloride (Farmácia Santa Paula, Araraquara, SP, Brazil) in each femur. Small cotton pads (Cottonbaby, Higie-Plus Cottonbaby Ind. Com. Ltda., São José, SC, Brazil) were soaked in a *C. albicans* cell suspension (4.5×10^7 CFU/mL) in such a way that the entire oral cavity of the anesthetized mice was swabbed to produce oral infections.

Photodynamic therapy and microbiological evaluation

On day 5 (4 days after *Candida* inoculation), mice were anesthetized by an intramuscular injection of 100 mg/kg body weight ketamine (União Química Farmacêutica Nacional S/A., Embu-Guaçu, SP, Brazil) and 10 mg/kg body weight xylazine (Produtos Veterinários J. A. Ltda., Patrocínio Paulista, SP, Brazil). Each animal was placed in a supine position on a pad in

a device fitted with stainless steel wires that were looped around the incisors to hold the mouth open. With mandible and cheeks retracted, the tongue was gently taken out of the mouth as far as it would go, to expose it without causing any injury to the tissue. Then, $50\ \mu\text{L}$ of Photogem at concentration of 400, 500, or 1000 mg/L was pipetted onto the dorsum of the tongue and mice were kept in the dark for 30 minutes (pre-irradiation time). During this period, the tongue of each animal was kept in the oral cavity and the photosensitizer was not swallowed, as the animals were anesthetized. After this period, the tongue was gently taken out of the mouth again to expose it for illumination. The solution was not rinsed off or removed; it remained in the oral cavity and the dorsum of the tongue was completely wet with Photogem. For illumination, the LED device was placed onto the dorsum of the tongue, which was illuminated for 20 minutes, resulting in a total fluence of $305\ \text{J}/\text{cm}^2$ (P+L+ groups). Therefore the P+L+ groups corresponded to 6 combinations of the 3 PS concentrations (400, 500, or 1000 mg/L) and the 2 wavelengths of LED light (455 or 630 nm) evaluated (6 groups). The effect of PS alone was tested by application of Photogem at the same concentrations for the same period of pre-irradiation time and irradiation but in the absence of light (P+L-, total of 3 groups). The groups that received light only (blue or red) were exposed to the same LED dose mentioned previously (P-L+, 2 groups). The positive control group did not receive any PS or light (P-L-). A negative control group of animals was immunosuppressed as described but did not receive *C. albicans* inoculation or any treatment. Each experimental group consisted of 5 animals. As mice from positive control were evaluated concurrently with the animals from all other experimental groups, in order to compare the results, a higher number of animals in the positive control group was tested ($n = 7$). Two additional mice were not immunosuppressed, and did not receive *C. albicans* inoculation or any treatment, and were considered as overall control.

The occurrence of *Candida* infection was microbiologically confirmed in the positive control group (P-L-) on day 5. The dorsum of the tongue was swabbed for 1 minute with a cotton pad. The end of the cotton pad was then cut off and placed in a tube containing 1 mL sterile saline. After mixing on a vortex mixer for 1 minute to release *C. albicans* cells from the swab into the saline, duplicate $25\text{-}\mu\text{L}$ aliquots from the 10-fold serial dilutions were spread over the surface of SDA with 5 mg/L gentamicin. All plates were aerobically incubated at 37°C for 48 hours. After incubation, the yeast colony counts of each plate were quantified using a digital colony counter (CP 600 Plus, Phoenix



*Tetracycline hydrochloride was given in drinking water during days 0 to 6;
 *The six PDT groups corresponded to the combinations of three PS concentrations and two wavelengths;
 †The two P-L+ groups corresponded to evaluation of LED light alone (blue and red);
 ‡The three P+L- groups corresponded to evaluation of the three concentrations of PS alone;

Fig. 2. Study design.

Ind Com Equipamentos Científicos Ltda, Araraquara, SP, Brazil). The number of CFU/mL was determined. The same procedures of swabbing and plating samples were performed in animals from experimental groups (P+L+, P+L-, P-L+) after treatments. These animals were not swabbed before treatment to avoid removing *Candida* cells from the tissue, which could potentially interfere in the results, decreasing the

CFU/mL values. Sampling (recovery of *C. albicans* from tongues of mice and yeast culture) was done at only one time point, on day 5 immediately after PDT, in order to assess the yeast viability immediately after treatment.

Histopathological study

On day 6, all mice from all groups were killed with a lethal dose of ketamine. Tongues were surgically



Fig. 3. White patches or pseudomembranes found on the dorsal tongue of all immunosuppressed mice 4 days after *C. albicans* inoculation (P+L+, P-L+, P+L-, and P-L- groups). For animals from P+L+ group, the white patches were present before and after PDT.

removed, fixed in 10% formalin fixative solution at pH 7.2 and embedded in paraffin. Five-micrometer-thick serial sections were cut, mounted on glass slides, and stained with periodic acid-Schiff and hematoxylin (PAS-H) stain for histopathological examination and fungal detection by light microscopy (Carl Zeiss 62774, Oberkochen, West Germany). Tissue reaction caused by *C. albicans* infection whether or not associated with the PDT was examined by a pathologist blinded to the all groups of mice. A descriptive analysis of the histological characteristics of the tissue with and without local inflammatory response of varied intensity was performed.

Characterization of LED light penetration into the tongue

Two additional animals were used for this evaluation. These mice were killed and tongues were surgically removed. Each tongue was cut into halves sagittally. Each LED handpiece was positioned perpendicularly to the dorsum of the tongue sample. A CCD (charge-coupled device) camera (DSC-F828 Cybershot Digital Style Camera, Sony Corp, Tokyo, Japan) placed on the side of the tissue recorded the whole image formed by the light scattered within the tissue. The image was saved to a PC computer as a file.

Statistical analysis

The \log_{10} (CFU/mL) data of *C. albicans* isolated from the tongues of mice in the different groups were compared using 1-way analysis of variance (ANOVA).

P values of less than .05 were considered significant. If a significant difference was found between the groups as a whole, further analyses were performed with the Holm-Sidak test to determine where these differences occurred. All calculations were made using a statistical software program (SigmaStat 3.1, Systat Software, Inc., Point Richmond, CA).

RESULTS

The experimental model of oral candidosis described by Takakura et al.²⁹ used in the present investigation showed reproducible results. White patches or pseudomembranes were macroscopically observed on the dorsum of the tongue of animals from groups P+L+, P+L-, P-L+, and P-L- (positive control) 4 days after *Candida* inoculation (Fig. 3). Moreover, appreciable number of *C. albicans* (10^{4-5} CFU/mL) was recovered from the tongues of mice from the positive control group (P-L-). Throughout the course of the experiment, mice showed no weight loss and none died.

The white patches or pseudomembranes observed were still present after PDT. Nevertheless, significant reduction in the viable counts of *C. albicans* was achieved after PDT, despite the PS and light combination used ($P < .05$). When compared with the control group (P-L-), the photosensitization with 400, 500, and 1000 mg/L associated with illumination of 305 J/cm² promoted significant reductions in the viability of *C. albicans* recovered from mouse tongue. There were no significant differences in effectiveness among the 400, 500, and 1000 mg/L concentrations. However,

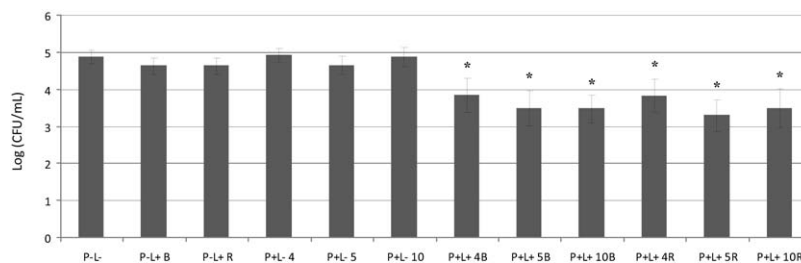


Fig. 4. Mean number of viable *C. albicans* taken from the dorsum of the tongue of immunosuppressed mice 4 days after yeast inoculation. P+L+ represents animals treated with PDT; 4, 5, and 10 represents 400, 500, and 1000 mg/L of PS, respectively; B and R represents Blue (455 nm) and Red (630 nm) LED light, respectively; P-L+ B and P-L+ R represent animals treated with Blue and Red light alone, respectively; P+L- 4, P+L- 5 and P+L- 10 represent animals treated with PS alone at 400, 500, and 1000 mg/L respectively; P-L- represents animals that were not treated with PS or light (positive control group). Each experimental group consisted of 5 animals, except the positive control group (P-L-) that consisted of 7 mice. *Significant difference ($P < .05$) when compared with control group (P-L-). Error bars indicate standard deviations.

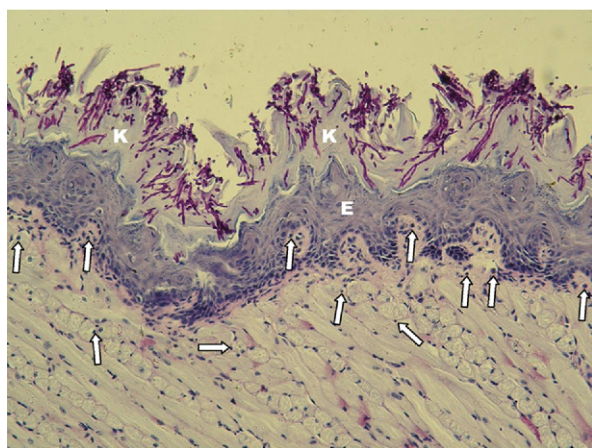


Fig. 5. Histological section of the tongue of immunosuppressed mice infected with *C. albicans* following PDT (mice were killed 5 days after yeast inoculation). Animals whether submitted to PDT or not showed a high number of pseudohyphae in the keratinized (K) layer without invasion in deep layers of the continuous epithelium (E). Mild inflammatory reaction in the subjacent connective mediated by mononuclear cells (arrows) was observed (PAS-H, $\times 200$).

Fig. 4 shows that lower CFU/mL counts were achieved with the use of 500 and 1000 mg/L of PS when compared with 400 mg/L. These results were observed for both light wavelengths tested, as no significant difference was verified between blue and red lights (Fig. 4). At concentrations of 400, 500, and 1000 mg/L of PS, blue LED light promoted reductions of 1.04, 1.39, and 1.41 \log_{10} respectively, whereas red LED light promoted reductions of 1.05, 1.59, and 1.40 \log_{10} respectively. The use of PS (400, 500, and 1000 mg/L) in the absence of light had no effect on the viable count. In addition, LED light alone (blue or red) caused no effect on the viability of *C. albicans*.



Fig. 6. Blue and red LED light scattering within the tongue tissue. Two additional animals, which were not immunosuppressed, did not receive *C. albicans* inoculation or any treatment, were used for this analysis. Note that the blue light is completely absorbed in the superficial tissue layers and the red light penetrates deeper into the tongue.

Histological examination of the mouse tongue infected with *C. albicans* showed the presence of yeast and pseudohyphae limited to the keratinized layer on the dorsum of the tongue (Fig. 5). No histological evidence of invasion of yeast and pseudohyphae into deep layers of the continuous epithelium was observed. However, the subjacent connective tissue exhibited mild inflammatory response mediated by mononuclear cells. Mice from negative control and overall control groups showed no fungi on the dorsum of the tongue and the epithelial and connective tissues exhibited normal histological characteristics. The epithelium was continuous, with an intact basal layer and a thin keratin layer as an external lining. The subjacent connective tissue exhibited a balance among extracellular matrix components and cells, with a notable presence of intact muscular tissue in the area.

Light scattering within the tongue sample is shown in Fig. 6. This image illustrates that blue light presented a lower scattering than red light in the tongue tissue, thus a higher penetration into the tissue was observed with

red light. This result is because of a higher absorption by the biomolecules of photons in the blue spectrum.

DISCUSSION

This investigation demonstrated that PDT was significantly effective in reducing the viability of *C. albicans* in an experimental model of oral candidiasis. In the present study, candidiasis was verified by the presence of white patches or pseudomembranes on the dorsal tongue associated with a significant number of CFU/mL of *C. albicans* (10^{4-5} CFU/mL). These results are in agreement with Takakura et al.,²⁹ who observed white patches on the tongues of mice, although a higher number of CFU of *Candida* (10^5 - 10^6) was recovered from each murine oral cavity. The difference between the CFU values of the 2 studies may be attributed to the strains of *C. albicans* used for oral inoculation. Although Takakura et al.²⁹ used a clinical strain isolated from a patient with cutaneous candidiasis, in the present study a reference strain (ATCC; isolated from blood) was used. Previous studies have demonstrated that strains isolated from patients with HIV infection or acquired immunodeficiency syndrome (AIDS) adhered to buccal epithelial cells in significantly higher numbers than isolates from healthy individuals.^{30,31} Another investigation showed that isolates of *Candida* obtained from denture wearers with signs of denture stomatitis were more adherent to epithelial cells than those obtained from healthy patients.³² In addition, Sanitá et al.³³ suggested that clinical isolates of *Candida* spp. can have significantly higher adherence to denture base materials than ATCC strains.

Although yeasts are more difficult to kill by PDT than bacteria,^{22,34} several in vitro investigations have demonstrated the photoinactivation of *C. albicans*.¹⁷⁻²⁴ The results of the present investigation demonstrated significant reduction of CFU/mL of *C. albicans* from tongues of mice after 400, 500, and 1000 mg/L of Photogem associated with LED light (305 J/cm^2) (455 and 630 nm). There is only one report describing a dose-dependent photoeradication of *C. albicans* in an immunodeficient murine model.²⁷ However, the CFU values obtained by the murine model of oral candidosis used in the present investigation (mean of 2.05×10^3 for the positive control group) are higher than those described by Teichert et al.²⁷ (1.94×10^2). In this study, concentrations of methylene blue from 250 to 400 mg/L decreased fungal growth, whereas complete kill was achieved using 450 and 500 mg/L with a 664-nm diode laser irradiation for 687.5 seconds.²⁷ These findings partially agree with those obtained in the present investigation, in which total inactivation of *C. albicans* was not observed. Although reduction of CFU from $2.74 \log_{10}$ to 0 was achieved by Teichert et al.²⁷

with 500 mg/L of methylene blue and red laser light, in the present investigation $1.59 \log_{10}$ was the highest reduction of CFU/mL values achieved, with the use of 500 mg/L of Photogem with 305 J/cm^2 of red LED light. The difference between the CFU reductions may be attributed to the different PS and light source combinations used in the 2 studies. The molecule size of methylene blue is smaller than that of porphyrins^{12,13,35} and a smaller molecule can more easily penetrate the inner layers of the biofilm and promote a better sensitization of the cells. This may, therefore, explain the different results of the 2 investigations. Furthermore, in their manuscript, Teichert et al.²⁷ stated that mice with oral candidosis were treated using PDT, in which they only observed a dose-dependent eradication of *C. albicans*, but the regression of lesions, which is an important clinical sign of treatment, was not reported. In addition, no dose-dependent inactivation was observed in the present investigation. This may be attributed to the higher concentrations of the PS used. At high concentrations, PS may suffer a self-aggregation process in solution, reducing the singlet oxygen yield.³⁶

Although no complete inactivation was observed in this study, in vitro investigations have demonstrated the photokilling of *C. albicans* using porphyrins. In these studies, low concentrations of PS and low light fluences were used. Bertoloni et al.¹⁷ showed that concentrations of hematoporphyrin above 1 mg/L in the incubation medium were able to significantly decrease the viability of *C. albicans* irradiated by 6 mW/cm² tungsten lamps for 10 minutes. A dose-dependent inhibition of metabolic activity was observed after treatment of *C. albicans* strains with Photofrin (0.01 to 10 mg/L) and illumination with a light fluence of 9 J/cm^2 from an Hg arc lamp.²⁰ Chabrier-Roselló et al.²³ also observed a dose-dependent reduction of metabolic activity of *C. albicans* biofilms mediated by 10 mg/L of Photofrin and with broadband illumination by visible light at fluences ranging from 0.9 to 18 J/cm^2 . However, it has been shown that photosensitization of *Candida* and subsequent susceptibility to PDT can be reduced dramatically by culture conditions, such as the type of culture medium and the presence of albumin.^{20,37} Moreover, on epithelial surfaces *C. albicans* grows as a biofilm,³⁸ which is less susceptible to PDT than its planktonic counterpart.^{24,28} It has been suggested that incomplete kill of PDT on biofilms might be because of the failure of PS and light penetration into the inner regions of the biofilm.^{39,40} Thus, in comparison with these in vitro studies, the higher PS concentrations and light fluence required for photoinactivation in the present investigation may be explained by the physiological responses of *C. albicans* to the in vivo environment.

Effective results of photoinactivation of *C. albicans* have also been reported using phenothiazine dyes, toluidine blue O, and methylene blue (TBO and MB respectively). The results obtained by Wilson and Mia¹⁸ demonstrated photokilling of *C. albicans* by a number of PS in association with light from low-power laser, with TBO and helium/neon gas laser being the most effective combination. These authors also verified photosensitization of *C. albicans* under conditions resembling those that would be encountered in vivo (in saliva, serum, saline, and broth at a pH ranging from 4.0 to 7.0).⁴¹ Jackson et al.¹⁹ found that the hyphal form of *C. albicans* was more susceptible to photoinactivation, requiring a lower TBO concentration than the yeast form. The germ tube formation of *C. albicans*, a transition state from budding to hyphal cells and an essential phase to virulence, was also inhibited by PDT using MB (0.027 to 13.37 mM) and laser light (28 J/cm²).⁴² In contrast to bacterial species, *C. albicans* sensitized by 100 mg/L MB was killed by visible light from a slide projector (42 mW/cm²) after only 20 minutes of irradiation.³⁴ Giroldo et al.⁴³ verified inhibition of *C. albicans* CFUs to approximately 50% in the presence of 50 mg/L MB and laser light irradiation of 28 J/cm². These authors also verified that cell death promoted by this combination can be related to damage to the plasma membrane of the yeast. However, Demidova and Hamblin²² showed that 5 μM of poly-L-lysine-chlorine(e6) conjugate was more effective in killing *C. albicans* than 50 μM of TBO and 200 μM of rose bengal after illumination at fluences ranging from 0 to 200 J/cm². Although the effectiveness of MB as a PS was also verified in vivo,²⁷ dyes have the undesirable effect of staining teeth, lips, tongue, buccal mucosa, and prosthetic devices. For this reason, a nondye photosensitizer would be more useful in the oral cavity.

Laser light sources are usually chosen to perform PDT. However, their high cost makes the appliances inaccessible to many institutions. Recently, alternative light sources, such as LED, have been successfully used in PDT.^{25,26,44} In the present investigation, LED was used as a light source because of its ability to irradiate larger areas than is possible with collimated laser light. Moreover, LED technology is simpler and has a lower cost than laser. The results of this study showed no significant difference between the LED light wavelengths used (blue and red) for photoinactivation of *C. albicans*. It was an unexpected result, because the maximum absorption band of Photogem is closer to 455 nm (blue) and 630 nm (red) matches the lowest Q-band. However, the chosen energy doses for both wavelengths were effective even for the red LED. Probably, the deeper penetration of red light into the biofilm, and therefore a higher-treated volume, may compensate its

weak absorption by Photogem. This may explain the lack of difference between blue and red LED lights observed in the present investigation.

Light propagation in biological tissues has been evaluated using direct measurement and complex mathematical equations.⁴⁵⁻⁴⁷ Absorption and scattering coefficients of tissues are factors responsible for several effects of light-tissue interaction; however, the optical properties of the same tissue may differ among patients.⁴⁸ Despite these considerations, it is known that light penetration into the tissue is proportional to its wavelength when considering the UV-near infrared range, i.e., the longer the wavelength, the deeper is the light penetration.⁴⁹⁻⁵² The shorter wavelengths are better absorbed by biological molecules, i.e., the greater part of light intensity is absorbed by the superficial tissue layers and as a consequence, lower penetration is observed. Whereas, photons in the red and infrared range are less absorbed by the biological chromophores and a higher light penetration is achieved. In addition, noncoherent light sources, such as LED lights, would be expected to achieve less optical penetration because of increased scattering.⁵⁰ Light scattering can be seen as a type of light diffusion as the penetration proceeds along the tissue. In the present investigation, it was observed that red LED light showed higher scattering than blue LED light in the tongue tissue.

Clinically, a 20-minute irradiation may be considered too long. However, a previously conducted pilot study demonstrated that shorter times (2.45, 10.00, and 15.00 minutes, which correspond to 37.5, 152.0, and 229.0 J/cm², respectively) were not effective when combined with 500, 300, 100, and 50 mg/L of Photogem (data not shown). As light fluence is proportional to exposure time and power output, increasing the power output of the light source would result in the same light fluence in a shorter exposure time. Nonetheless, a higher power output led to greater heating of the light source, which may not be clinically safe. On the other hand, when topical or systemic antifungal drugs are used, the medicament should be applied more than once a day, for several days, weeks, and, sometimes, months. The medicine should be taken for as long as recommended by the professional, because if the drug is stopped too soon, the symptoms may return. Thus, in comparison with antifungal agents, 20 minutes of illumination during a PDT session may be clinically applicable. Nonetheless, further clinical trials should be conducted to investigate whether the parameters found in this investigation would be effective in humans.

The histological evaluation of the animals' tongues was performed after they were killed on day 6, which corresponds to 24 hours after PDT for the animals from P+L+, P+L, and P-L+ groups. This analysis showed

that PDT had no adverse effects on the adjacent tissue. This finding is in agreement with that demonstrated by Kömerik et al.,¹⁵ who also verified no damage to periodontal tissues of rats submitted to PDT. Junqueira et al.⁵³ described fewer epithelial alterations and lower chronic inflammatory response in rats submitted to PDT and more intense lesions in rats treated only with laser. In the present study, yeast and pseudohyphae were observed only in the keratinized layer without invasion of the epithelium and the inflammation in the subjacent connective tissue was scored as mild for all mice infected with *C. albicans*, whether or not they were submitted to PDT. Therefore, the inflammation observed in the connective tissue may be associated with *Candida* infection but not with PDT. Nevertheless, the murine model of oral candidosis used in this investigation has previously shown the destruction of several epithelial layers.²⁹ This finding could be attributed to the strain used by these authors, as clinical strains isolated from infections showed increased virulence factors.³² The findings of the present investigation partially corroborate those of Teichert et al.,²⁷ as these authors also observed yeast and pseudohyphae limited to the keratinized layer, but lack of inflammatory changes in mice not submitted to PDT and subepithelial inflammatory infiltrate and neutrophilic exocytosis after PDT. Moreover, the different mouse strain, time of assays, PS, and light source used may also explain the divergence between the histological findings in these studies.

As regards the limitations of this study, different light fluences were not investigated, because it was previously found that shorter illumination times were not effective, as was explained previously, and longer illumination times were considered inapplicable. Moreover, the long-term effect of PDT was not evaluated. Sampling (recovery of *C. albicans* from tongues of mice and yeast culture) was done at only one time point, immediately after PDT, to assess the yeast viability immediately after treatment. However, sampling could be performed in different periods and a long-term effect of PDT on the yeast viability would be evaluated. In the present investigation, decrease of *C. albicans* counts was verified, but the macroscopic regression of white patches or pseudomembranes was not assessed. The partial or total disappearance of these lesions could have been evaluated if the mice had been killed at different intervals of time (longer periods than 24 hours after PDT).

In conclusion, the results of this study demonstrated that Photogem-mediated PDT promoted significant reduction in the viability of *C. albicans* biofilm without harming the tongue tissue. These results indicate the critical importance of determining effective in vivo

PDT parameters before clinical applications. However, the results cannot be extrapolated to a clinical situation, as the oral environment of humans is different (microbiota and biofilm composition, salivary flux, food habits, and so forth). Further in vivo studies are still necessary to investigate the parameters required for complete inactivation of *Candida* biofilms and the consequence of a repeatable therapy. Clinical trials are also required to evaluate the effect of PDT as a treatment of oral candidosis.

REFERENCES

1. Totti MA, dos Santos EB, de Almeida OP, Koga-Ito CY, Jorge AO. Oral candidosis by *Candida albicans* in normal and xerostomic mice. *Braz Oral Res* 2004;18:202-7.
2. Scully C, eL-Kabir M, Samaranayake LP. *Candida* and oral candidosis: a review. *Crit Rev Oral Biol Med* 1994;5:125-57.
3. Egusa H, Soysa NS, Ellepola AN, Yatani H, Samaranayake LP. Oral candidosis in HIV-infected patients. *Curr HIV Res* 2008;6:485-99.
4. Samaranayake LP, MacFarlane TW, editors. *Oral candidosis*. London: Wright; 1990. p. 265.
5. Allen CM. Animal models of oral candidiasis. A review. *Oral Surg Oral Med Oral Pathol* 1994;78:216-21.
6. Samaranayake YH, Samaranayake LP. Experimental oral candidiasis in animal models. *Clin Microbiol Rev* 2001;14:398-429.
7. Rex JH, Rinaldi MG, Pfaller MA. Resistance of *Candida* species to fluconazole. *Antimicrob Agents Chemother* 1995;39:1-8.
8. Cannon RD, Lamping E, Holmes AR, Niimi K, Tanabe K, Niimi M, et al. *Candida albicans* drug resistance: another way to cope with stress. *Microbiology* 2007;153:3211-7.
9. Mishra NN, Prasad T, Sharma N, Payasi A, Prasad R, Gupta DK, et al. Pathogenicity and drug resistance in *Candida albicans* and other yeast species. A review. *Acta Microbiol Immunol Hung* 2007;54:201-35.
10. Johnson EM, Warnock DW, Luker J, Porter SR, Scully C. Emergence of azole drug resistance in *Candida* species from HIV-infected patients receiving prolonged fluconazole therapy for oral candidosis. *J Antimicrob Chemother* 1995;35:103-14.
11. Hitchcock CA. Resistance of *Candida albicans* to azole antifungal agents. *Biochem Soc Trans* 1993;21:1039-47.
12. Donnelly RF, McCarron PA, Tunney MM. Antifungal photodynamic therapy. *Microbiol Res* 2008;163:1-12.
13. Konopka K, Goslinski T. Photodynamic therapy in dentistry. *J Dent Res* 2007;86:694-707.
14. Paulino TP, Ribeiro KF, Thedei G Jr, Tedesco AC, Ciancaglini P. Use of hand held photopolymerizer to photoinactivate *Streptococcus mutans*. *Arch Oral Biol* 2005;50:353-9.
15. Kömerik N, Nakanishi H, MacRobert AJ, Henderson B, Speight P, Wilson M. In vivo killing of *Porphyromonas gingivalis* by toluidine blue-mediated photosensitization in an animal model. *Antimicrob Agents Chemother* 2003;47:932-40.
16. Zeina B, Greenman J, Corry D, Purcell WM. Antimicrobial photodynamic therapy: assessment of genotoxic effects on keratinocytes in vitro. *Br J Dermatol* 2003;148:229-32.
17. Bertoloni G, Reddi E, Gatta M, Burlini C, Jori G. Factors influencing the hematoporphyrin-sensitized photoinactivation of *Candida albicans*. *J Gen Microbiol* 1989;135:957-66.
18. Wilson M, Mia N. Sensitisation of *Candida albicans* to killing by low-power laser light. *J Oral Pathol Med* 1993;22:354-7.
19. Jackson Z, Meghji S, MacRobert A, Henderson B, Wilson M. Killing of the yeast and hyphal forms of *Candida albicans* using

- a light-activated antimicrobial agent. *Lasers Med Sci* 1999; 14:150-7.
20. Bliss JM, Bigelow CE, Foster TH, Haidaris C. Susceptibility of *Candida* species to photodynamic effects of photofrin. *Antimicrob Agents Chemother* 2004;48:2000-6.
 21. Lambrechts SA, Aalders MC, Van Marle J. Mechanistic study of the photodynamic inactivation of *Candida albicans* by a cationic porphyrin. *Antimicrob Agents Chemother* 2005;49:2026-34.
 22. Demidova TN, Hamblin MR. Effect of cell-photosensitizer binding and cell density on microbial photoinactivation. *Antimicrob Agents Chemother* 2005;49:2329-35.
 23. Chabrier-Roselló Y, Foster TH, Pérez-Nazario N, Mitra S, Haidaris CG. Sensitivity of *Candida albicans* germ tubes and biofilms to photofrin-mediated phototoxicity. *Antimicrob Agents Chemother* 2005;49:4288-95.
 24. Donnelly RF, McCarron PA, Tunney MM, David Woolfson A. Potential of photodynamic therapy in treatment of fungal infections of the mouth. Design and characterisation of a mucoadhesive patch containing toluidine blue O. *J Photochem Photobiol B* 2007;86:59-69.
 25. Zanin IC, Gonçalves RB, Junior AB, Hope CK, Pratten J. Susceptibility of *Streptococcus mutans* biofilms to photodynamic therapy: an in vitro study. *J Antimicrob Chemother* 2005;56:324-30.
 26. Peloi LS, Soares RR, Biondo CE, Souza VR, Hioka N, Kimura E. Photodynamic effect of light-emitting diode light on cell growth inhibition induced by methylene blue. *J Biosci* 2008;33:231-7.
 27. Teichert MC, Jones JW, Usacheva MN, Biel MA. Treatment of oral candidiasis with methylene blue-mediated photodynamic therapy in an immunodeficient murine model. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002;93:155-60.
 28. Dovigo LN, Pavarina AC, Mima EG, Giampaolo ET, Vergani CE, Bagnato VS. Fungicidal effect of photodynamic therapy against fluconazole-resistant *Candida albicans* and *Candida glabrata*. *Mycoses* 2009 [Epub ahead of press].
 29. Takakura N, Sato Y, Ishibashi H, Oshima H, Uchida K, Yamaguchi H, et al. A novel murine model of oral candidiasis with local symptoms characteristic of oral thrush. *Microbiol Immunol* 2003;47:321-6.
 30. Sweet SP, Cookson S, Challacombe SJ. *Candida albicans* isolates from HIV-infected and AIDS patients exhibit enhanced adherence to epithelial cells. *J Med Microbiol* 1995;43:452-7.
 31. Schwab U, Milatovic D, Braveny I. Increased adherence of *Candida albicans* to buccal epithelial cells from patients with AIDS. *Eur J Clin Microbiol Infect Dis* 1997;16:848-51.
 32. Lyon JP, de Resende MA. Correlation between adhesion, enzyme production, and susceptibility to fluconazole in *Candida albicans* obtained from denture wearers. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006;102:632-8.
 33. Sanitá PV, Vergani CE, Giampaolo ET, Pavarina AC, Machado AL. Growth of *Candida* species on complete dentures: effect of microwave disinfection. *Mycoses* 2009;52:154-60.
 34. Zeina B, Greenman J, Purcell WM, Das B. Killing of cutaneous microbial species by photodynamic therapy. *Br J Dermatol* 2001; 144:274-8.
 35. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). *J Antimicrob Chemother* 1998;42:13-28.
 36. Sternberg ED, Dolphin D. Porphyrin-based photosensitizers for use in photodynamic therapy. *Tetrahedron* 1998;54:4151-202.
 37. Lambrechts SA, Aalders MC, Verbraak FD, Lagerberg JW, Dankert JB, Schuitmaker JJ. Effect of albumin on the photodynamic inactivation of microorganisms by a cationic porphyrin. *J Photochem Photobiol B* 2005;79:51-7.
 38. Douglas LJ. *Candida* biofilms and their role in infection. *Trends Microbiol* 2003;11:30-6.
 39. Soukos NS, Mulholland SE, Socransky SS, Doukas AG. Photodestruction of human dental plaque bacteria: enhancement of the photodynamic effect by photomechanical waves in an oral biofilm model. *Lasers Surg Med* 2003;33:161-8.
 40. de Beer D, Srinivasan R, Stewart PS. Direct measurement of chlorine penetration into biofilms during disinfection. *Appl Environ Microbiol* 1994;60:4339-44.
 41. Wilson M, Mia N. Effect of environmental factors on the lethal photosensitization of *Candida albicans* in vitro. *Lasers Med Sci* 1994;9:105-9.
 42. Munin E, Giroldo LM, Alves LP, Costa MS. Study of germ tube formation by *Candida albicans* after photodynamic antimicrobial chemotherapy (PACT). *J Photochem Photobiol B* 2007; 88:16-20.
 43. Giroldo LM, Felipe MP, de Oliveira MA, Munin E, Alves LP, Costa MS. Photodynamic antimicrobial chemotherapy (PACT) with methylene blue increases membrane permeability in *Candida albicans*. *Lasers Med Sci* 2009;24:109-12.
 44. Soares BM, da Silva DL, Sousa GR, Amorim JC, de Resende MA, Pinotti M, et al. In vitro photodynamic inactivation of *Candida* spp. growth and adhesion to buccal epithelial cells. *J Photochem Photobiol B* 2009;94:65-70.
 45. Maschesini R, Bertoni A, Andreola S, Mellari E, Sichirollo AE. Extinction and absorption coefficients and scattering phase functions of human tissue in vitro. *Appl Opt* 1989;28:2318-24.
 46. Cheong WF, Pahl SA, Welch AJ. A review of the optical properties of biological tissues. *IEEE J Quant Electr* 1990;26: 2166-85.
 47. Arnfield MR, Mathew RP, Tulip J, McPhee MS. Analysis of optical coefficients using an approximate equation valid for comparable absorption and scattering. *Phys Med Biol* 1992;37: 1219-30.
 48. Star WM. Light dosimetry in vivo. *Phys Med Biol* 1997;42: 763-87.
 49. Stolik S, Delgado JA, Pérez A, Anasagasti L. Measurement of the penetration depths of red and near infrared light in human "ex vivo" tissues. *J Photochem Photobiol B* 2000;57:90-3.
 50. Alexiades-Armenakas M. Laser-mediated photodynamic therapy. *Clin Dermatol* 2006;24:16-25.
 51. Wei HJ, Xing D, Wu GY, Jin Y, Gu HM. Optical properties of human normal bladder tissue at five different wavelengths of laser and their linearly polarized laser irradiation in vitro. *Spectroscopy and Spectral Analysis* 2004;24:1039-41.
 52. Taroni P, Pifferi A, Torricelli A, Comelli D, Cubeddu R. In vivo absorption and scattering spectroscopy of biological tissues. *Photochem Photobiol Sci* 2003;2:124-9.
 53. Junqueira JC, da Silva Martins J, Faria RL, Colombo CE, Jorge AO. Photodynamic therapy for the treatment of buccal candidiasis in rats. *Lasers Med Sci* 2009;24:877-84.

Reprint requests:

Ana Cláudia Pavarina, DDS, MSc, PhD
Faculdade de Odontologia de Araraquara-UNESP
Rua Humaitá, n° 1680
CEP: 14801-903, Araraquara, SP, Brazil
pavarina@foar.unesp.br