Photochemical & Photobiological Sciences

PAPER

Cite this: *Photochem. Photobiol. Sci.*, 2015, **14**, 1319

In vivo evaluation of photodynamic inactivation using Photodithazine® against *Candida albicans*

J. C. Carmello,[a] L. N. Dovigo,[b] E. G. Mima,[a] J. H. Jorge,[a] C. A. de Souza Costa,[c] V. S. Bagnato[d] and A. C. Pavarina*[a]

Received 3rd October 2014,
Accepted 16th May 2015

DOI: 10.1039/c4pp00368c

www.rsc.org/pps

This study describes the photoinactivation of *Candida albicans* in a murine model of oral candidosis, mediated by Photodithazine® (PDZ). Six-week-old female Swiss mice were immunosuppressed, and inoculated with *C. albicans* to induce oral candidosis. After five days, photodynamic inactivation (PDI) mediated by PDZ at concentrations of 75, 100, 125 and 150 mg L^{-1} was applied on the tongue of mice. Next, microbiological evaluation was performed by recovering *C. albicans* from the tongue *via* colony forming units (CFU mL^{-1}). After 24 h of treatment, the animals were killed and the tongues were surgically removed for histological analysis. PDI was effective in reducing *C. albicans* on the tongue of mice using 100 mg L^{-1} of PDZ, when compared to the positive control group (without treatment). No adverse effect on the tongue tissue was verified after PDI. Therefore, PDI was effective for inactivation of *C. albicans* without causing any harmful effects on host tissues, which is promising for future clinical trials.

1. Introduction

Candida albicans is the most prevalent pathogen in humans and is responsible for diseases ranging from oropharyngeal candidosis (OPC) overgrowth to disseminated forms of infections.[1] The expression of *C. albicans* virulence can be associated with a deficiency in the host immune response and other systemic conditions, such as hyposalivation, diabetes mellitus, prolonged use of broad-spectrum antibiotics and transplantation.[2] Many medications are available for the treatment of *Candida* infection such as local antifungal agents (polyenes and azoles) and systemic drugs (amphotericin B, itraconazole). However, the use of these medications can promote hepatotoxicity and development of antifungal resistance.[3] Another aspect related to recurrent infection and antifungal resistance is the ability of *Candida* spp. to form biofilms on surfaces.[4] Biofilms are structured, coordinated and functional community of microorganisms enclosed in a self-produced polymeric matrix and adhered to biotic or abiotic surfaces.[5] The biofilm structure provides survival advantage to the yeast and increased resistance to antifungal drugs.[3,5] Due to these difficulties associated with the use of conventional drugs and antifungal resistance, photodynamic therapy (PDI) has been suggested as an alternative method to inactivate *Candida* species and consequently to treat oral candidosis.

PDI uses light energy to activate a photosensitizer (PS) in the presence of oxygen.[6] This process initiates a cascade of photo-chemical events that causes the PS to undergo a transition to a higher energy state, culminating in the production of free radicals and other reactive species, such as singlet oxygen. These reactive species induce cytotoxicity and are responsible for irreversible damage on microorganisms.[7] The major advantages of PDI are the reduced chances of collateral effects, since it can be applied specifically to the target cells, and its effectiveness against both antifungal-susceptible and resistant microbial cells.[8] However, photoinactivation of yeast seems to be harder to achieve because the larger cell size and structural characteristics of these eukaryotic organisms demand a greater amount of oxygen singlets for cell metabolism inactivation.[9]

Chlorine e6 is the second-generation PS derived from oxidation of chlorophyll *a*[10] and a promising compound, characterized by shorter periods of photosensitization, longer activation wavelengths and higher yields of singlet oxygen.[11]

[a]*Department of Dental Materials and Prosthodontics, Araraquara Dental School, UNESP – Univ Estadual Paulista. Rua Humaitá, 1680, 14801-903 Araraquara, SP, Brazil. E-mail: cabrini.juliana@gmail.com, ewerton_mima@hotmail.com, janainahj@foar.unesp.br, pavarina@foar.unesp.br; Tel: +55 16 33016544*
[b]*Department of Social Dentistry, Araraquara Dental School, UNESP – Univ Estadual Paulista. Rua Humaitá, 1680, 14801-903 Araraquara, SP, Brazil. E-mail: lidovigo@foar.unesp.br; Tel: +55 16 33016552*
[c]*Department of Physiology and Pathology, Araraquara Dental School, UNESP – Univ Estadual Paulista Rua Humaitá, no 1680, 14801–903 Araraquara, SP, Brazil. E-mail: casouzac@foar.unesp.br*
[d]*Physics Institute of São Carlos, USP – University of São Paulo, Av. Trabalhador São-carlense, 400, CEP: 13566-590 São Carlos, SP, Brazil. E-mail: vander@ifsc.usp.br; Tel: +55 16 33739810*

Photodithazine® (PDZ) is a glucosamine salt of chlorine (e6), which is water soluble and an amphiphilic molecule.[12] These molecules are able to penetrate into the biological membranes, which usually improves PDI action.[13] Previous *in vitro* studies showed that PDI mediated by PDZ and other chlorine e6 derivatives, and red light-emitting diode, effectively inactivated planktonic cells of *C. albicans* and *Candida guilliermondii*.[14–16] Moreover PDZ was also effective in reducing the viability of clinical isolates of *Candida* spp.[17] and microorganisms grown in multispecies biofilms.[18] However, *in vivo* investigation demonstrating the effectiveness of PDZ against *Candida* species has not been performed yet. The use of animal models for PDI evaluation is an important step to prove the *in vivo* effectiveness of the treatment and may provide outcomes that are more closely correlated to clinical situations, in comparison with *in vitro* studies. In addition, there are some advantages in studying animals instead of humans beings, such as the evaluation of standardized samples, in which the animal and its environment can be manipulated and controlled in order to allow universally comparable data on the etiopathology, and the management of candidosis.[19]

Therefore, this study aimed to contribute to *in vivo* antimicrobial PDI by reporting the use of PDZ for the inactivation of *C. albicans* in a murine model of oral candidosis when associated with LED light.

2. Materials and methods

2.1 Photosensitizer (PS) and light source

The PS used in this investigation was a chlorine e6 derivative, as PDZ, which is obtained by the reduction of a pyrrole double bond on the porphyrin periphery, and these classes of molecules have band I absorption maxima at longer wavelengths (λ_{max}) 650–670 nm.[12] Photodithazine® is produced by VETA-GRAND Co., (Moscow, Russia), and the molecular structure was changed by the addition of the solubilizing and stabilizing agent *N*-dimethyl-D-glucosamine.[12] This compound is water soluble, stable and has a homogeneous chemical composition (60% of *N*-dimethyl-D-glucosamine complex of chlorine e6, chlorine p6 and purpurins 7 and 18).

The ability of PDI to inactivate microorganisms was evaluated using PDZ at concentrations of: 75, 100, 125 and 150 mg L^{-1}.[17] Stock solutions of PDZ were prepared in microcentrifuge tubes wrapped in an aluminum foil, and mixed vigorously for 1 minute to complete homogenization. The entire procedure was performed prior to the beginning of each experiment and the stock solutions were obtained from the commercial solution of PDZ (5000 mg L^{-1}). The solutions were kept in the dark until use.

One handpiece with a red (660 nm) light emitting diode (LXHL-PR09, Luxeon® III Emitter, Lumileds Lighting, San Jose, California, USA) was designed by the 'Physics Institute of São Carlos, USP' (Univ. de São Paulo, São Carlos, SP, Brazil). The light output power delivered at the end of the handpiece (5 mm in diameter) was 44.6 mW cm^{-2}.

2.2 *Candida* strains and culture conditions

A reference strain of *C. albicans* (ATCC 90028, Rockville, MD) was used in this study. The strain was maintained in microtubes with solid yeast–peptone–glucose medium and frozen at −70 °C. Prior to each experiment, the yeast was aerobically cultured in Sabouraud's Dextrose Agar (SDA, Acumedia Manufacturers Inc., Baltimore, MD) containing 5 μg mL^{-1} gentamicin at 37 °C for 48 h. The yeast was inoculated in 5 mL of Tryptic Soy Broth (TSB, pH 7.2, Acumedia Manufacturers Inc., Baltimore, MD) and grown overnight aerobically at 37 °C for 24 h. To obtain the standardized suspensions of *Candida* each culture was harvested after centrifugation at 2000 rpm for 10 minutes, washed with sterile distilled water and resuspended in 2 ml of sterile saline to 10^7 CFU ml^{-1} by adjusting the optical density of the suspension to 0.38 at 520 nm in a spectrophotometer (Biospectro, Equipar Ltda, Curitiba, Paraná, Brazil).[17]

2.3 Animals and induction of experimental candidosis

The study was performed in compliance with the relevant laws and institutional guidelines and was approved by the Animal Ethics Committee (Araraquara Dental School, São Paulo State University). Fifty-five 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 ± 2 °C). Standard chow and tap water were given *ad libitum*.

The methodology described by Takakura *et al.*[20] was used to induce oral candidosis in mice. The study design is shown in Fig. 1. The animals were immunosuppressed with 2 subcutaneous injections of prednisolone (Depo-Medrol, Laboratórios Pfizer Ltda., Guarulhos, SP, Brazil) at a dose of 100 mg per kg body weight 1 day before and 3 days after the infection with *Candida*. The mice were given tetracycline hydrochloride (Farmácia Santa Paula, Araraquara, SP, Brazil) in their drinking water at the concentration of 0.83 mg mL^{-1}, which began on day 1 of the experiment until the last day of the experiment. On day 2, the mice were anesthetized by an intramuscular injection with 50 μL of 2 mg mL^{-1} chlorpromazine chloride (Farmácia Santa Paula, Araraquara, SP, Brazil) in each femur and small cotton pads (Cotton-baby, Higie-Plus Cottonbaby Ind. Com. Ltda., São José, SC, Brazil) soaked in a fungal cell suspension (10^7 CFU mL^{-1}) were used to swab the entire oral cavity for 30 seconds to produce oral candidosis. The swabs contained 9×10^6 CFU mL^{-1} of *C. albicans*, approximately.

2.4 Photodynamic therapy and microbiological evaluation

According to Takakura *et al.*[20] five days after *Candida* inoculation mice were anesthetized by an intramuscular injection of 100 mg per kg body weight of ketamine (União Química Farmacêutica Nacional S/A., Embu-Guaçu, SP, Brazil) and 10 mg per kg body weight of xylazine (Produtos Veterinários J. A. Ltda., Patrocínio Paulista, SP, Brazil). To perform the treatment, each animal was placed in a supine position and the tongue was gently taken out of the mouth without causing any injury to the tissue. Next, 70 μL of PDZ solution was topically pipetted on the tongue dorsum at concentrations of 75, 100, 125 or

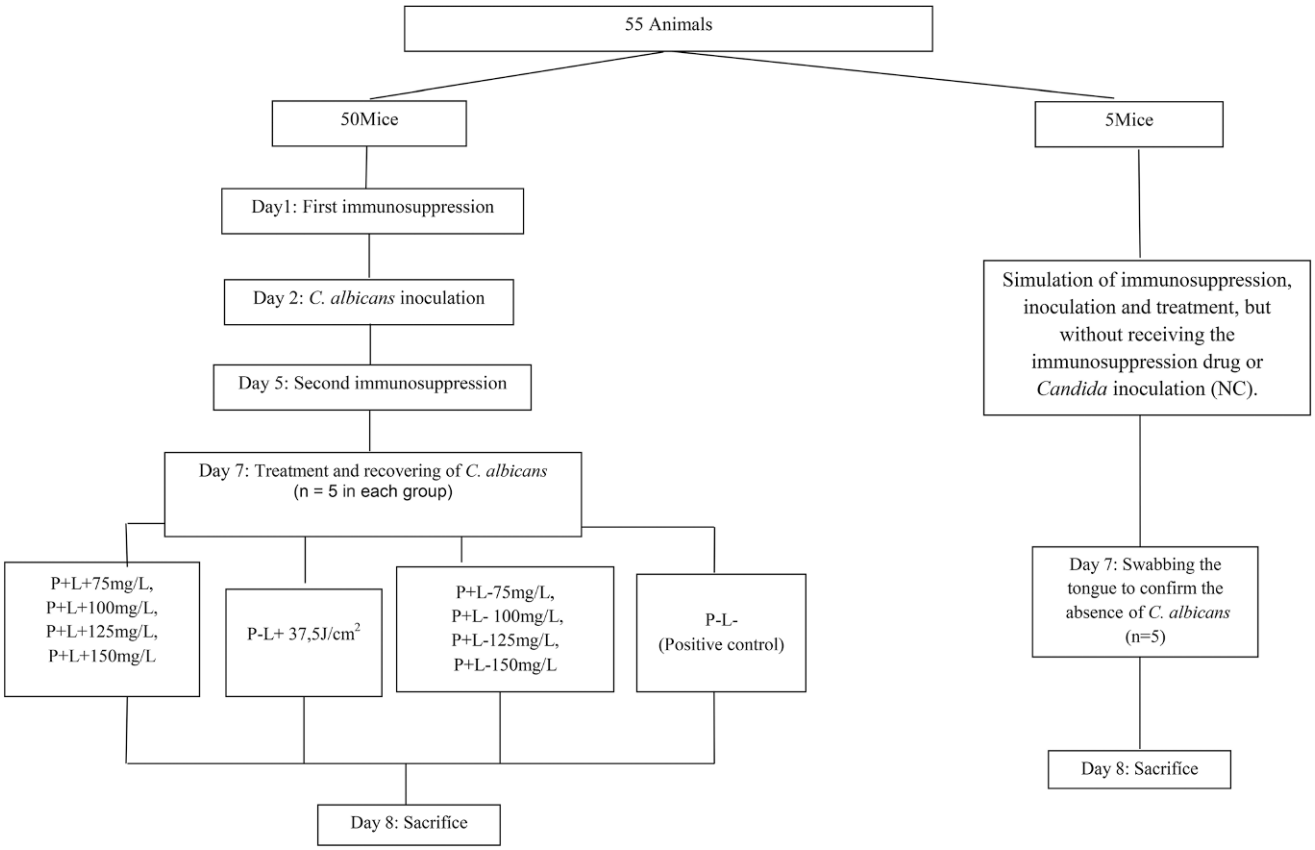

Fig. 1 Study design: * Tetracycline hydrochloride was given in drinking water during days 1 to 7; P+L+ groups corresponded to the animals treated with PS and LED light; ** P−L+ group corresponded to evaluation of LED light alone; *** P+L− groups corresponded to evaluation of PS alone.

150 mg L^{-1} and the mice were kept in the dark for 20 minutes (pre-irradiation time). Next, the LED device was placed onto the dorsum of the tongue, and the irradiation time for each application was 14 minutes, resulting in a total fluence of 37.5 J cm^{-2} (P+L+ groups). The effect of PDZ alone was tested by applying PDZ for the same periods of pre-irradiation and irradiation times and at the same concentrations that were used for the P+L+ group, without the LED illumination (P+L−, 4 groups). To verify only the effect of light, animals were exposed to the same LED light dose mentioned earlier (P−L+, 1 group). The positive control did not receive any PS or light (P−L−). The negative control group (NC) was evaluated with a simulation of immunosuppression, inoculation and treatment in the same way as performed in the P+L+ group, but without receiving the immunosuppression drug or *Candida* suspension. Each experimental group consisted of 5 animals (n = 5).

Macroscopic evaluation of *Candida* infection was confirmed by white lesions on the tongue surface. The dorsum of the tongue was swabbed for 1 minute with a cotton pad. The end of the cotton pad was cut off and placed in a tube containing 1 mL of sterile saline. The tubes were mixed with a vortex mixer for 1 minute to release *C. albicans* cells from the swab into the saline. Duplicate 25 μL aliquots from the 10-fold serial dilutions were spread over the surface of SDA with 5 mg L^{-1} gentamicin. All plates were aerobically incubated at 37° for 48 hours. The yeast colony counts were quantified and the number of CFU mL^{-1} was determined. This procedure of swabbing and plating samples was performed for each experimental group after treatment. The recovery of *C. albicans* from the tongues of mice and yeast culture was done immediately after PDI, in order to assess the yeast viability and the local inflammatory response immediately after treatment.

2.5 Histopathological study

In order to evaluate whether PDI may harm the host tissue and based on the fact that immune system of mice would restore itself in a few days, animals were killed 24 hours after treatment and histological analysis was performed.

One day after PDI treatment, all mice from all groups were killed with a lethal dose of ketamine. The tongues from the euthanized mice were surgically 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 PDI was examined by an oral pathologist blinded to all groups of mice. This pathologist made a descriptive analysis of the histological characteristics of the tissue with and without local inflammatory response of varied intensity without knowing which group the images belonged to. The main characteristics observed on the histological images were the epithelium integrity and the presence of inflammatory cells in the adjacent connective tissue according to a 5-point scoring system (0: inflammation absent; 1: mild inflammation; 2: moderate inflammation; 3: severe inflammation and 4: abscess formation – ISO 7405:1997).

2.6 Statistical analysis

C. albicans counts (CFU mL^{-1}) were transformed into base-10 logarithms (Table 1). A parametric analysis was conducted given that this type of analysis is robust to ANOVA-assumptions violation in the scenario presented (balanced groups within each factor).[21] Thus, log_{10}-transformed CFU mL^{-1} were analyzed with a two-way ANOVA with PDZ (0; 75; 100; 125 and

Table 1 Log_{10}-transformed CFU mL^{-1} and summary values obtained from each group for statistical analysis

PDZ concentration	Light fluence	CFU mL^{-1}	Log_{10} (CFU mL^{-1})	Mean	Standard deviation	Standard error
0.0	0.0	4.80×10^5	5.681242	4.93	0.64	0.28501
		3.20×10^4	4.505164			
		3.74×10^5	5.572873			
		2.46×10^4	4.390953			
		3.24×10^4	4.510558			
0.0	37.5	2.18×10^5	5.338458	4.85	0.37	0.16679
		6.76×10^4	4.829953			
		6.20×10^4	4.792399			
		2.00×10^4	4.301052			
		9.19×10^4	4.963320			
75.0	0.0	2.52×10^5	5.401402	5.42	0.21	0.0954
		1.58×10^5	5.198660			
		5.32×10^5	5.725912			
		3.26×10^5	5.513219			
		1.76×10^5	5.245515			
75.0	37.5	1.92×10^5	5.283303	4.71	0.52	0.23433
		1.70×10^4	4.230474			
		6.26×10^4	4.796581			
		1.38×10^5	5.139882			
		1.32×10^4	4.120607			
100.0	0.0	2.74×10^4	4.437766	5.06	0.36	0.16014
		2.20×10^5	5.342425			
		1.62×10^5	5.209518			
		1.54×10^5	5.187524			
		1.40×10^5	5.146131			
100.0	37.5	3.40×10^2	2.532754	1.05	1.44	0.64572
		0.00×10^0	0.000000			
		0.00×10^0	0.000000			
		$5.40E \times 10^2$	2.733197			
		0.00×10^0	0.000000			
125.0	0.0	2.38×10^4	4.376595	4.69	0.39	0.17388
		3.54×10^4	4.549016			
		2.28×10^4	4.357954			
		8.48×10^4	4.928401			
		1.80×10^5	5.255275			
125.0	37.5	0.00×10^0	0.000000	2.34	2.50	1.11965
		0.00×10^0	0.000000			
		1.46×10^5	5.164356			
		5.70×10^4	4.755882			
		6.00×10^1	1.785330			
150.0	0.0	9.00×10^2	2.954725	4.30	1.20	0.53529
		1.08×10^3	3.033826			
		1.86×10^5	5.269515			
		1.84×10^5	5.264820			
		9.30×10^4	4.968464			
150.0	37.5	1.20×10^2	2.082785	2.26	1.39	0.61952
		6.20×10^2	2.793092			
		0.00×10^0	0.000000			
		4.66×10^3	3.668479			
		6.00×10^2	2.778874			

150 mg L^{-1}) and light (0 and 37.5 J cm^{-2}) as main effects, followed by Games–Howell *post-hoc* tests for multiple comparisons. The significance level adopted was 5%. A qualitative analysis of the histopathological images was performed.

3. Results

White patches or pseudomembranes were observed on the tongue dorsum of all animals which received *Candida*, 5 days after inoculation (Fig. 2A). The number of *C. albicans* recovered from infections before any treatment ranged between 10^4 and 10^5 CFU mL^{-1}. Histological evaluation confirmed the presence of the infection (Fig. 2B).

As shown in Table 2, both light and PDZ concentrations had a significant effect on yeast viability, as well as the interaction of the two factors. The most effective photodynamic protocol in reducing *C. albicans* viability was the use of 100 mg L^{-1} of PDZ associated with LED light, which was equivalent to 4.36 $\log_{10}$ of reduction ($p = 0.027$ when compared to the P–L– group; Fig. 2), and three animals belonging to this group showed no viable *Candida* after PDI. The use of higher PDZ concentrations (125 and 150 mg L^{-1}) and light also led to some reduction in $\log_{10}$ values (CFU mL^{-1}) that were equivalent to 2.72 and 2.43 $\log_{10}$ reductions, respectively, with no evidence of *C. albicans* on the tongue dorsum of two animals and one animal treated with 125 and 150 mg L^{-1}, respectively, and light (P+L+ groups). However, the mean values of these groups were considered statistically similar to the P–L– group ($p \geq 0.105$). The use of 75 mg L^{-1} PDZ showed 0.22 $\log_{10}$ of reduction after PDI. The mean value of this group was considered statistically similar to the P–L– group ($p \geq 0.324$).

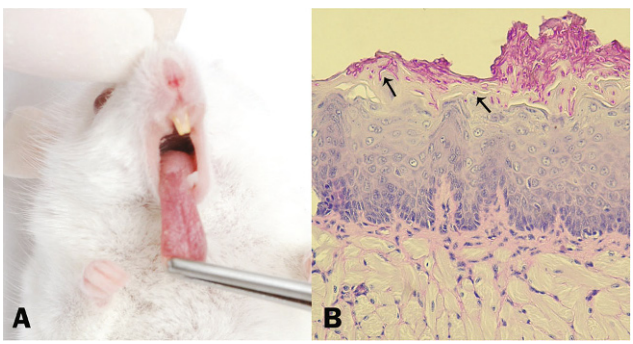

Fig. 2 (A) White patches or pseudomembranes found on the dorsum tongue of all immunosuppressed mice, five days after *C. albicans* inoculation (P + L+, P–L+, P+L– and P–L– groups). (B) Representative histological section of the tongue of immunosuppressed mice infected with *C. albicans*, five days after *C. albicans* inoculation (P+L+, P–L+, P+L– and P–L– groups); arrows are showing the presence of yeast and hyphae on the keratinized layer.

The use of PDZ (75, 100, 125 and 150 mg L^{-1}) in the absence of light had no significant effect on the viable count of *C. albicans* ($p \geq 0.610$; Fig. 3) and the mean values of these groups were statistically similar to the P–L– group. Similarly, the use of red LED light alone (without photosensitization with PDZ) caused no effect on the yeast viability ($p = 0.247$). In summary, the use of PDZ or light alone was not toxic to *C. albicans* cells. Histological analysis of the tongue of the three animals from P+L+ groups with 100 mg L^{-1} of PDZ revealed the absence of yeast and pseudohyphae on the keratinized layer (Fig. 4A). This finding corroborated with the previous observation that total inactivation of viable *C. albicans* (CFU mL^{-1}) was achieved in three animals from this experimental group. For the animals of P+L+ groups treated with 75, 125 and 150 mg L^{-1} of PDZ, the presence of yeast and pseudohyphae limited to the keratinized layer on the dorsum of the tongue was observed (Fig. 4B). No histological evidence of invasion of yeast and pseudohyphae into deep layers of the continuous epithelium was noticed. For the positive control (P–L–) and other groups (P–L+ and P+L– with 75, 100, 125 and 150 mg L^{-1} of PDZ) a high number of yeast and pseudohyphae/hyphae was found limited to the keratinized layer on the dorsum of the tongue, but no evidence of microbiological invasion into deep layers of the continuous epithelium was observed (Fig. 4C). In addition, the keratinized layer of these specimens was thinner than those without *Candida* inoculation, and the subjacent connective tissue exhibited mild inflammatory response mediated by mononuclear cells (score 1). The mice from the negative control group showed no fungi on the dorsum of the tongue. The epithelial tissue was continuous with an intact keratin and basal layer. The subjacent connective tissue demonstrated a balance between extracellular matrix components and cells, with the presence of intact muscle tissue in the area (Fig. 4D).

Table 2 Summary of two-way ANOVA (light × PDZ) for data obtained after photodynamic treatment of *C. albicans*-induced infection in mice[a]

Source	SS	df	MS	*F*	*p*	Effect size	Power
Light	42.204	1	42.204	32.845	<0.001	0.451	1.000
PDZ	35.529	4	8.882	6.913	<0.001	0.409	0.988
Light × PDZ	23.453	4	5.863	4.563	0.004	0.313	0.916
Error	51.397	40	1.285				
Total	937.599	50					

[a] SS = sum of squares, df = degrees of freedom, MS = mean square, $F = F$ value, $p = p$ value.

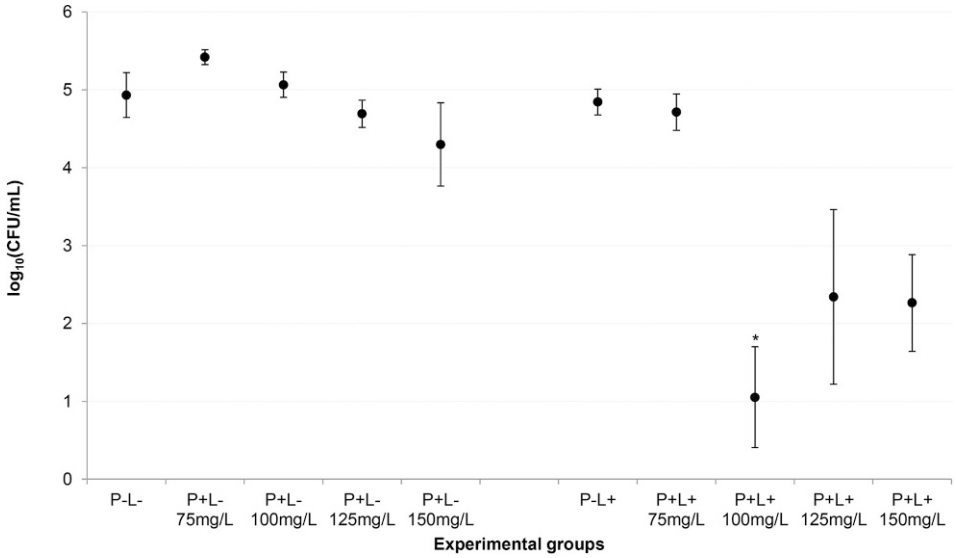

Fig. 3 Mean values and standard error observed for experimental groups. Asterisks (*) represent statistically significant differences among groups according to Games–Howell *post-hoc* test ($p < 0.05$).

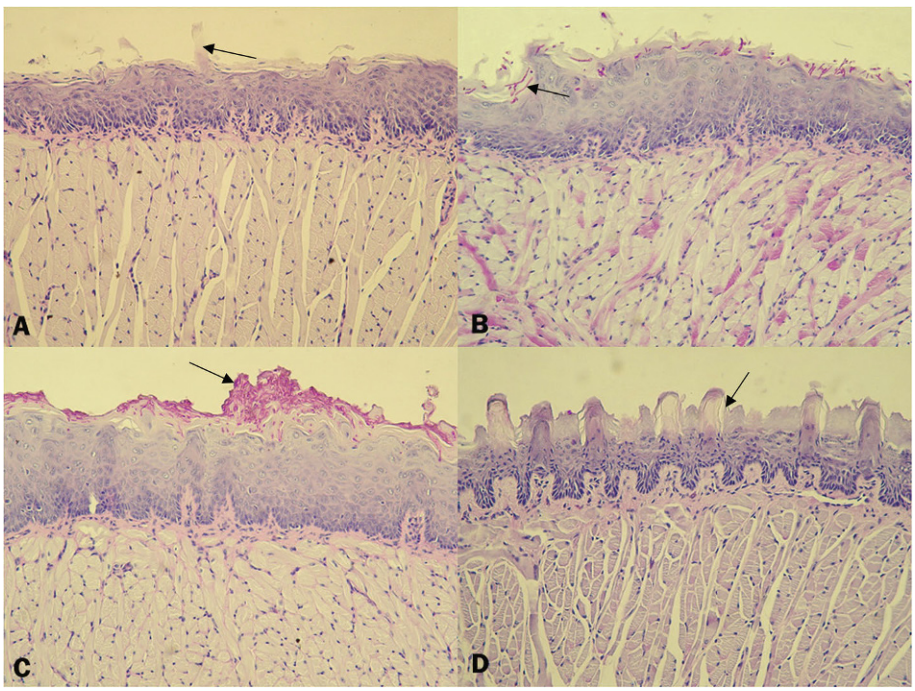

Fig. 4 Histological sections of the tongue of mice (PAS-H, 200). (A) Animals from the P+L+ group with 100 mg L^{-1}, the arrow indicates the absence of yeast and pseudohyphae/hyphae on the keratinized layer; (B) animals from the P+L+ group with 125 mg L^{-1} of PDZ, the arrow shows some yeast and hyphae on the keratinized layer; (C) animals from the positive control group (untreated animals), arrow is showing numerous yeast and pseudohyphae/hyphae limited to the keratinized layer on the dorsum of the tongue; (D) animals from the negative control group (NC), arrow shows the continuous epithelial tissue with an intact keratin and basal layer.

4. Discussion

In recent years several *in vitro* investigations have pointed out that antifungal PDI could be an interesting approach for the treatment of oral candidosis.[8,9] Different *in vitro* analyses of photosensitizers have been carried out, including the use of chlorine e_6 derivatives that have already shown promising results against *Candida* cells.[14–16] However, a limited number of

in vivo studies, such as animal evaluations or clinical trials, have been conducted to confirm the effectiveness of PDI as an antifungal therapy against *Candida* infections.[22–28] In the present study, animals with induced oral candidosis were exposed to PDI, mediated by the chlorine e_6 derivative PDZ, and the survival rate of the yeast was assessed immediately after the therapy. The results demonstrated that PDI promoted significant reductions on *C. albicans* viability, in comparison with the control group (P−L−). Data from previous *in vivo* investigations are comparable with our results, since they also showed that PDI is effective to inactivate *Candida* in localized infections.[22,26,28]

Statistical analysis did not reveal significant differences among the PDI groups 75, 125 and 150 mg L^{-1} of PDZ. However, the use of 100 mg L^{-1} resulted in the highest $\log_{10}$ reduction (4.36 $\log_{10}$) and three animals belonging to this group showed no viable *Candida* after PDT. Promising results of photoinactivation of *C. albicans* using methylene blue,[22] porphyrin,[28] erythrosine[27] and curcumin[26] have been published using animal models of oral candidosis. However, a high concentration of methylene blue and porphyrin was reported in the cited studies.[22,28] In order to obtain the eradication of *C. albicans*, Teichert *et al.*[22] used high concentrations of methylene blue (450 and 500 μg mL^{-1}) and the overall reduction was of approximately 3 $\log_{10}$. In the investigation conducted by Mima *et al.*[28] Photogem® was used at concentrations of 400, 500 and 1000 mg L^{-1} and the *Candida* reductions achieved were of 1.05, 1.59 and 1.40 $\log_{10}$, respectively. In addition, in these studies, higher light fluences were used to obtain photoinactivation, in comparison with the present investigation. Costa *et al.*[27] reported reduction of only 0.73 $\log_{10}$ on *C. albicans* counts recovered from immunosuppressed mice with oral candidosis after erythrosine-mediated PDI, which reduced by 35% the *in vitro* yeast adherence to buccal epithelial cells. The different PS concentrations and fluences of light required for photoinactivation in each study may be justified by the structure of the PS used. Chlorine derivatives are efficient PS with a very high singlet oxygen yield, so low concentrations of the drug may be required to promote the PDI effectiveness.[14,29] In addition, due to the high singlet oxygen yield the illumination time can be shortened. Corroborating the outcomes of the present investigation, Dovigo *et al.*[26] reported 4 $\log_{10}$ of reduction using curcumin at 80 μM, and some mice also showed complete eradication of *C. albicans* after PDI. On the other hand, it was interesting to observe that the use of high PDZ concentrations, such as 125 and 150 mg L^{-1}, did not improve the effectiveness of PDI. Similar result was observed by Mima *et al.*[28] with the use of a porphyrin derivative. This may be explained by the possibility of formation of aggregates at high PS concentrations. As seen with different types of PS in water-soluble solutions, the formation of dimers and higher aggregates are a common problem in the photodynamic field. Aggregation has a direct influence on photophysical behavior, rendering normally active PS inactive through self-quenching.[30] Since the use of 100 mg L^{-1} of PDZ was adequate to promote the photoinactivation of *Candida*, increasing the PS concentration is not recommended in order to avoid the aggregation of the drug and consequently the failure of the treatment.

On the last day of the experiment, all animals were sacrificed and tongues were removed to perform the histological analysis of the specimens. The findings suggest that no adverse effect on the adjacent tissue was induced by PDI, since the tissue exhibited histological characteristics of normality. A low inflammatory response was observed in the subjacent connective tissue of all mice infected with *C. albicans*, regardless the type of treatment received. Only the animals from the negative control group, which did not receive *Candida* inoculation, showed no signs of inflammation in the subjacent connective tissue. Therefore, the inflammation verified in the mice subjected to PDI may be associated with the *Candida* infection and not be related to the PDT treatment. Similarly, Mima *et al.*[28] and Dovigo *et al.*[26] also verified a mild inflammatory response in the subjacent connective tissues of all mice infected with *C. albicans*, subjected or not to PDI. In addition, these results agree with the investigation conducted by Fernandes *et al.*[31] who demonstrated the effectiveness of PDI as an adjunctive treatment of induced periodontitis without any harmful effects on the tissues. On the other hand, Junqueira *et al.*[23] who evaluated the efficacy of PDI on buccal candidosis in rats, observed fewer epithelial alterations and lower chronic inflammatory response in the PDI groups, compared with the groups treated with light only. The different PS, exposure time and experimental model for oral candidosis used may explain the divergence between such findings in these studies. An important observation was that the histological sections from PDI groups (P+L+) revealed a small number of hyphae in the keratin layer, compared to the positive control group. In addition, for the P+L−, P−L+ and P−L−groups, yeast and pseudohyphae were verified only in the keratinized layer without an invasion of the epithelium. The results of the present study were comparable with the findings reported by Teichert *et al.*[22] Mima *et al.*[28] Junqueira *et al.*[23] and Dovigo *et al.*[26] These authors also verified hyphae and pseudohyphae in the keratinized layer without destruction of the epithelial layers. On the other hand, in the investigation conducted by Takakura *et al.*[20], the histological analysis performed demonstrated numerous hyphae on the epithelium of the tongue dorsum and destruction of the epithelial layers. The divergent outcomes of these investigations may be attributed to the strain used to induce oral candidosis, since Takakura *et al.*[20] employed a clinical isolate of *C. albicans*, which may show increased virulence factors.

It is important to mention that the experimental model of oral candidosis used in this investigation showed reproducible results. The presence of white patches or pseudomembranes was verified on the tongue of all animals evaluated and the mean number of *C. albicans* recovered was approximately 10^{4-5} CFU mL^{-1}. These CFU values were similar with those observed by Mima *et al.*[28] and Dovigo *et al.*[26] but lower in comparison with those described by Takakura *et al.*[20] (10^{5-6} CFU mL^{-1}). The similarity between the CFU values observed in the present study and those verified by Mima *et al.*[28] and Dovigo *et al.*[26]

may be attributed to the use of a reference strain to perform *Candida* inoculation. On the other hand, Takakura *et al.*[20] used a clinical strain isolated from a patient with cutaneous candidosis. Some investigations have shown that strains isolated from patients with acquired immunodeficiency syndrome (AIDS) and denture stomatitis can be more adherent to epithelial cells than isolates from healthy individuals.[32] Other methodologies were also proposed to induce oral candidosis in animals.[22,33] Teichert *et al.*[22] used a protocol of severe combined immunodeficiency disease (SCID) to induce oral candidosis and recovered approximately 2×10^2 CFU mL^{-1} from infections. Another method to induce oral candidosis was used by Totti *et al.*[33] In this study, the major salivary glands of rats were surgically removed to induce this infection. The infection was identified by the presence of lesions on the dorsum of the tongue which were confirmed by the histopathological analysis. Nakajima *et al.*[34] determined the amount of *Candida* spp. in the oral cavity of individuals with oral lesions. The mean value of CFU mL^{-1} obtained in the saliva of these individuals was 1.58×10^5. Thus, it may be suggested that the experimental model used in the present investigation was able to promote an average amount of *Candida* carriage similar to those found in the clinical situations.

In this study there was no statistical difference between the positive control group (P−L−) with P+L− groups (with 75, 100, 125 and 150 mg L^{-1} of PDZ) and P−L+. The use of PDZ alone and the use of LED alone did not result in toxicity to yeast cells. In the studies carried out by Mima *et al.*[28] and Dovigo *et al.*[26] the authors also used a LED as a light source similar to our investigation, and they verified that the use of LED only or PS only did not cause any damage to the yeast cells.

In conclusion, the results of our investigation showed that PDI mediated by PDZ promoted an expressive reduction in *C. albicans* counts and significant differences were observed between PDI (P+L+ 100 mg L^{-1}) and the control group (P−L−). Our results suggested that PDZ may be considered as a potential PS with adequate efficacy in antifungal PDI. Moreover, the experimental animal model of *Candida* infection used in this study has promoted valuable information with respect to the process of this disease. However, it is important to point out that the experiment was stopped after 24 h in order to assess the immediate effect of PDI on yeast viability and on the local inflammatory response. If the experiment would be prolonged for some more days, probably the immune response of the animals would restore itself, the *Candida* growth would be inhibited and the animal would be healthy. *C. albicans* is not a constituent of the normal microflora of mice.[19] Considering our results together with the findings from the cited studies, it is possible to suggest that PDI is an effective method to reduce the viability of *C. albicans* in oral lesions. However, the results of this investigation cannot be extrapolated to a clinical situation, since the oral environment of human beings is different (microbiota composition, dietary habits and salivary flux). Nonetheless, previous clinical studies demonstrated that the association of porphyrin and LED light improved the palatal inflammation and reduced *Candida* spp. counts from palates and dentures from patients with denture stomatitis after six sessions of PDT,[24,25] which showed clinical and microbiological efficacies comparable to conventional antifungal therapy with topical nystatin.[25] However, clinical trials are still required to evaluate the effect of PDI mediated by more effective PSs, such as PDZ, for the treatment of oral candidosis.

5. Conclusion

It is possible to conclude that PDI is an effective therapy for fungal inactivation, and our results may contribute to the development of more effective protocols for *in vivo* photoinactivation of *Candida* spp. However, further studies are necessary for a safe and effective future clinical application in the treatment of *Candida* infections.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported by São Paulo Research Foundation (FAPESP) (grants number 2008/03994-9and 2008/00601-6), Centro de Pesquisa em Óptica e Fotônica-CEPOF-CEPID (13/07276-1) and Fundação para o Desenvolvimento da UNESP-FUNDUNESP-PROINTER (0454/011/14 – PROPe/CDC).

References

1 P. Eggimman, J. Garbino and D. Pittet, Epidemiology of *Candida* species infections in critically ill non-imunosupressed patients, *Lancet Infect. Dis.*, 2003, **3**, 685–702.

2 M. Ortega, F. Marco, A. Soriano, M. Almela, J. A. Martnez, J. Lopez, C. Pitart and J. Mensa, Candida species bloodstream infection epidemiology and outcome in a single institution from 1991 to 2008, *J. Hosp. Infect.*, 2011, **77**, 157–161.

3 J. C. O. Sardi, L. Scorzoni, A. Bernardi, A. M. Fusco-Almeida and M. J. S. Mendes Gianini, *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options, *J. Med. Microbiol.*, 2013, **62**, 10–24.

4 S. Silva, M. Negri, M. Henriques, R. Oliveira, D. W. Willians and J. Azeredo, *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis*: biology, epidemiology, pathogenicity and anti-fungal resistance, *FEMS Microbiol. Rev.*, 2011, **36**, 288–305.

5 C. J. Seneviratne, W. J. Silva, Y. H. Samaranayake and L. P. Samaranayake, Architectural analysis, viability assess-

ment and growth kinetics of *Candida albicans* and *Candida glabrata*, *Arch. Oral Biol.*, 2009, **54**, 1052–1060.
6 J. P. Lyon, L. M. Moreira, P. C. G. Moraes, F. V. Santos and M. A. Resende, Photodynamic therapy for pathogenic fungi, *Mycoses*, 2011, **54**, 265–271.
7 L. M. Moreira, F. V. Santos, L. P. Lyon, M. Maftoum-Costa, C. Pacheco-Soares and N. S. Silva, Photodynamic therapy: porphyrins and phthalocyanines as photosensitizers, *Aust. J. Chem.*, 2008, **61**, 741–754.
8 L. N. Dovigo, A. C. Pavarina, E. G. Mima, E. T. Giampaolo, E. C. Vergani and V. S. Bagnato, Fungicidal effect of photodynamic therapy against fluconazole-resistant, *Mycoses*, 2011, **54**, 123–130.
9 G. Jori, C. Fabris, M. Soncin, S. Ferro, O. Coppellotti, D. Dei, *et al.*, Photodynamic therapy in the treatment of microbial infections: basic principles and perspective applications, *Lasers Surg. Med.*, 2006, **38**, 468–481.
10 M. R. Detty, S. L. Gibson and S. J. Wagner, Current clinical and preclinical photosensitizers for use in photodynamic therapy, *J. Med. Chem.*, 2004, **16**, 3897–3915.
11 S. Banfi, E. Caruso, L. Caprioli, L. Mazzagatti, G. Canti, R. Ravizza, M. Gariboldi and E. Monti, Photodynamic effects of porphyrin and chlorine photosensitizers in human colon adenocarcinoma cells, *Bioorg. Med. Chem.*, 2004, **12**, 4853–4860.
12 L. Pires, S. de M. Bosco, M. S. Baptista and C. Kurachi, Photodynamic therapy in Pythiuminsidiosum - an in vitro study of the correlation of sensitizer localization and cell death, *PLoS One*, 2014, **21**, 9.
13 F. M. Engelmann, Interactions of Cationic Meso-Porphyrins with biomembranes, *J. Bioenerg. Biomembr.*, 2007, **39**, 175–185.
14 M. G. Strackhovskaya, N. S. Belenikina, E. V. Ivanova, Y. K. Chemeris and E. F. Stranadko, The photodynamic inactivation of the yeast *Candida guilliermondii* in the presence of photodithazine, *Microbiology*, 2002, **71**, 345–348.
15 M. G. Strakhovskaya, V. G. Zhukhovitskii, A. F. Mironov, A. M. Seregin, E. F. Stranadko and Corresponding Member of the RAS A. B. Rubin, Fungicidal Activity of Khlorin Photosensitizers, *Dokl. Biochem. Biophys.*, 2002, **384**, 155–158.
16 G. P. Tegos, M. Anbe, C. Yang, T. N. Demidova, M. Satti, P. Mroz, S. Janjua, F. Gad and M. R. Hamblin, Protease-stable polycationic photosensitizer conjugates between polyethyleneimine and chlorin (e6) for broad-spectrum antimicrobial photoinactivation, *Antimicrob. Agents Chemother.*, 2006, **50**, 1402–1410.
17 L. N. Dovigo, J. C. Carmello, M. T. Carvalho, E. G. Mima, C. E. Vergani, V. S. Bagnato and A. C. Pavarina, Photodynamic inactivation of clinical isolates of *Candida* using Photodithazine®, *Biofouling*, 2013, **29**, 1057–1067.
18 C. C. Quishida, J. C. Carmello, E. G. Mima, V. S. Bagnato, A. L. Machado and A. C. Pavarina, Susceptibility of multi-species biofilm to photodynamic therapy using Photodithazine®, *Lasers Med. Sci.*, 2015, **30**, 685–694.
19 Y. H. Samaranayake and L. P. Samaranayake, Experimental oral candidosis in animal models, *Clin. Microbiol. Rev.*, 2001, **14**, 398–429.
20 N. Takakura, Y. Sato, H. Ishibashi, H. Oshima, K. Ushida, H. Yamaguchi and S. Abe, A novel murine model of oral candidosis with local symptons characteristic of oral thrush, *Microbiol. Immunol.*, 2003, **47**, 321–326.
21 J. P. Maroco, Análise Estatística com o PASW Statistics (ex-SPSS). Report Number, Pêro Pinheiro., 2010, p. 963.
22 M. C. Teichert, M. D. Jones, M. N. Usacheva and M. A. Biel, Treatment of oral candidosis with methylene blue-mediated photodynamic therapy in an immunodeficient murine model, *Oral Surg., Oral Med., Oral Pathol., Oral Radiol., Endod.*, 2002, **93**, 155–160.
23 J. C. Junqueira, J. S. Martins, R. L. Faria, C. E. D. Colombo and A. O. C. Jorge, Photodynamic therapy for the treatment of buccal candidosis in rats, *Lasers Med. Sci.*, 2009, **24**, 877–884.
24 E. G. Mima, A. C. Pavarina, M. M. Silva, D. G. Ribeiro, C. E. Vergani, C. Kurachi and V. S. Bagnato, Denture stomatitis treated with photodynamic therapy: five cases, *Oral Surg., Oral Med., Oral Pathol., Oral Radiol., Endod.*, 2011, **112**, 602–608.
25 E. G. Mima, C. E. Vergani, A. L. Machado, E. M. Massucato, A. L. Colombo, V. S. Bagnato and A. C. Pavarina, Comparison of Photodynamic Therapy versus conventional antifungal therapy for the treatment of denture stomatitis: a randomized clinical trial, *Clin. Microbiol. Infect.*, 2012, **18**, 380–388.
26 L. N. Dovigo, J. C. Carmello, C. A. de Souza Costa, C. E. Vergani, I. L. Brunetti, V. S. Bagnato and A. C. Pavarina, Curcumin-mediated photodynamic inactivation of *Candida albicans* in a murine model of oral candidosis, *Med. Mycol.*, 2013, **51**, 243–251.
27 A. C. Costa, V. M. Campos Rasteiro, E. S. da Sil Hashimoto, C. F. Araújo, C. A. Pereira, J. C. Junqueira and A. O. Jorge, Effect of erythrosine- and LED-mediated photodynamic therapy on buccal candidosis infection of immunosuppressed mice and *Candida albicans* adherence to buccal epithelial cells, *Oral Surg., Oral Med., Oral Pathol., Oral Radiol.*, 2012, **114**, 67–74.
28 E. G. Mima, A. C. Pavarina, L. N. Dovigo, C. E. Vergani, C. A. S. Costa, C. Kurashi and V. S. Bagnato, Susceptibility of *Candida albicans* to photodynamic therapy in a murine model of oral candidosis, *Oral Surg., Oral Med., Oral Pathol., Oral Radiol., Endod.*, 2010, **109**, 392–401.
29 J. Ferreira, P. F. C. Menezes, C. Kurachi, C. Sibata, R. R. Allison and V. S. Bagnato, Photostability of different chlorine photossensitizers, *Laser Phys. Lett.*, 2008, **5**, 156–161.
30 M. C. Derosa and R. J. Crutchley, Photosensitzed singlet oxygen and its applications, *Coord. Chem. Rev.*, 2002, **233**, 351–371.
31 L. A. Fernandes, J. M. de Almeida, L. H. Theodoro, A. F. Bosco, M. J. H. Nagata, T. M. Martins, T. Okamoto and V. G. Garcia, Treatment of experimental periodontal

disease by photodynamic therapy in immunosuppressed rats, *J. Clin. Periodontol.*, 2009, **36**, 219–228.

32 J. P. Lyon and M. A. de Resende, 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**, 632–638.

33 M. A. G. Totti, E. B. Santos, O. P. Almeida, C. Y. Koga-Ito and A. O. C. Jorge, Oral candidosis by *Candida albicans* in normal and xerostomic mice, *Braz. Oral Res.*, 2004, **18**, 202–207.

34 J. Nakajima, P. Papaah, M. Yoshizawa, F. Marotta, T. Nakajima, S. Mihara and E. Minelli, Effect of a novel phyto-compound on mucosal candidosis: Further evidence from an ex vivo study, *J. Dig. Dis.*, 2007, **8**, 48–51.

