Photodynamic therapy in *Candida* spp. and epithelial cell cultures: An *in vitro* study

.

ABSTRACT

Aims: The objective of this study was to determinate the photodynamic effect using methylene blue and diode laser on *Candida* spp. and epithelial cells *in vitro.*
Study Design: An *in vitro* study was carried on using cultures of *Candida* spp. and HEp-2 cells.
Place and Duration of Study: This study was developed at the School of Biosciences of the Department of Microbiologic Sciences and at the Laboratory of Pneumology of the Biomedical Research Institute at São Lucas Hospital, both at the Pontifical Catholic University of Rio Grande do Sul, located in Porto Alegre, Brazil, between January and December.
Methodology: Cultures of *Candida* spp. and HEp-2 cells were submitted to PDT with methylene blue (100 µg/mL) and indium-gallium-aluminum-phosphide (InGaAlP) diode laser at 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2.
Results: All of these three doses caused significant inactivation of *Candida* spp. ($P<0.05$). At 450 J/cm^2, the viability of *Candida* spp., based on colony forming units (CFUs), was reduced by 72.42%, followed by lesser effects with 270 J/cm^2 and 100 J/cm^2, 45.87% and 22.83%, respectively. PDT decreased CFUs by 50.44% in *C. albicans*, while other *Candida* species showed a 41.18% decline in CFUs ($P<0.05$). With regard to the average effect of the three doses tested in PDT group, HEp-2 cell viability, based on trypan blue exclusion, declined to 70.81%, which was significantly lower than that observed in the control group (86.21%).
Conclusion: Methylene blue plus laser exposure (100 J/cm^2, 270 J/cm^2 and 450 J/cm^2) caused significant inactivation of *Candida* spp. Photodynamic inactivation of the epithelial cells based on cell viability was 2.24-fold lower than the inactivation of *Candida* spp., which suggests a safety margin for *in vivo* application.

Keywords: Candida; Photochemoterapy;Lasers; Methylene Blue.

1. INTRODUCTION

The fungus *Candida* sp., mainly the species *C. albicans*, is frequently found as a commensal in the digestive and vaginal tracts of the human host. *C. albicans* is part of the normal microbiota of individuals in more than 60% of the population, who do not show any clinical manifestation of disease, thereby considered as carriers of the fungus [1,2]. Oral candidosis is considered the "illness of the ill patient"[3], because it needs one or more predisposing factors for its establishment [4]. The effective treatment of candidosis requires the correction of the predisposing factor, since the absence of this factor makes it difficult for the fungal disease to occur [5]. Species of *Candida* have become very prevalent in systemic infections. In addition, resistance of *Candida* spp. to traditional antifungals (as polyenes, azoles, and echinocandins) has increased, because of the excessive and uncontrolled use of these drugs. The number of pathogenic fungi has been increasing due to the high frequency of hospital infections in immunocompromised patients, and currently there are few safe antifungicals, where most of them are fungistatic agents. Moreover, the intensive use of antibiotic therapy leads progressively to the appearance of resistant strains [6-9].
Antimicrobial photodynamic therapy (PDT) consists of the combination of a photosensitizing agent, generally exogenous, administered topically or systemically, and a source of visible light, with the objective of causing microbial inactivation [10,11]. This technique should not

be considered as a substitute for antibiotics, but as a new treatment modality for localized infections and as an effective complementary treatment for oral infections [12,13], especially in cases of resistant microorganisms. The sensitivity of microorganisms to PDT indicates the applicability of the technique *in vivo*, for localized and superficial infections of known microbiota [14].

Isolated application of LASER did not affect the viability of fungal cells [15-17], where the application of 21 J of energy to *Candida* sp. yeasts and hyphae did not change their viability [16].However isolated application of the laser caused a reduction in the CFUs of *C. tropicalis*, which suggested the susceptibility of this species to laser light[18,19] and also a susceptibility of*C. krusei* [19].

The aim of the present study was to determine the antifungal efficacy *in vitro* of PDT with methylene blue and an indium gallium aluminum phosphide (InGaAlP) diode laser in cultures of *Candida* spp., and its effect on cultures of epithelial cells as well.

2. MATERIAL AND METHODS

2.1 Sample

The sample comprised cultures of *Candida* spp. and cultures of epithelial cells. *Candida* cultures were obtained from 38 adult patients, of both genders with diagnosis of oral candidosis[20]. The specimens were collected with a dry, sterile swab, the material was cultivated in Sabouraud-4% dextrose agar (Merck, Darmstadt, Germany) with chloramphenicol (16 mg/mL, Neo Química, Anapolis, Brazil), and incubated at 30^{o}C for 48 h. The species were identified by phenotypic tests (germ tube formation, microculture in rice agar, CHROMAgar^{TM}*Candida* and assimilation of carbohydrates). In the identification tests, *C. albicans* ATCC (American Type Culture Collection) 28367, *C. dubliniensis* CBS (Centraalbureauvoor Schimmelcultures) 7987 and *C. krusei* ATCC 6250 served as standards.

The identification of C. albicans was confirmed by PCR (Polymerase Chain Reaction), using the oligonucleotides CAL5 (TGTTGCTCTCTCGGGGGCGGCCG) and NL4CAL (AGATCATTATGCCAACATCCTAGGTTAAA), in the 5' to 3' sequence, which produced an amplification product of 175 bp. [21,22].

HEp-2 ATCC CCL-23® cell line was grown in DMEM medium (Dulbecco's Modified Eagle Medium, Sigma, St Louis, USA), supplemented with penicillin (100 UI/mL, Sigma), streptomycin (100 μg/mL, Sigma), gentamycin (50 μg/mL, Sigma) and 10% fetal bovine serum (Gibco, Grand Island, USA). The cultures were incubated and maintained in a humidified incubator at 37°C, 5% CO_2 atmosphere. After 7 days, the cells were washed with PBS and trypsinized with trypsin/EDTA solution (0.25% trypsin/0.02% EDTA) for 10 min at 37°C and afterward centrifuged (8500 x g for 3 min) to obtain a cell suspension [23].

2.2 Laser and photosensitizer

The diode laser (InGaAlP) used was Thera Lase (DMC Equipment, São Carlos, Brazil): 685 nm, continuous emission, 35 mW. The doses were 100 J/cm^2 (2.8 J, 1 min 21 s), 270 J/cm^2 (7.5 J, 3 min 37 s) and 450 J/cm^2 (12.6 J, 6 min), considering an area of 0.028 cm^2. The photosensitizer used was methylene blue (Sigma), at a concentration of 100 μg/mL [24], with an incubation time of 5 min.These three doses could provide a viable treatment time*in vivo*.

2.3 PDT in *Candida* spp.

After growth in YPD medium (yeast peptone dextrose, Sigma) at 30°C for 48 h, serial dilutions were made with *Candida* spp. cultures using sterile saline. Aliquots of 100 μL of the 1:1000 dilution were used for the application of the treatments, carried out in duplicate in a 96-well culture plates, with single application. The doses were: 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2.

2.3.1 PDT test groups: 100 µL of 1:1000 dilution of *Candida* sp., 90 µL of PBS and 10 µL of methylene blue (100 µg/mL, 5 min).
2.3.2 Control groups:100 µL of 1:1000 dilution of *Candida* sp. and 100 µL of PBS, where no treatment was applied. Each laser dose had its corresponding control group, where they were handled and evaluated simultaneously.
Just after the treatments, the cell suspensions were centrifuged (3 min, 8500 x g) and washed 3 times to remove residual methylene blue. After centrifugation, aliquots of 100 µL of each microtube were seeded on Sabouraud dextrose agar and incubated at 30^{o}C for 48 h, and CFUs were counted. Viability (percentage survival) was determined considering the percentage of CFUs of the test group in relation to that of the corresponding control group.
During the treatments, the cultures were protected from light using an opaque plastic box.

2.4 PDT in epithelial cells

After cultivation, HEp-2 cells were resuspended in PBS, and the suspension was transferred to 96-well culture plates, in aliquots of 100 µL at a density of 2 x 10^5 cells/mL.
The samples were distributed into groups, and the treatments performed in triplicate:
PDT group (application of laser and methylene blue) and *Control group – methylene blue* (application of methylene blue without laser): 100 µL of the epithelial cell suspension, 90 µL of PBS and 10 µL of methylene blue (100 µg/mL, 5 min).
Control group - laser (application of laser without methylene blue) and *Control group - untreated*: 100 µL of the epthelial cell suspension, 100 µL of PBS.
The laser doses employed were 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2. During the treatments, the cultures were protected from light using an opaque plastic box. After the treatments, the samples were centrifuged (3 min, 8500 x *g*) and washed 3 times to remove residual methylene blue. Afterward, 100 µL of trypan blue (0.4%, w/v) were added to the cell suspension and the cells counted in a Neubauer chamber (Optik Labor, Darmstadt, Germany) using a light microscope (MC80DX, Zeiss, Germany) with a 40x objective. Viability of the epithelial cells was determined by trypan blue exclusion, counting stained and unstained cells in 16 fields.

2.5 Statistical analysis

The results obtained were analyzed by descriptive statistics, Student's *t*-test for paired samples and analysis of variance (ANOVA), complemented by Tukey's test for multiple comparisons, at the 5% level of significance.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1.Viability of Candida sp.

Of the total of 38 samples of *Candida* spp. collected from patients, 24 were identified as *C. albicans* and 14 as *C. non-albicans*. Comparing the number of CFUs between the test and control groups, significant difference (P<0.05) was found for the three doses employed (Table 1, Figure 1).

By calculating the mean percentage of viable *Candida sp*. CFUs after PDT at different doses, the following results were obtained: 77.17% (±18.32) of viable CFUs for 100 J/cm^2; 54.16% (±19.73) of viable CFUs for 270 J/cm^2 and 27.58% (±12.26) of viable CFUs for 450 J/cm^2.
When considering the different *Candida* species, the mean viability of *C. albicans* was 49.56% (±26.12), while for *C. non-albicans*, this value was 58.82% (±26.35). These values showed statistically significant differences, that is, regardless of dose, *C. albicans*presented a significantly lower mean percentage of CFUs than *C. non-albicans* species (Figure 2).

The species *C. albicans* was more sensitive to PDT compared to *C.non-albicans* species. No significant difference (P>0.05) in viability was observed between the two groups when compared at different doses (Table 2).

3.1.2 Viability of Epithelial Cells

At 100 J/cm^2, the mean viability of epithelial cellsin PDT group was 77.23% (±3.31). The minimum and maximum values of cell viability were, respectively, 75.07% and 81.04%. In control group 1 (laser), the minimum and maximum viability values were, respectively, 77.1% and 96.19%, with mean 86.53% (±9.55). Control group 2 (methylene blue) presented a mean viability of cells of 78.83% (±6.30), with minimum value of 73.66%, and a maximum of 85.84% of cell viability. In control group 3 (without laser and without methylene blue), the mean viable cells were90.23% (±0.70), and the minimum and maximum values were respectively, of 89.59% and 90.97% of viable epithelial cells.
At 270J/cm^2 the mean viability in PDT group was 69.82% (±6.08), and the minimum and maximum values of cell viability were64.09% and 76.19%. In control group 1 (laser), the minimum and maximum viability values were, respectively, 68.32% and 93.51%, with a mean of 79.29% (±12,91) of viable cells. Control group 2 (methylene blue) presented a mean of viable cells of 80.27% (±4.08), with a minimum value of 76.59%, and a maximum of 84.66% of cell viability. In control group 3 (without laser and without methylene blue), the mean of viable cells was 80.56% (±14,58), with minimum and maximum values being respectively, 64.67% and 93.33% of viable epithelial cells.
At 450J/cm^2 the epithelial cells presented a mean viability of 65.39% (±1.77) in PDT group. The minimum and maximum values of cell viability were63.58% and 67.12% in this group. In control group 1 (laser), the minimum and maximum viability values were, respectively, 74.18% and 93.09%, with a mean of 85.65% (±10.08) of viable cells. Control group 2 (methylene blue) presented a minimum value of 53.66%, and a maximum of 84.64% of cell viability, with a mean of cell viability of 66.47% (±16.17). In control group 3 (without laser and without methylene blue), minimum and maximum values were respectively, of 79.67% and 96.11% of viable epithelial cells, with a mean of 87.85% (±8.22) of viable cells.

Figure 3 shows the viability of epithelial cells in different treatments at 100 J/cm^2,270 J/cm^2 and 450 J/cm^2.
In comparing the different treatments, independent of dose, a significant difference in viability (P<0.05) was found for the epithelial cells, but there was not significant difference (P>0.05) among the doses used (Table 3).

3.2 Discussion

Antimicrobial PDTacts to inactivate *Candida spp.*causing irreversible damage and cell death. LASER and photosensitizerassociation generates reactive oxygen species (ROS), with two types of photochemical reactions: type I, which occursafter photosensitizer degradation by direct action of light and generation of free radicals or superoxide ions that interact with biomolecules. Type II reaction occurs when the photosensitizer reacts with molecular oxygen and forms singlet oxygen, highly reactive and the main cytotoxic agent of antifungal PDT. ROS can affect many types of organic molecules (nucleic acids, lipids and amino acids) and they are responsible for irreversible damage to cell membranes (cytoplasmic, mitochondrial, lysosomal and nuclear).Many of the cellular responses are centered on mitochondria, inducing cellular apoptosis through the release of cytochrome c and activation of caspases. ROS disrupt *Candida* cytoplasmatic membrane and cause an increase in cellular permeability and subsequent damage to intracellular targets[24-29].
The effect of PDT on cultures of *Candida* spp. was determined, and cytotoxicity was found to be directly proportional to the dose of the laser applied. PDT at a single dose of 450 J/cm^{2}was considered effective in the inactivation of *Candida* spp.Kömerik et al. [30] showed that PDT at a dose of 340 J/cm^2 did not cause clinical alterations or histological changes in

the oral mucosa of rats. In humans, doses of 250 to 300 J/cm^2 have been applied to the skin in anticancer PDT [31] with recovery of the epithelium after a few weeks. However, in the oral cavity, these authors did not apply more than 200 J/cm^2. PDT at a dose of 100 J/cm^2 was found to be less than effective causing a 22.83% decrease in CFUs of *Candida* spp., in the present study. Perhaps, successive application of 100 J/cm^2 would lead to a greater degree of inactivation of *Candida* spp. Guidolin et al. [32] in their systematic review about PDT (mainly using Photofrin as a photosensitizer) and colorectal cancer in case series of humans (n=137), observed that light dose used in the studies varied from 32J/ cm^2 until 500 J/cm^2 with the most common light dose falling around at aproximatelly 200 J/cm^2. Complications observed was a skin photosensitivity reaction. Cure/complete ablation of the tumor was reported in 40% and partial response was reported in 43.2% of the cases.

The energy provided by the highest dose in the present study was 12.6 J. Wilson et al. [33] applied an energy of 12.2 J on bacterial biofilms *in vitro* and demonstrated effective inactivation of Streptococcus sanguinis. Langmack et al. [34] utilized 108 J in humans, by applying a dose of 12.6 J/cm^2, for the treatment of basocellular carcinoma. Side effects observed were moderate erythema and slight desquamation. Despite that the dose employed was low (12.6 J/cm^2), the energy applied was high (108 J), due to the long period of application (30 min). As another treatment option, in cases of denture stomatitis (a commum oral fungal infection caused by upper oral dentures) the application of PDT using Light-emitting diode - LED (the most popular light source) on acrylic dentures could prevent or treat this fungal infection [35], instead of applying PDT to the oral mucosa.

In the present study, PDT was applied to yeasts of *Candida* spp. (culture in YPD medium). According to Jackson et al. [16], yeasts as well as hyphae of *Candida* spp. are sensitive to this treatment, although hyphae are more sensitive. The authors employed an energy of 21 J, that is, 1.66 times higher than that of 450 J/cm^2 in the present study, and determined that the sensitivity of hyphae was 175 times greater than for yeasts. The greater susceptibility may be associated with differences between hyphae and yeasts in the expression of sites that are targets of cytotoxic substances and of protective antioxidants [25]. As 72.42% of the yeasts in the present study were sensitive to PDT at a dose of 450 J/cm^2, it is possible that hyphae which are the pathogenic form of candidosis are even more sensitive. Such supposition should be investigated by in vitro studies with the induction of hyphae. Also, according Costa et al. [36],*Candida* in biofilms have been shown to be less susceptible to PDT than fungi in the planktonic phase, which could be due the heterogeneity of the biofilm, protection of yeasts by the extracellular matrix material, and the reduced penetration of the photosensitizer in a biofilm. A systematic review made by Wiench et al. [37]showed that studies conducted with planktonic cells, only one showed eradication of *C. albicans* and the others showed only partial elimination. In yeast biofilms, there are partial and statiscally significant cell growth inhibition. *In vivo*, antimicrobial PDT with toluene blue exhibited some effects against *Candida*spp, but its clinical use for oral infections still requires further investigations. Zhang et al. [38] observed a rapid inactivation of mature mixed biofilm (*C. albicans* and *C. tropicalis*) *in vitro* using toluene blue (1 mg/mL) and LED (500 or 750mW illumination for 1 or 2 minutes), inactivating more than 90% and 99% of the fungi respectively.

In the present study, regardless of the dose used, *C. albicans* was shown to be significantly more sensitive to PDT compared to non-albicans species, with inactivation levels of 50.44% and 41.18%, respectively. This is a favorable result since *C. albicans* is the predominant species of fungus in the oral cavity [1,39].Strakhovskaya et al. [40] found that *C. guilliermondii* was 1.6 to 1.7 times more sensitive than *C. albicans*. Dovigo et al. [41] found that PDT presented a fungicidal effect on fungal suspensions, being effective in the inactivation of *C. albicans, C. dubliniensis and C. tropicalis* species and promoved a significant reduction in the cell viability of *C. krusei.* Contrarily, Souza et al. [18]observed that PDT (10.5 J, 28 J/cm^2) decreased the number of CFUs by 91.6% for *C. krusei*, 88.6% for *C. albicans*, 84.8% for *C. dubliniensis* and 82.3% for *C. tropicalis*. This result differs from the

findings of the present study, in which a greater of amount energy was applied, 12.6 J, supplied by a dose of 450 J/cm^2, eliminated 76.73% of *C. albicans* and 65.02% of *C.* non-*albicans*. Lavaee et al. [42] showed that PDT using methylene blue as photosensitizer (1.92 J/cm^2 for 60 seconds) showed antifungal effect only on *C. albicans*. The combination of methylene blue and silver nanoparticles did not have any effect on *C. albicans*, but has on *C. glabrata and C. parapsilosis*, concluding the effect of antifungal PDT was strain and photosensitizer dependents.

For the three different doses employed, the mean viability of the epithelial cells was 70.81%, that is, an inactivation of 29.19% the cells by PDT. PDT of epithelial cells at a dose of 450 J/cm^2 resulted in mean viability of 65.39%. That is, the most effective dose in the inactivation of *Candida* spp. in the present study also appears to allow a margin of safety for its application *in vivo*.

In the present study, despite having an effect similar to that of methylene blue alone, PDT caused more damage to epithelial cells than with no treatment.Zeina et al. [23]found that the application of visible light alone at an energy of 452 J did not affect the proliferation of keratinocytes [24]. The viability of epithelial cells in the present study did not exhibit a significant difference between the untreated group (86.21%) and laser group (83.82%), indicating that the application of laser alone did not affect the viability of the cells, even in the dose 450 J/cm^2 dose, while PDT did.

As the yeast *Candida* sp. is a eukaryotic microorganism, like epithelial cells, and both have a greater number of target organelles that bind the photosensitizer, compared to bacteria, this could explained the higher sensitivity of bacterial cells in relation to yeast and host cells. The cell volume should also be considered, since species of *Candida* and keratinocytes are 25 to 50 times larger than bacteria such as *Staphylococcus* spp. and *Streptococcus* spp. The mortality rate of keratinocytes was on average 18 times less than that of *Candida* spp. and 200 times less than that of bacteria [17].Such finding is in agreement with the results of the present study, since HEp-2 epithelial cells were more resistant than *Candida* spp. to PDT.Also, further studies are need to prove PDT is valuable to use in daily clinical practice, since PDT against *Candida* spp has superior performance compared to conventional antifungal therapies [25].

4. CONCLUSION

PDT with methylene blue and InGaAlP laser at doses of 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2 caused substantial inactivation of *Candida* spp., where *C. albicans* was more sensitive compared to other species. HEp-2 epithelial cells were less sensitive to PDT than were *Candida* spp., showing photodynamic inactivation that was 2.24 times less than that for fungal cells, which suggests the existence of a safety margin for *in vivo* application. Further studies are warranted *in vitro* and in animal models for a better understanding of the basis of the results obtained.

REFERENCES

1.Sidrim JJC, Moreira JLB. [Clinical and laboratory fundaments of medical micology].Rio de Janeiro:GuanabaraKoogan; 1999. Portuguese.

2. Lalla R, Patton L, Dongari-Bagtzoglou A. Oral candidiasis: Pathogenesis, clinical presentation, diagnosis and treatment strategies. J Calif Dent Assoc. 2013;41(4):263-8.

3. McCarthy GM, Mackie ID, Koval J, Sandhu HS, Daley TD. Factors associated with increased frequency of HIV-related oral candidiasis. J Oral Pathol Med.1991;20(7):332-6.doi: 10.1111/j.1600-0714.1991.tb00940.x.

4. Aguirre Urizar JM. [Oral Candidiasis]. Rev IberoamMicol. 2002;19(1):17-21. Spanish.

5. Oksala, E. Factors predisposing to oral yeast infections. ActaOdontol Scand. 1990;48(1):71-4.doi: 10.3109/00016359009012736.

6.Carré V, Gaud O, Sylvain I, Bourdon O, Spiro M, Blais J, et al. Fungicidal properties of meso-arylglycosylporphyrins: influence of sugar substituents on photoinduced damage in the yeast *Saccharomyces cerevisiae*. J PhotochemPhotobiol B.1999;48(1):57-62.doi: 10.1016/s1011-1344(99)00009-3.

7.Prasad R, Shah AH, Rawal MK, Prasad R, Shah AH, Rawal MK. Antifungals: Mechanism of action and drug resistance. In:Yeast membrane transport.Adv Exp Med Biol. 2016;14:327-49. doi: 10.1007/978-3-319-25304-6_14.

8. Wiederhold NP. Antifungal resistance: current trends and future strategies to combat.Infect Drug Resist.2017;10:249-59. doi: 10.2147/idr.s124918.

9. Zhu C, Liao B, Ye X, Zhou Y, Chen X, Liao M. Artemisinin elevates ergosterol levels of Candida albicans to synergise with amphotericin B against oral candidiasis. Int J Antimicr Agents. 2021;58(3): 106394. doi: 10.1016/j.ijantimicag.2021.106394.106394.

10.Salva KA. Photodynamic therapy: Unapproved uses, dosages or indications. Clin Dermatol.2002;20(5):571-81.doi: 10.1016/s0738-081x(02)00266-3.

11.Bapat PS, Nobile CJ. Photodynamic therapy is effective against *Candida auris* biofilms. Front Cell Infect Microbiol. 2021;11:713092. doi: 10.3389/fcimb.2021.713092.

12. Fontana CR, Abernethy AD, Som S, Ruggiero K, Doucette S, Marcantonio RC et al. The antibacterial effect of photodynamic therapy in dental plaque-derived biofilms. J Period Res. 2009;44(6):751-9.doi: 10.1111/j.1600-0765.2008.01187.x.

13. Pérez-Laguna V, Barrena-López Y, Gilaberte Y, Rezusta A. *In Vitro* effect of photodynamic therapy with different lights and combined or uncombined with chlorhexidine on *Candida* spp. Pharmaceutics. 2021;13(8):1176. doi: 10.3390/pharmaceutics13081176.

14. Marques Meccatti V, Souza Moura L, Guerra Pinto J, Ferreira-Strixino J, Abu Hasna A, Alves Figueiredo-Godoi LM et al. *Curcuma longa*L. extract and photodynamic therapy are effective against *Candida* spp. and do not show toxicity*in vivo*. Int J Dent. 2022;2022:5837864. doi: 10.1155/2022/5837864.

15. Wilson M, Mia N. Sensitisation of Candida albicans to killing by low-power laser light. J. Oral Pathol. Med. 1993;22(8):354-7.

16.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(2):150-7. doi: 10.1007/s101030050037.

17. Hidalgo KJR, Carmello JC, Jordão CC, Barbugli PA, Costa CAS, Mima EGO et al. Antimicrobial Photodynamic Therapy in Combination with Nystatin in the Treatment of Experimental Oral Candidiasis Induced by *Candida albicans* Resistant to Fluconazole. Pharmaceuticals (Basel). 2019 Sep 18;12(3):140. doi: 10.3390/ph12030140.

18. Souza SC, Junqueira JC, Balducci I, Koga-Ito CY, Munin E, Jorge AO. Photosensitization of different *Candida* species by low power laser light. J PhotochemPhotobiol B.2006;83(1):34-8. doi: 10.1016/j.jphotobiol.2005.12.002.

19.Tavangar A, Khozeimeh F, Razzaghi-Abyaneh M, Sherkat S. Sensitivity of four various Candida species to photodynamic therapy mediated by indocyanine green, an in vitro study. J Dent (Shiraz). 2021;22(2):118-24. doi: 10.30476/DENTJODS.2020.81817.0.

20. Holmstrup P,Axéll T. Classification and clinical manifestations of oral yeast infections. ActaOdontol Scand.1990;48(1):57-9.doi: 10.3109/00016359009012734.

21. Mannarelli BM, Kurtzman CP. Rapid identification of *Candida albicans* and other human pathogenic yeasts by using short oligonucleoides in a PCR. J Cli. Microbiol.1998;36(6),1634-41.doi: 10.1128/JCM.36.6.1634-1641.1998.

22. Marinho SA, Teixeira AB, Santos OS, et al. Identification of Candida spp. by phenotypic tests and PCR. Braz J Microbiol. 2010;41(2):286-94. doi:10.1590/S1517-83822010000200004

23. Zeina B, Greenman J, Corry D, Purcell WM. Cytotoxic effects of antimicrobial photodynamic therapy on keratinocytes *in vitro.*Br J Dermatol.2002;146(4):568-73.doi: 10.1046/j.1365-2133.2002.04623.x.

24. Zeina B, Greenman J, Purcell WM, Das B. Killing of cutaneous microbial species by photodynamic therapy. Br J Dermatol.2001;144(2):274-8. doi: 10.1046/j.1365-2133.2001.04013.x.

25.Rodríguez-Cerdeira C, Martínez-Herrera E, Fabbrocini G, Sanchez-Blanco B, López-Barcenas A, El-Samahy M et al. New applications of photodynamic therapy in the management of candidiasis. J Fungi (Basel). 2021;7(12):1025. doi: 10.3390/jof7121025.

26 Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy: part one- photosensitizers, photochemistry and cellular localization. Phothodiag. Photodyn. Ther. 2004;1(4):279-93.

27 Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy: part two- cellular signaling, cell metabolism and modes of cell death. Phothodiag. Photodyn. Ther. 2005;2(1):1-23.

28 Freire FMS, Ferraresi C, Jorge AO, Hamblin MR. Photodynamic therapy of oral Candida infection in a mouse model. J Photochem Photobiol B. 2016; 159:161-8.doi: 10.1016/j.jphotobiol.2016.03.049.

29 Soares BM, Silva DL, Sousa GR, Amorim JCF, Resende MA, Pinotti M et al. In vitro photodynamic inactivation of Candida spp. growth and adhesion to buccal epithelial cells. J Photochem Photobiol B: Biology. 2009; 94(1):65-70.

30.Kömerik N, Curnow A, MacRobert AJ, Hopper C, Speight PM, Wilson M. Fluorescence biodistribution and photosensitising activity of toluidine blue O on rat buccal mucosa. Lasers Med Sci. 2002;17(2):86-92. doi: 10.1007/s101030200015.

31. Bagnato VS, Kurachi C, Ferreira J, Marcassa LG,SibataCH,Allison RR. PDT experience in Brazil: a regional profile. PhotodiagPhotodynTher.2005;2(2):107-18. doi: 10.1016/S1572-1000(05)00058-X.

32.Guidolin K, Ding L, Yan H, EnglesakisHba M, Chadi S, Quereshy F et al. Photodynamic Therapy for colorectal cancer: A systematic review of clinical research. SurgInnov. 2022;29(6):788-803. doi: 10.1177/15533506221083545.

33. Wilson M, Burns T, Pratten J. Killing of *Streptocuccussanguis* in biofilms using light-activated antimicrobial agent. J AntimicrobChemother. 1996;37(2):377-81. doi: 10.1093/jac/37.2.377.

34. Langmack K, Mehta R, Twyman P, Norris P. Topical photodynamic therapy at low fluence rates: theory and practice. J PhotochemPhotobiol B.2001;60(1):37-43.doi: 10.1016/s1011-1344(01)00116-6.

35. Al-Qahtani MA. Efficacy of antimicrobial photodynamic therapy in disinfection of Candida biofilms on acrylic dentures: A systematic review. PhotodiagnosisPhotodynTher. 2022;40:102980. doi: 10.1016/j.pdpdt.2022.102980.

36. Costa AC, Campos Rasteiro VM, Pereira CA, Silva Hashimoto ES, Beltrame M Jr, Junqueira JC et al. Susceptibility of Candida albicans and Candida dubliniensis to erythrosine- and LED-mediated photodynamic therapy. Arch Oral Biol. 2011; 56:1299-1305. doi:10.1016/j.archoralbio.2011.05.013

37.Wiench R, Skaba D, Matys J, Grzech-Leśniak K. Efficacy of toluidine blue-mediated antimicrobial photodynamic therapy on *Candida* spp.: A systematic review. Antibiotics (Basel). 2021;10(4):349. doi: 10.3390/antibiotics10040349.

38.Zhang L, Hu Q, Zhang Y, Wang Y, Liu N, Liu Q. Rapid inactivation of mixed biofilms of *Candida albicans* and *Candida tropicalis* using antibacterial photodynamic therapy: Based on PAD™ Plus. Heliyon. 2023;9(4):e15396. doi:10.1016/j.heliyon.2023.e15396.

39.d'Enfert C, Kaune AK, Alaban LR, Chakraborty S, Cole N, Delavy M et al. The impact of the fungus-host-microbiota interplay upon *Candida albicans* infections: Current knowledge and new perspectives. FEMS Microbiol Rev. 2021;45(3):fuaa060. doi: 10.1093/femsre/fuaa060.

40.Strakhovskaya MG,Zhukhovitskii VG,Mironov AF,Seregin AM,Stranadko EF, Rubin AB. Fungicidal activity of khlorin photosensitizers. DoklBiochemBiophys.2002;384:155-8.doi: 10.1023/a:1016072130789.

41. Dovigo LN, Pavarina AC, Ribeiro DG, Adriano CS, Bagnato VS. Photodynamic inactavaction of four *Candida* species induced by Photogem®. Br J Microbiol. 2010;41(1):42-9.doi: 10.1590/S1517-83822010000100009.

42.LavaeeF, Yousefi M, Haddadi P. Comparison of the fungicidal efficacy of photodynamic therapy with methylene blue, silver nanoparticle, and their conjugation on oral *Candida* isolates using cell viability assay. Curr Med Mycol. 2020;6(4):35-40. doi: 10.18502/cmm.6.4.5332.

Table 1. Colony-forming units (CFUs) of *Candida* sp. after PDT application at doses of 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2.

Dose J/cm^2	*Candida* sp. (n=38)				*P**
	Test group		Control group		
	Mean	Standard deviation	Mean	Standard deviation	
100	78.28	27.79	99.78	23.52	**<0.05**
270	52.93	19.54	100.74	29.71	**<0.05**
450	28.87	15.71	103.54	26.30	**<0.05**

**t-test for paired samples*

Table 2. Viability of *Candida* sp. after PDT application at doses of 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2.

PDT Dose	Viability				Total *Candida* sp.	
	C. albicans (n=24)		*C.* non-*albicans* (n=14)			
	Mean (%)	Standard deviation	Mean (%)	Standard deviation	Mean (%)	Standard deviation
100 J/cm^2	75.60	17.68	79.88	19.74	77.17 ^{A}	18.32
270 J/cm^2	49.82	18.03	61.60	20.95	54.16 ^{B}	19.73
450 J/cm^2	23.27	6.34	34.98	16.22	27.58 ^{C}	12.26
Mean	49.56^{b}	26.12	58.82 ^{a}	26.35	52.97	26.47

Means followed by different uppercase letters and means followed by different lowercase letters differ significantly. ANOVA in randomized blocks, Tukey's test at the 5% level of significance.

Table 3. Viability of epithelial cells at laser doses of 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2.

Treatment	Dose			Total
	100 J/cm^2	270 J/cm^2	450 J/cm^2	

	Mean (%)	Standard deviation	Mean (%)	Standard deviation	Mean (%)	Standard deviation	Mean (%)	Standard deviation
PDT	77.23	3.31	69.82	6.08	65.39	1.77	70.81^{B}	6.29
Laser	86.53	9.55	79.29	12.91	85.65	10.08	83.82^{A}	10.08
Methylene blue	78.83	6.30	80.27	4.08	66.47	16.17	75.19^{AB}	11.07
Control	90.23	0.70	80.56	14.58	87.85	8.22	86.21^{A}	9.44
Total	83.20	7.57	77.49	10.02	76.34	14.07	79.01	11.00

PDT: methylene blue and laser; Laser: application of laser without methylene blue; Methylene blue: application of methylene blue without laser; Control: without methylene blue or laser. Means followed by different uppercase letters differ significantly. ANOVA, Tukey's test at the 5% level of significance.

Figure 1-Mean of *Candida* sp. colony-forming units (CFUs) after PDT application at doses of 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2

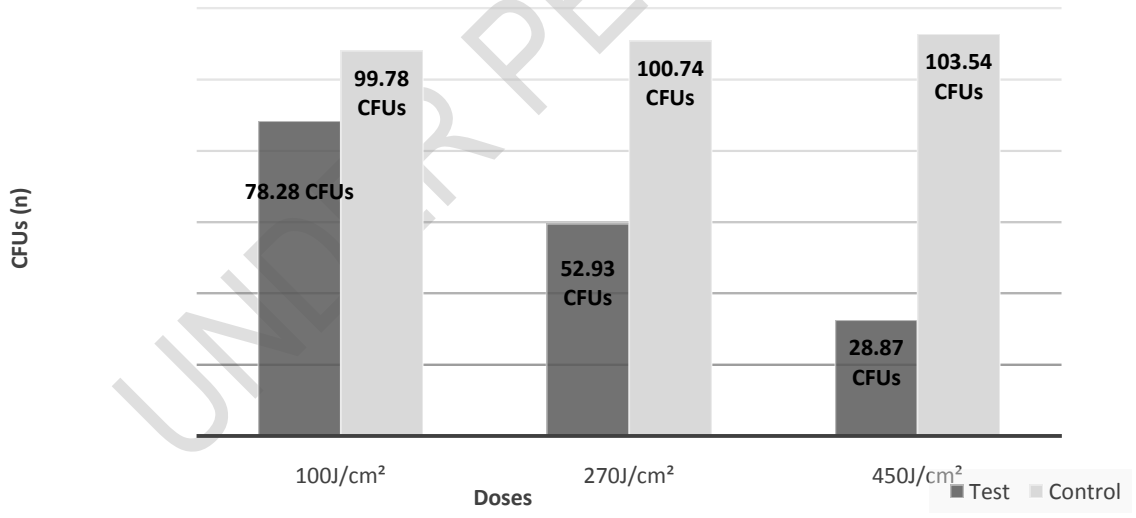

Figure 2- Viability of *Candida* sp. after PDT at doses of 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2.

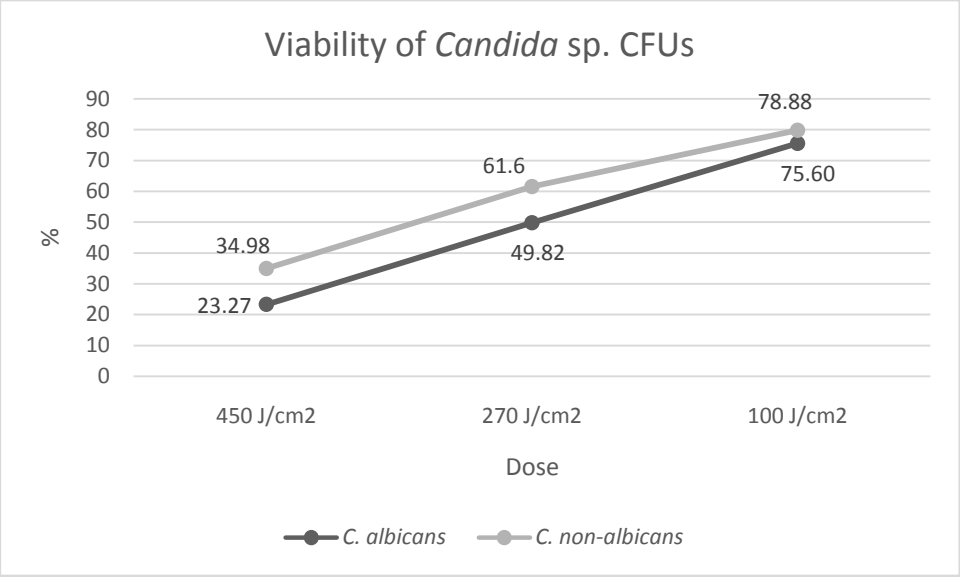

Figure 3- Viability of epithelial cells in different treatments at 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2.