Original Research Article

Cellular Structural Changes in two Candida species Caused by Photodynamic Therapy

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INTRODUCTION

Management of infections caused by clinically relevant fungal pathogens is a challenge due to the incidence of resistance that can develop during therapy, especially in immunocompromised individuals (Cowen et al., 2002). The increasing need for prolonged use of antifungal drugs, longer than usually recommended for antibiotics, is accompanied by a corresponding increased incidence of side effects. Furthermore, the limited number of available antifungal compounds and the need to determine the susceptibility profile of the organism also complicate the treatment of these infections (Gomez et al., 2008).

Candida albicans is commensal yeast from the oral cavity and is the most virulent species of the genus. A pathogenic phase that produces superficial to systemic infections by disrupting the balance between microorganism and host can result from alterations in the host environment (Naglik et al., 2008). In immunosuppressed individuals, such as those with acquired immunodeficiency syndrome (AIDS), oral candidosis is the most common fungal manifestation; 84–100% of HIV infected individuals develop at least one episode of colonization by Candida spp., and up to 90% develop pseudomembranous candidiasis (de Souza et al., 2009). The treatment of oral candidosis in HIV-positive individuals is complicated by its recurrent nature; previous exposure reduces its susceptibility to conventional antifungals. C. albicans and other Candida species can develop resistance to antifungals used to treat oral candidosis, such as fluconazole (Deldago et al., 2009).

The other Candida species are part of the commensal flora of the human gastrointestinal and genitourinary tracts and can cause superficial infections of the mucosa, nails and skin. The infection depends on imbalances between increased C. albicans virulence attributes and impaired host defense systems. In immune-compromised individuals, however, C. albicans may invade deeper tissues, penetrate the blood vessels, and cause life-threatening systemic infections (Gualco et al., 2007).
Photodynamic therapy (PDT) is a light-based treatment platform that has been under development for several applications in oncology, dermatology, and ophthalmology, and it has recently been investigated as an antimicrobial therapy. The combination of non-toxic dyes referred to as photosensitizers (PS), these are generally macrocyclic compounds that exhibit no or minimal inherent toxicity but result in the generation of cytotoxic reactive oxygen species when excitation occurs with light of the appropriate wavelength. Also, harmless low-intensity visible light generates reactive oxygen species (ROS) that are toxic to microorganisms. The potent and broad-spectrum antimicrobial effect has highlighted this therapy as a promising alternative treatment for localized infections. The photodynamic effect depends on the type and concentration of PS employed, combined with the irradiation parameters that activate the dye (Munin et al., 2007).

Many reports in the literature have confirmed efficient antimicrobial photodynamic inactivation (APDI) of various yeast and bacterial species following the proper light and PS dosimetries delivered to the cells. The production of ROS in APDI has been implicated in two important aspects of microbial physiology: (i) changes in the expression of virulence determinants of yeasts (Soares et al., 2009) and (ii) the impact of APDI on the overall survival of microorganisms. Moreover, some types of PS are able to penetrate the microbial cell and bind to cytoplasmic components and nuclear material. Methylene blue (MB), a widely studied PS (Dai et al., 2009), has an affinity to guanine bases of DNA (Lin et al., 2007).

The purpose of the present investigation is to characterize with the aid of scanning (SEM) and transmission (TEM) electron microscopies the surface and ultra-structures alterations of C. parapsilosis and C. tropicalis cells after in vitro exposure to PDT treatment using different wavelengths of laser light and methylene blue dye as photosensitizers.

MATERIALS AND METHODS

Organisms

Two Candida species used in this study C. parapsilosis and C. tropicalis were previously isolated from patients suffering nails infection and identified in the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University.

Inoculum preparation

Yeasts were sub-cultured from vial stock onto Sabouraud dextrose agar (Difco, Detroit, USA) in air atmosphere for 48 h at 37°C. The inoculum initial quantification was confirmed by cells growing in the control group.

Irradiation source

A GaAlAs diode laser (Photon Lase III, DMC, São Carlos, Brazil) with wavelength 445; 532; 660 and 808 nm were used in this study. Yeast strains were continually irradiated from the top of a flat bottomed micro-titer plaque and the laser beam passed through all the suspensions at 1.0 cm² spot size, which was coincident for all groups. The output power was maintained constant at 300 mW/cm² for all laser wavelength at 5 and 10 min of irradiation. The fluences of light equal 90 at 5 min while at 10 min equal 180 J/cm².

Photosensitizer

For the sensitization of Candida species the solution of methylene blue dye was (0.1 mg/mL) was used as photosensitizer (PS). The solution of methylene blue was prepared by the dissolution of the powder (Sigma Chem. Co., St. Louis, USA) in physiologic solution (0.85% NaCl). Then, the solution was filtered through a sterile filter membrane (0.22 μm Millipore, Sao Paulo, Brazil) After filtration, the photosensitizer solutions were stored in the dark (Prates et al., 2009).

Experimental conditions

For each microorganism, twenty experimental conditions were tested, obtained by crossing the one concentrations of PS and four doses of light at five and ten minutes. These experimental conditions were denominated PS+L+. Thus, using a 96-well plate, aliquots of 100 μL of the cell suspensions (10³ concentration) of each species were photosensitized with the same volume of methylene blue dye (100 μL). The well plate containing the resulting
suspending was left to rest in the dark for 30 minutes, and after that was placed under each laser wavelength at 5 and 10 minutes, the results tabulated in table 1 and 2. These procedures were performed to detect each laser light wavelength had effect on pathogenic yeast isolates.

Furthermore, the effect of the of methylene blue dye (PS+L-) and of each light wave length (PS-L+) was also evaluated. Additional samples of the control group were not photosensitized with methylene blue and did not subject to any of laser light (PS-L-), totaling nine experimental conditions and 1 control conditions for each Candida species.

For all the conditions evaluated, serial dilutions $10^3$ was obtained for each Candida species. This serial dilution was plated on Sabouraud Dextrose Agar with 5 µg/mL of gentamicin (SDA; Acumedia manufactures, Inc. Baltimore, Maryland 21220, EUA). In addition, aliquots of 10 µL were removed from the cavities of the well plates and transferred directly to the SDA, without being diluted. After 48 hours of incubation at 37 °C, the Petri plates were submitted to colony counting and the numbers of colony forming units were calculated (Dovigo et al., 2011).

**In vitro statistics**

The yeast colonies were counted and converted into CFU for analysis. All samples were submitted to this process and statistical analysis of the experimental data was performed using one-way analysis of variance (ANOVA). Mean comparisons were carried out with the student-Newman-keuls method performed multiple comparison procedures, which retains the overall significance level at 5% (P<0.05) (Pfaller et al., 1988).

**Scanning (SEM) and Transmission (TEM) Electron Microscopy**

Cells were harvested, washed in 0.1 M sodium phosphate buffer, pH 7.4, prefixed with glutaraldehyde (5% glutaraldehyde "Sigma, Germany" in 0.1 M sodium buffer, pH 7.4) for 1 h, washed twice in buffer and placed in 1% osmium tetroxide for 1 h at 4 °C. The material was subsequently washed in distilled water and dehydrated in a graded acetone series (35 to 100%).

The dehydrated cells, immersed in absolute acetone, were mounted on specimen holders; the specimens were then dried in the critical dryer and were mounted onto stubs by double-sided carbon tape. Samples were coated with a thin layer of gold by sputter coater, and visualized using high vacuum mode of (JEOL JSM-5500LV) Scanning Electron Microscope (SEM).

For the transmission electron microscope (TEM) observation, following the dehydration step, the fixed cells were embedded in Epon and the small blocks of samples were cut with an ultra-microtome (Leica Ultracut). The ultra-thin sections were then analyzed at 80 KV using (JEOL 1010) Transmission Electron Microscope.

**RESULTS**

**I-Anticandidal activity**

After incubation (48 hours/ 37°C), the control plates (PS-L-) of the two Candida species showed abundant growth of viable colonies. It was observed that the effect of the methylene blue dye (PS+L-) significantly alter the number of cfu/mL for the two Candida species, in comparison with the values obtained in the control groups (p<0.05). On the other hand, the effect of the four diode laser light wavelength (PS-L+) on the two species resulted in moderated significantly lower cfu/mL values (p<0.05) in comparison with those obtained in the control group.

For all the species evaluated, a significant reduction in the number of cfu/mL was observed after the application of PDT. The effectiveness of the therapy in reducing cell viability varied as a function of the microorganism and the association between photosensitizer and the wavelength of diode laser light (PDT). Table (1) and (2) showed variation on microbiologic growth of *C. parapsilosis* and *C. tropicalis*, respectively under PDT effect at 5 and 10 minutes, respectively, after 48 hours incubation time at 37 °C.

For both Candida species tested showed a significant reduction in cell viability after PDT at 660 nm and much better for 10 minutes than 5 min. When other wavelength was evaluated, the use of PS at the concentrations of 0.1 mg/mL resulted that 445 nm the best wavelength after 660 nm for *C. parapsilosis* while 808 nm the second best laser wavelength for *C. tropicalis*, which were statistically lower than the value obtained in the other wavelength, PS substance and control group (p<0.05).

**Scanning (SEM) and Transmission (TEM) electron microscope observations**

Scanning electron microscopy (SEM) was used to observe surface alterations or general morphological changes in *C. parapsilosis* and *C. tropicalis* cells after exposure to PDT. Comparisons were made between untreated yeasts (control) and cells in the similar inoculum treated with PS (Methylene blue dye) 0.1 mg/mL and PDT treatment at two different wavelength for each yeast species (660 and 445 nm for *C. parapsilosis* while 660 and 880 nm for *C. tropicalis*).

All of the *C. parapsilosis* cells in the control group generally had smooth-walled bodies, were semi-spherical to oval in shape, and were mostly present in the yeast form in large quantities (Figure 1A), after 10 min of
Table 1. Mean of standard deviation values of the colony forming units per millimeter (CFU/ml) of C. parapsilosis at the fluence rate of 300mW/cm² for the following studies groups: PS+ L+ = group treated with laser in the presence of photosensitizer (n = 3); PS-L+ = group treated only with laser (n = 3); PS+ L- = group treated only with photosensitizer (n = 3); PS- L- = group treated neither with laser nor with photosensitizer (n= 3).

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<th>Laser type</th>
<th>Viable count colony forming unit CFU/ml</th>
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<td>PS- L- (Group 1)</td>
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<td>5min</td>
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<td>445nm</td>
<td>350±2.11*</td>
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*aMean cfu count ±SD; **Percentage of cfu
Comparing to group 1, statistically significant with p < 0.05
Comparing to group 2 lasing time 5 min, statistically significant with p < 0.05
Comparing to group 3, statistically significant with p < 0.05
Comparing to group 2 lasing time 10 min, statistically significant with p < 0.05

Table 2. Mean of standard deviation values of the colony forming units per millimeter (CFU/ml) of C. tropicalis at the fluence rate of 300mW/cm² for the following studies groups: PS+ L+ = group treated with laser in the presence of photosensitizer (n = 3); PS-L+ = group treated only with laser (n = 3); PS+ L- = group treated only with photosensitizer (n = 3); PS- L- = group treated neither with laser nor with photosensitizer (n= 3).

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Comparing to group 3, statistically significant with p < 0.05
Comparing to group 2 lasing time 10 min, statistically significant with p < 0.05

Exposure to PS, we observed a significant reduction of the amount of yeasts, and the cells became enlarged in sizes (Figure 1B).

Diode laser at 660 nm exhibited aggregation of yeast cells and distortion in shape of many cells C. parapsilosis after 10 min "arrow" (Figure 1 C and D). On the other hand, High distortion was observed in yeast cells which became enlarge in sizes when treated with PDT at (660 nm and PS), also, a few remaining yeasts had irregularities on their surface "arrows", such as convolutions (Figure 1 E and F) which are not observed in Figure 1A.

On the other hand, 445 nm of diode laser showed elongation "arrows" and swollen of yeast cells (Figure 1G, H), on contrast PDT effect on size and shape of the same yeast species at the same wavelength and PS whereas, all cells became smaller and thinner than control (Figure 1 I, J).

Transmission electron microscopy (TEM) used to observe ultrastructure alterations or sub-organelles changes in C. parapsilosis and C. tropicalis cells after exposure to PDT. Figure (2A) revealed that the untreated yeast cells in the control group had typical C. parapsilosis morphology, with a uniform central density, structured nucleus, and cytoplasm with several elements of an endomembrane system that was enveloped by a regular,
Figure 1 (A). SEM micrographs of the untreated *Candida parapsilosis* cell

Figure 1 (B). SEM micrographs of PS effect on *Candida parapsilosis* cells

Figure 1 (C and D). SEM micrographs of 660 nm of diode laser effect on *Candida parapsilosis* cells

Figure 1 (E and F). SEM micrographs of PDT effect at 660 nm of diode laser and PS on *Candida parapsilosis* cells
intact cell wall and plasma membrane that lied close to the cell wall.

After *C. parapsilosis* cells were exposed to PS for 10 min, we observed the cell wall increase in diameter and the shape of cell became un-regular (Figure 2B).

660 nm of diode laser did not showed changes in ultrastructure of *Candida parapsilosis* except a separation of cell membrane and some lysis in sub organelles were observed (Figure 2 C and D). While great distortion on the shape of yeast cells and sub-organelles became distorted and not limited after exposure to PDT at the same wavelength (Figure 2 E and F).

In similar, 445 nm of diode laser showed the same effect of 660 nm where little effect was observed such as a little separation of cell membrane and some lysis sub organelles plus cell wall became thicker than the control (Figure 2G and H). On the other hand, PDT at this wavelength showed the presence of cell lysis, with loss of the cell wall and rupture of the cytoplasmic membrane, leading to the loss of intracellular material and cell wall irregularities, some cells showed cytoplasmic membrane invaginations that caused marked structural disorganization within the cytoplasm and reduced intracellular volume (Figure 2 I and J).

Many changes exhibited on *C. tropicalis* cells after PDT treatment, however, figure (3A) showed the SEM examination for untreated *C. tropicalis* cells (control) where oval to elongate in shape blastospores, generally smooth-walled bodies. Pseudomycelium forms were occasionally abundant. As well as figure (3B) no changes
Figure 2 (A). TEM micrographs of the untreated *Candida parapsilosis* cells

Figure 2 (B). TEM micrographs of PS effect on *Candida parapsilosis* cells

Figure 2 (C and D). TEM micrographs of 660 nm of diode laser effect on *Candida parapsilosis* cells

Figure 2 (E and F). TEM micrographs of PDT effect at 660 nm of diode laser and PS on *Candida parapsilosis* cells
on *C. tropicalis* cells after treated with PS for 10 minute except the cells became enlarged in size. The alterations of the surface micromorphology shown here were observed in most of the examined cells. However, 660 nm of diode laser showed large decrease of unicellular of yeast cells which became smaller than control (figure 3 C and D) while the same changes were observed after PDT treatment with this wavelength plus the pseudomycellium and plastosporis became distorted with rough walls "arrow" (figure 3 E and F).

In addition 808 nm of diode laser showed great enlargement of cells of *C. tropicalis* (figure 3 G and H). On the other hand, great reduction in number and the size became smaller in some yeast cells while aggregation and big circular "arrow", distorted in shape were observed in many other cells after PDT treatment (figure 3 I and J).

The ultrastructures of *C. tropicalis* were seen by TEM. Figure 4 (A) show the typical structure of *C. tropicalis* without any changes while the effect of PS on yeast cells was seen in Figure 4 (B), however, no remarkable changes were observed unless dye particulate inside the cell "arrow". Treated cells with 660 and 880 nm of diode laser...
Figure 3 (A). SEM micrographs of the untreated *Candida tropicalis* cells

Figure 3 (B). SEM micrographs of PS effect on *Candida tropicalis* cells

Figure 3 (C and D). SEM micrographs of 660 nm of diode laser effect on *Candida tropicalis* cells

Figure 3 (E and F). SEM micrographs of PDT effect at 660 nm of diode laser and PS on *Candida tropicalis* cells
Figure 3 (G and H). SEM micrographs of 808 nm of diode laser effect on *Candida tropicalis* cells

Figure 3 (I and J). SEM micrographs of PDT effect at 808 nm of diode laser and PS on *Candida tropicalis* cells

Figure 4 (A). TEM micrographs of the untreated *Candida tropicalis* cells

Figure 4 (B). TEM micrographs of PS effect on *Candida tropicalis* cells
Figure 4 (C and D). TEM micrographs of 660 nm of diode laser effect on Candida tropicalis cells

Figure 4 (E and F). TEM micrographs of PDT effect at 660 nm of diode laser and PS on Candida tropicalis cells

Figure 4 (G and H). TEM micrographs of 808 nm of diode laser on Candida tropicalis cells
Figure 4 (I and J). TEM micrographs of PDT effect at 808 nm of diode laser and PS on Candida tropicalis cells showed the same effects on C. tropicalis, such as, semi separation of cell membrane "arrows" and the cell wall increased in thickness Figure (4 C and D). Also, the same alterations of the surface micromorphology shown after PDT treatment with these wavelengths such as lysis and distortion of all sub organelles, shrinkage of cell membrane in middle of yeast were seen in Figure (4 E, F, G and H).

DISCUSSION

Candida species is a major opportunistic human pathogen that causes superficial and disseminated forms of infections. The increasing emergence of antifungal resistance among Candida spp. has resulted in a growing interest in the antimicrobial effects of PDT as a therapy for localized candidiasis. In this context, different types of PS with distinct physicochemical properties have been proposed. (Dovigo et al., 2011).

In spite of the wavelength suitable to the absorption beak of methylene blue is red (660 nm), we used other wavelengths to compare between the different effects of different wavelengths. The production of reactive oxygen species by PDT depends on the interaction the photosensitizer with photons of visible light at suitable wavelength. For this interaction to occur, the laser or LED must emit light at a wavelength that the photosensitizer is able to absorb. In the present work, diode laser with an emission of 450, 532, 660 and 808 nm were chosen for the photodynamic reaction so that the emission of the light source coincided with the absorption maximum (output power of 300 mW/cm² was adjusted for all wavelengths which the fluences at 5 and 10 min had energy equal 90 and 180 J/cm², respectively, of the 100 µL of photosensitizer. Recent data and the results published by another group using methylene blue (Prates et al., 2009) indicate that output power and/or time of exposure should be considered as important determinants of the photokilling of yeast cells.

This study evaluated the in vitro susceptibility of two Candida species to PDT by means of the association of four wavelengths at two fluences of LED light and 100 µL of methylene blue as photosensitizer. One of the most significant findings was the great inviability of the suspensions of C. tropicalis. In another study, methylene blue dye was also effective in photosensitizing C. albicans, promoting the complete elimination of this microorganism from the oral cavity of immunosuppressed rats after illumination with 275 J/cm² of diode laser (Teichert et al., 2002).

In the present study, a similar inactivation behavior was also observed between C. parapsilosis and C. tropicalis. The latter is a more recently described species of Candida that has greater incidence in patients with AIDS and is not always eliminated with the use of antifungal agents. The two species have almost identical phenotypical characteristics, as well as genetic similarities (Sullivan et al., 1995) which could explain the similar result obtained with the application of PDT.

Bearing in mind that the photodynamic action requires an association of PS and light, the application of light without the presence of a PS is not capable of promoting the formation of singlet oxygen. It is fundamental to have the presence of a substance to intermediate the process of reactive species formation. However, the results of condition PS-L⁺ showed statistically significant differences, when compared with the control group, suggesting a possible toxic effect of the light. Souza et al. (2006) observed that the isolated application of laser also caused a reduction in the cfu/mL values of C. tropicalis. It has been reported that cell irradiation with visible light and its consequent absorption by the tissue molecules could cause both a proliferative and inhibitive effect, and
is elucidated by two processes that involve electronic excitation. One of the processes is the transfer of energy to oxygen, since the cytchrome works as photosensitizer, enabling the photodynamic damage to occur without the presence of an external photosensitize.

For *C. parapsilosis*, no results similar to those of the other species were obtained, once this microorganism was not completely inactivated under any experimental condition. Nevertheless, under the conditions in which PDT was evaluated, significantly lower cfu/mL values were observed in comparison with the control group. The susceptibility of *C. parapsilosis* to PDT can be considered an important result since this microorganism has frequently been isolated in immunocompromised patients, particularly when there were reports of previous exposure to fluconazol (Muñoz et al., 2005).

Although it has previously been observed that *C. krusei* was more resistant to being photosensitized, the reasons that lead to this behavior have not yet been explained. Particularly with regard to *C. albicans*, the photochemical processes mediated by hematoporphyrin derivatives, initially appear to promote an alteration in the cytoplasmatic membrane of the microorganism. This effect possibly occurs through oxidative alterations in lipids and proteins present in the membrane (Bocking et al., 2000). Afterwards, PS is able to penetrate into the cell and cause irreversible damage to the intracellular organelles, which leads to cellular inactivation.

It is important to point out that the inactivation of the Candida species by PDT could present important advantages over conventional treatments considering that antifungal medications frequently do not completely eliminate the Candida species (Chandra et al., 2001). Recently, it has been shown that it possesses phototoxic affects against Candida cells; however, the results showed that PDT was able to promote antifungal effects against *Candida parapsilosis* and *C. tropicalis*. The association of 100 µL with light was able to produce significant reductions in viable Candida counts when compared to untreated sample (PS- L-). Although statistical analyses find significant differences between the PS-L- groups using 100 µL of methylene blue dye at 660 nm of diode laser was able to cause greater reduction of Candida cells of both species.

To our knowledge there is no previous published study investigating the *in vitro* and *in vivo* photodynamic effects of methylene blue dye against *Candida parapsilosis* and *C. tropicalis*. However, previous laboratory studies have shown the lethal photo-sensitization of microorganisms using some curcumin preparations (Haukvik et al., 2009).

Dovigo et al. (2011) obtained that curcumin represents a potential new PS against Candida cells, *in vivo*. A single application of PDT was sufficient to produce complete elimination of *C. albicans* from some of the evaluated mice. The most promising results were observed with the use of curcumin at 80 µM and illumination with 37.5J/cm². However, they believe that one possible limitation of the study could be that only one time point of observation was investigated regarding treatment outcomes. Thus, future animal investigations will be design to evaluate different follow-up time intervals, such as 3, 5 and 10 days. In addition, the protocol established in this investigation should expand further in order to evaluate the safety of repeated applications of the therapy and its efficacy in treating oral candidiasis in randomized clinical trials.

In contrast, the study of Costa et al. (2011) resulted in a greater microbial reduction at lower concentrations of photosensitizer than that reported by Peloi et al. (2008), these authors assessed the photodynamic action of a methylene blue photosensitizer at a concentration of 35.2 mM irradiated by a red LED (2– 12 J cm⁻²) for 10–60 min against planktonic cultures of *Staphylococcus aureus*, *Escherichia coli* and *C. albicans*, obtaining reductions of 2.34–3.71, 1.61–3.41 and 2.77–3.87 log10, respectively. However, the fluence of the LED used by Peloi et al. (2008) was approximately 3.5 times smaller than the fluence of the LED used here.

According to Lalla et al. (2010), the Infectious Diseases Society of America (IDSA) guidelines recommend the use of nystatin suspension as first line therapy for the management of mild oropharyngeal candidiasis and fluconazole for moderate and severe conditions. Although this investigation did not include animals treated with standard drugs, such as these two antifungals, it is possible to suggest that curcumin-mediated PDT can have important advantages over traditional treatments of oral candidiasis. First, illumination was only 7 min in duration, indicating that PDT rapidly inactivated Candida cells while antifungals can require days to be effective.

Using the same animal model of oral candidiasis, Takakura et al. (2003) evaluated systemic treatment with fluconazole (in drinking water for 3 days) and topical application of amphotericin B (for 3 days). Candida was not detected in animals treated with fluconazole, while only a reduction of *C. albicans* cells was obtained after topical treatment. In an experimental model of esophageal candidiasis, administration of itraconazole (oral solution once a day for 3 consecutive days) reduced the number of Candida cells from 6 to 4 log (CFU/ml) (Ishibashi et al., 2007). Chami et al. (2004) reported that topical application of nystatin (twice a day for 8 consecutive days), for the treatment of induced oral candidiasis in rats reduced the mean viable counts of *C. albicans* from 3.64 – 2.71 log (CFU/ml).

Over the years, a number of investigations have been designed to evaluate the effects of antimicrobial PDT, especially in the dental field. The growing evidence from *in vivo* studies has resulted in the clinical approval of some PDT systems for treating endodontic infections, periodontitis and dental caries (Dai et al., 2009). However, there are relatively few studies concerning the
topical treatment of oral candidiasis by PDT. Previous *in vivo* reports on photoactivation of *C. albicans* using methylene blue (Martins et al., 2011) and porphyrins (Mima et al., 2010) have been published. Teichert et al. (2002) used high concentrations of methylene blue (450 and 500 mg/l) and reported an overall reduction of 3 log 10 in *C. albicans* viability. While, Martins et al. (2011) described the use of a lower methylene blue concentration (100 mg/l), but the number of *C. albicans* recovered from lesions remained similar to untreated animals. The use of porphyrin derivatives to treat oral candidiasis also required high concentrations of PS and only a limited antifungal effect (1.40 log 10 reduction) was reported (Mima et al., 2010).

Jori et al. (2006) suggested that the efficiency of a PS can be expressed as the minimal concentration which induces a 4 log decrease in the survival of microorganisms. In the present study, the use of 100 µL of methylene blue associated with 180 J/cm² of LED light was sufficient to reduced 31% in *Candida parapsilosis* and 16% in *C. tropicalis* viability. Thus, compared to the previous published studies on Candida photoactivation, the findings presented here can clearly contribute to the development of effective protocols of antifungal PDT. However, it is important to mention that some animal models of oral candidiasis promote less than 4logs of Candida recovery before treatment, which would make Jori et al. (2006) statement not applicable under all circumstances.

The SEM observations presented in this study clearly confirm the potent fungistic action exerted by PDT. The surface alterations are most probably due to a change in cell permeability, which is in agreement with earlier ultrastructural observations, showing that the first changes are localized at the plasmalemma and cell wall before any alteration can be detected in the cell interior. According to Jori et al. (2006), the increase in the permeability of the membrane during the initial photochemical processes is fundamental for accentuating the photodynamic effect on fungal cells, as inactivation effectively occurs after the uptake of PS into the cell interior. It can be supposed that some of the peculiarities inherent to *C. krusei* could act as resistance to cell inactivation by photochemical processes, as is the case with PDT. It has been suggested that *C. krusei* has greater superficial hydrophobicity in comparison with *C. albicans*. This characteristic is responsible for *C. krusei* cells to have a greater tendency to adhere to each other when in the yeast form. In order for the photodynamic effect to occur, it is necessary for singlet oxygen to be formed close to its target, due to its short life time and low diffusion capacity in water (Dovigo et al., 2013). Therefore, the phenomenon of co-adhesion among the *C. krusei* cells adhering to each other could be associated with difficulty singlet oxygen in attaining the surface of fungal cells, thus diminishing the effectiveness of the therapy. Nevertheless, no specific information was found in the literature.

Studies with miconazole, done by Sreedhara et al. (1974), showed that the permeability of the plasmalemma was altered, as evidenced by the leakage of cations, amino acids, and proteins. It is obvious that this permeability change provokes an osmotic imbalance, which could explain the indentations of the walls in collapsed cells. The folds in the cell wall, as noted in most of the cells exposed to the lower doses, may correspond to the focal areas where membranous material is deposited between the plasmalemma and the cell wall or in the cell wall itself, thereby causing a bulged appearance of the cellular contour.

As evidenced by the TEM study, the treatment caused an accumulation of membranous material in the cell wall, especially at sites of bud-formation. The SEM observations show a clear increase in buds and bud scars at several regions of the cell wall. This implies that the drug affects the normal division process of the yeasts and in such a way that one cell makes multiple attempts to divide. Moreover, the inability of cells to separate, resulting in the formation of clusters of interconnected cells, points to the fungistatic activity of the drug. As for the cells treated with the fungicidal dose, some have a completely smooth surface fully comparable to those seen in the controls, though the surfaces of most cells were partly covered with vesicles. The presumptive origin of the vesicular material, i.e., cytoplasmic components derived from broken cells, is suggested by the presence in large amounts of fragmented cell walls.

Further evidence is given by TEM observations on similarly treated cells, demonstrating the presence of membrane-limited cytoplasmic material sticking to or in the vicinity of intact cell walls. The apparent quantitative differences in the amount of vesicles on the cell surface as seen between the samples of SEM and TEM can be merely attributed to the great difference that exists between the two methods of approaching a quantitation problem.

Of further interest are the discrepant findings between SEM and TEM observations concerning the viability of the Candida cells, i.e., cells with a normal-looking smooth surface as seen with SEM, are found inside to be completely necrotic upon TEM examination. This means that normal shape and size of a cell may not be taken as a criterium of viability persent. The considerable shape changes which accompany cell necrosis as observed with minimal fungicidal doses may be attributed to the fact that the process of cellular necrosis progressed more slowly with these doses and involved a progressive degradation of the cell wall as well. On the contrary, with the fungicidal dose as shown here the lytic changes probably appear faster and do not affect the cell walls at least in part of the population.

Considering the limitations of the present study, it was concluded that PDT presented a fungicidal effect on fungal suspensions, being effective in the inactivation of
the *Candida parapsilosis* and *C. tropicalis* species, and in the significant reduction in the cell viability of *C. tropicalis* and *Candida parapsilosis*.

**CONCLUSION AND FUTURE PERSPECTIVE AND DIRECTIONS**

The worldwide rise in antifungal resistance has driven research into the development of novel antimicrobial strategies that have new mechanisms of action in comparison with the routinely used agents and that do not induce fungal resistance. The problem is further exacerbated because Candida infections are mostly not induced fungal resistance. The problem is further compared with the routinely used agents and that do not induce microbial resistance and can be repeated many times at the same site in case of infection recurrence are urgently needed. The advantage of aPDT in relation to the conventional antimicrobial treatment is that its local action only affects areas of PS and light.

EM has been an essential technique to acquire knowledge about intracellular protein trafficking and the cells ultrastructural organization in high eukaryotes. This approach is particularly important in yeast as well when investigating the compartments of the endosomal and secretory systems because in EM they appear as puncta. This experimental need, has been hindered by the lack of however procedures allowing satisfactory results. An emerging field in EM is the use of the electron tomography to study the three-dimFensional projection of a structure or an organelle. Although this technique has already been applied to yeast, it has not yet been combined with immunological reactions as done for mammalian cells.

**REFERENCES**


Physics 19:1038–44.