Photosensitization of different *Candida* species by low power laser light

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Abstract

The aim of this study was to evaluate the effects of the laser radiation (685 nm) associated with photosensitizers on viability of different species of *Candida* genus. Suspensions of *Candida albicans*, *Candida dubliniensis*, *Candida krusei* and *Candida tropicalis*, containing $10^6$ viable cells per milliliter were obtained with the aid of a Neubauer’s chamber. From each species, 10 samples of the cell suspension were irradiated with diode laser (685 nm) with 28 J/cm $^2$ in the presence of methylene blue (0.1 mg/ml), 10 samples were only treated with methylene blue, 10 samples were irradiated with laser in the absence of the dye, 10 samples were treated with the dye and irradiated with laser light and 10 samples were exposed to neither the laser light nor to the methylene blue dye. From each sample, serial dilutions of $10^{-2}$ and $10^{-3}$ were obtained and aliquots of 0.1 ml of each dilution were plated in duplicate on Sabouraud dextrose agar. After incubation at 37 °C for 48 h, the number of colony-forming units (CFU/ml) was obtained and data were submitted to ANOVA and Tukey’s test ($p < 0.05$). Laser radiation in the presence of methylene blue reduced the number of CFU/ml in 88.6% for *C. albicans*, 84.8% for *C. dubliniensis*, 91.6% for *C. krusei* and 82.3% for *C. tropicalis*. Despite of this, only laser radiation or methylene blue did not reduce significantly the number of CFU/ml of *Candida* samples, except for *C. tropicalis*. It could be concluded that the photo activation of methylene blue by the red laser radiation at 685 nm presented fungicide effect on all *Candida* species studied.

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1. Introduction

*Candida* genus yeasts are of common occurrence in the oral cavity of healthy individuals and *Candida albicans* is the most prevalent species, totaling 60–70% of the isolates, followed by *Candida tropicalis* and *Candida glabrata* [1]. These microorganisms are usually commensal but among certain individuals and under specific situations they can transform into parasitary form, causing oral candidosis [2–4].

In the last decades, candidosis became an human disease of increasing importance due to the high number of immunocompromised patients associated to acquired human immunodeficiency virus (HIV), use of immunosuppressants after organ transplantations and antineoplasic therapies [5]. Oropharyngeal candidosis is the most common manifestation of HIV infection, occurring among 84% of the patients [6].

Most of the oral infections caused by *C. albicans* are treated by the simple topical application of polyene antifungals, such as nystatin and amphotericin B. Meanwhile, in HIV-associated oral candidosis, these agents may be
not effective and the administration of ketoconazole, fluconazole or itraconazole may be necessary [5].

The widespread use of topical and systemic antifungals among HIV-infected patients resulted in an alarming increase in the number of isolates resistant to this therapy. This resistance is mainly associated to severe immunosuppression, recidivation of infections and long-period treatment [6]. Approximately 81% of all AIDS patients are colonized by antifungal resistant Candida [7].

Considering this, it is necessary the development of alternative therapies for oral candidosis associated to HIV virus. Promising studies have been performed with a new method of treatment: the photodynamic therapy (PDT) [6,8].

Photodynamic therapy is a technique based on the photosensitization of dyes by low-power laser. This technique promotes the destruction of the target-cell by oxidation mechanisms that lead to cell membrane lysis and protein inactivation [6].

Photodynamic therapy has been successfully applied to the treatment of malignant neoplasia, particularly among head and neck related tumors. Nowadays, the use of photodynamic therapy to treat other pathologies such as systemic reumatoid arthritis, fungal and bacterial infections have been investigated [6,9–11].

Soukos et al. [12] observed that some oral bacteria are susceptible to death by red light after their sensitization with toluidine blue. The sensitization depends on the parameters related to the laser such as wavelength, power density or light intensity that arrives to the tissue, and the energy density that is responsible for the desired radiation effect. This effect takes to alterations in the mitochondrial gradient of ionic concentration, interfering in the respiratory chain, inhibiting or stimulating ATP synthesis, and at the same time producing singlet oxygen that is toxic to cell [13].

Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Porphyromonas gingivalis, among other microorganisms were exposed to helium-neon lasers (632.8 nm) with 30 mW, diode laser (665 nm) with 100 mW and diode laser (830 nm) with 100 mW in the presence or absence of methylene blue as photosensitizer. The results indicated that the exposition to 100 mW laser was able to eliminate 40% of the bacteria and higher effect was observed in the presence of methylene blue [14].

In another published study, P. gingivalis, F. nucleatum and A. actinomycetemcomitans were treated with different photosensitizers and only toluidine and methylene blue were effectively lethal for all the target-microorganisms [8].

C. albicans yeasts were sensitized in vitro by toluidine blue, thionin and crystal violet associated with helium–neon laser. The higher microbial reduction was observed with toluidine blue [8]. Moreover, photodynamic therapy associated with methylene blue was able to eradicate C. albicans from the oral cavity of mice that were previously inoculated with this microorganism [6].

Our study aims to contribute with the search for alternative therapies in the treatment of infections by Candida, evaluating the effects of photosensitization of methylene blue by laser radiation in different species of Candida genus.

2. Materials and methods

2.1. Candida yeasts

Initially, standardized suspensions (10⁶ viable cells/ml) of C. albicans (ATCC 18804), Candida dubliniensis (NCPF 3108), C. tropicalis (ATCC 13803) and Candida krusei (ATCC 6258) were prepared.

The strains were plated on Sabouraud dextrose agar (Difco, Detroit, USA) and incubated at 37°C for 48 h. After this period, growth was suspended in 5 ml of sterile physiological solution (0.85% NaCl) and centrifuged at 1300 g for 10 min. This procedure was repeated once and the pellet was resuspended in 5 ml of physiological solution (NaCl 0.85%). The number of viable cells in the suspension was obtained by the aid of a Neubauer’s chamber by exclusion method with 0.05% methylene blue [15].

From the standardized suspension of each species, 40 samples were obtained: 10 were irradiated with laser light in presence of photosensitizer (Group L+P+), 10 were treated only with the laser radiation (Group L+P−), 10 were treated with the photosensitizer (Group L−P+) and 10 samples were exposed to neither the laser light nor to the methylene blue dye (Group L−P−).

2.2. Photosensitizer and laser

For the sensitization of Candida species the methylene blue dye (0.1 mg/ml) was used as photosensitizer. The solution of methylene blue was prepared by the dissolution of the powder (Synth, São Paulo, Brazil) in physiologic solution (0.85% NaCl). Then, the solution was filtered through a sterile filter membrane (0.22 μm, Millipore, São Paulo, Brazil). The light source used was a diode laser InGaAlP (Photon Lase, DMC, São Carlos, Brazil), with output power of 0.035 W and wavelength of 685 nm, for which the employed dye presents high absorption. The laser beam illuminated an area of 0.38 cm² and the irradiation time was 5 min, resulting in an energy dosage of 28 J/cm² for each sample.

2.3. In vitro photosensitzation

In a sterile 96 well flat-bottomed microtitulation plate, 0.1 ml of Candida suspension and 0.1 ml of the photosensitizer or physiologic solution were added. Then, the plate containing the samples was agitated for 5 min in orbital shaker platform (Solab, Piracicaba, Brazil). After this period, the containing of each well was irradiated according to the previously described groups. The irradiation of the samples was performed under aseptic conditions in laminar...
air flow chamber and laser operating at an energy density of 28 J/cm² for 5 min.

After irradiation, serial dilutions of $10^{-2}$ and $10^{-3}$ were obtained from each sample in physiological solution and aliquots of 0.1 ml were plated in duplicate on Sabouraud dextrose agar (Difco, Detroit, USA). After the incubation at 37 °C for 48 h, the number of colony forming units per milliliter (CFU/ml) was obtained. Data were analyzed by ANOVA and Tukey’s tests ($p < 0.05$). The percentage of CFU/ml reduction for each species was calculated, considering the groups L+P+ and L−P−.

During all the experiment the samples were manipulated in the dark. A dull and dark screen with an aperture with the diameter coincident with the well entrance was used, avoiding light spreading. The area of the aperture was 0.38 cm².

### 3. Results

The group irradiated with laser in the presence of photosensitizer (Group L+P+) presented the lowest mean

![Graph](image)

**Table 1**

Mean and standard deviation values of the logarithm of colony forming units per milliliter (CFU/ml) for the following studied groups: L+P+ = group irradiated with laser in the presence of photosensitizer ($n = 10$); L+P− = group treated only with laser ($n = 10$); L−P+ = group treated only with photosensitizer ($n = 10$); L−P− = group treated neither with laser nor with photosensitizer ($n = 10$).

<table>
<thead>
<tr>
<th>Species</th>
<th>L+P+</th>
<th>L+P−</th>
<th>L−P+</th>
<th>L−P−</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>4.68 ± 0.29&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.56 ± 0.09&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.69 ± 0.10&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.69 ± 0.08&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. dubliniensis</td>
<td>4.43 ± 0.58&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.40 ± 0.11&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.43 ± 0.12&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.46 ± 0.10&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. krusei</td>
<td>4.20 ± 0.36&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.50 ± 0.11&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.60 ± 0.19&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.53 ± 0.06&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>4.72 ± 0.53&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.26 ± 0.09&lt;sup&gt;C&lt;/sup&gt;</td>
<td>5.65 ± 0.06&lt;sup&gt;B&lt;/sup&gt;</td>
<td>5.68 ± 0.05&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
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A, B and C: statistically significant difference (Tukey’s test: $p < 0.05$).

### 4. Discussion

Several studies that were performed with different *Candida* species contribute to the development of treatment options for different types of infectious diseases that can occur in different sites of the human body, and for a better comprehension of the transmissibility of that fungus [16–18]. Among the medically important species, *C. albicans*, *C. tropicalis*, *C. krusei* and *C. dubliniensis* are frequently cited. *C. albicans* is the most frequently isolated species followed by *C. tropicalis* [17]. The choice of the species for this study was motivated by those published studies.

The treatment of *Candida* infections is troublesome because the effects of antifungal drugs are usually species-dependent. For instance, a drug that affect *C. albicans*, the most susceptible species, may not present any effect on *C. tropicalis* that exhibit a weak response to the majority of the antifungal drugs. Despite of the lower incidence of *C. krusei* (1–2%), it has important clinical significance due to the resistance to fluconazole and low susceptibility to all the antifungal drugs. *C. dubliniensis* is susceptible to most of the antifungal drugs [17].

Several studies pointed to photodynamic therapy as a treatment alternative for several infectious or not diseases. The motivation to these studies is the cytotoxic effect to the yeast cells due to the sensitization with a substance that is innocuous to host tissues [14,19–25].
In vitro studies showed that the use of laser associated with a photosensitizer is very effective against bacteria, yeasts, virus and parasites [8,21,26]. However, a great number of variables may influence the number of microorganisms affected by this technique including: type and concentration of the photosensitizer, microorganism’s physiologic stage, photosensitizer incubation period before the irradiation, exposure period and density of laser energy [8].

In this study, the photo activation of methylene blue at a concentration of 0.1 mg/ml with 685 nm laser light (28 J/cm²) reduced the number of C. albicans CFU/ml by 88.6%. On the other hand, a reduction of 42% of C. albicans CFU/ml has been reported [8] when 0.1 mg/ml methylene blue and 660 nm laser light with an energy dose of 2.04 J/cm² was used. Possibly, the differences between these results can be attributed to the parameters used for the laser irradiation.

C. krusei was the species that presented higher percentage of CFU/ml reduction (91.6%), followed respectively by C. albicans (88.6%), C. dubliniensis (84.8%) and C. tropicalis (82.3%). The effects of photodynamic therapy on different Candida species have also been studied by others [8]. After the photo activation of toluidine blue with the He–Ne (632.8 nm) laser, the authors in reference [8] found higher UFC/ml reduction percentage for C. albicans (77%), followed respectively by C. tropicalis (65%), C. stellatoidea (63%) and C. kefyr (40%).

Moreover, Bliss et al. [27], using photofrin as photosensitizer in photodynamic therapy, verified similar reduction of the metabolic activity of C. albicans and C. krusei. However, C. glabrata showed resistance to this type of therapy.

The results of these studies suggest that Candida species have different responses to the photodynamic therapy, indicating the need of new studies related with the effects of the photosensitization of Candida species.

Bacteria seem to be more susceptible to photodynamic therapy in relation to yeasts. Photo-activation of toluidine blue (25 µg/ml) presented lethal effect on several Gram-positive and Gram-negative bacteria. The same technique applied on yeasts was not able to induce cell death [8,19].

O’Neill et al. [28] observed a reduction of 97.4% of the microorganisms present in the dental biofilm when toluidine blue and He–Ne laser (632.8 nm) was employed. Sarkar et al. [21] also verified significant reduction in the viability of both aerobes bacteria (death percentage of 91.6%) and anaerobes ones (96.6%) present in subgingival biofilm, after use of toluidine blue and He–Ne laser.

In this study, the isolate effect of the photosensitizer (Group L–P+) and of laser (Group L+P−) on Candida yeast was also studied. When the photosensitizer was used without the laser (Group L–P+) no reduction in the number of CFU/ml for all the studied species was observed, suggesting that methylene blue (0.1 mg/ml) do not present cytotoxic effect on yeasts. The absence of cytotoxicity of methylene blue without light has been related in studies including bacteria [20,29–31]. However, published work [8] reports a reduction of 17% and of 35% in C. albicans CFU/ml number when methylene blue at 0.1 mg/ml and 1 mg/ml concentrations, respectively, was used without irradiating with laser light.

In relation to the effects of laser in the absence of the photosensitizer (Group L+P–), C. tropicalis was the unique species, among those investigated in the present work, that presented significant reduction in the number of CFU/ml, suggesting a possible native susceptibility of this species to laser light.

In summary, our results showed that all the studied Candida species were susceptible to the tested PDT protocol. These findings encourage further in vivo studies, maybe by using animal model, to explore the potential application of this protocol for candidosis treatment in immunocompromised patients.

References