Original Article

Susceptibility of *Candida albicans* and *Candida dubliniensis* to Photodynamic Therapy Using Four Dyes as the Photosensitizer

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ABSTRACT

Statement of the Problem: Oral candidiasis is the most common opportunistic infection affecting the human oral cavity. Photodynamic therapy, as one of its proposed treatment modalities, needs a distinct dye for achieving the best effect.

Purpose: The purpose of this study was to evaluate photosensitization effects of four distinct dyes on standard suspension of *Candida albicans* (*C. albicans*) and *Candida dubliniensis* (*C. dubliniensis*) and biofilm of *C. albicans* considering the obtained optimum dye concentration and duration of laser irradiation.

Materials and Method: In this *in vitro study*, colony forming units (CFU) of two sets of four groups of Laser plus Dye (L+D+), Dye (L-D+), Laser (L+D-) and No Laser, No Dye (L-D-) were assessed individually with different methylene blue concentrations and laser irradiation period. The photodynamic therapy effect on standard suspension of *Candida* species (using methylene blue, aniline blue, malachite green and crystal violet) were studied based on the obtained results. Similar investigation was performed on biofilm of *C. albicans* using the spectral absorbance. Data were imported to SPSS and assessed by statistical tests of analysis of variance (ANOVA) and Tukey test (α= 0.05).

Results: CFU among the different dye concentration and irradiation time decrease in dose- and time-dependent manner (p> 0.05), all of which were significantly lower than the control groups (p< 0.05). Among the examined photosensitizers, there was no statistically significant difference, (p> 0.05) though all of them were significantly decrease CFU compared with the control groups (p< 0.05). In L+D- and L+D+ groups, biofilm was significantly destroyed more than that of L-D- (p< 0.05).

Conclusion: Photodynamic therapy might be used as an effective procedure to treat *Candida* associated mucocutaneous diseases and killing biofilm in the infected surfaces such as dentures.

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Introduction

Oral candidiasis is the most common opportunistic infection affecting the human oral cavity caused by an overgrowth of *Candida* species, the most prevalent

being *Candida albicans*). [1-2] The incidence of candidiasis has been reported in 45% of neonates, [3] 45%–65% of healthy children, [4] 30%–45% of healthy adults, [5-6] 50%–65% of removable denture wearers, [6] 65%–88% of those in acute and long term care facilities, [6-9] 90% of patients with acute leukemia on chemotherapy, [10] and 95% of patients with acquired immunodeficiency syndrome (AIDS) [11] has been reported. In immune compromised patients, oral candidiasis can lead to systemic candidiasis. The mortality rate of which is reported to be 71% to 79%. [12] The ability of *Candida* to form antifungal-resistant biofilms seems to be an important determinant factor of the disease, in addition to immune status of the individual. [13-15] Along with *C. albicans*, *C. dubliniensis* has emerged as another etiologic agent in oral candidiasis, known for its azole resistance. *C. dubliniensis* is phenotypically similar to *C. albicans*. [16] Various treatment modalities have been proposed for oral candidiasis. Earlier options include the use of an oral or topical polyene agent (Nystatin and Amphotericin B), and generally systemic azoles (Fluconazole, Ketoconazole, and Itraconazole) is being used. However, as it was stated earlier, drug-resistant species have emerged such as *C. dubliniensis* or *C. glabrata*. [17] Resistance rate of *Candida* species to Fluconazole and Itraconazole in special groups such as HIV positive patients have been reported at 35% and 38%, respectively. [18] As a result of widespread use of various types of azole antifungals in immunocompromised patients, the rate of resistance to these drugs is alarmingly on the rise, which is associated with episodic treatment, longer durations of treatment, and severe immunosuppression. [19] Therefore, new treatment modalities in this regard should be considered. One such promising therapeutic approach is photodynamic therapy (PDT). PDT applies a low intensity visible light and non-toxic dye, called a photosensitizer (PS) which combines to produce cytotoxic species in the presence of oxygen. As PS can be targeted and the illumination source can be focused on the lesion, PDT has the advantage of dual selectivity. [20] Low cytotoxicity, appropriate antimicrobial activity, water solubility, molecular size and penetration ability to microbial cells, stability and cost-effectiveness are factors may influence the selection of PS. [21]

PDT has been suggested as an antibacterial agent in many studies. [22-25] Its antifungal application has also been reported in several researches. [26-28] Pasyechnikova and *et al.* suggested that; 0.05% concentration of methylene blue to have the most growth restriction efficacy on *C. albicans*. [29] In another study by Souza and *et al.*, assessing PDT by methylene blue, toluidine blue and malachite green, the optimum duration of laser irradiation was 8 minutes. [30] In Wilson and colleagues' study, crystal violet was applied as a photosensitizer and it showed to have comparable effects to methylene blue and toluidine blue. [31] In a few studies, PDT was assessed *in vitro* on *Candida* biofilm, especially *C. dubliniensis*. [32] Hence, in this study, we aimed to evaluate photosensitization effects of four distinct dyes (methylene blue, aniline blue, malachite green and crystal violet) on standard suspension and biofilm of *C. albicans* and *C. dubliniensis* considering the obtained optimum dye concentration and duration of laser irradiation.

Materials and Method

In this *in vitro* study, standard species of *C. albicans* (ATCC 5314) and *C. dubliniensis* (ATCC 6144) were evaluated.

Preparation of standard suspension of *C. albicans* and *C. dubliniensis*

Cells were seeded onto Sabouraud dextrose agar (Merck, Germany) and were incubatedat 35±2°C between 18 to 24 hours. After incubation, some of the colonies were selected and suspended in 5 ml sterile distilled water and placed in an orbital shaker (Solab; Piracicaba, Brazil) for 15 S. The cell densities were then adjusted to 0.5 McFarland standards at 530 nm using a spectrophotometric method (this yielded stock suspension of 1-5 $\times 10^6$ CFU/mL)

Preparation of photosensitizer

Methylene blue solution was prepared by dissolution of 10 mg methylene blue powder (Calbiochem; Merck, Germany) in 1 mL of normal saline (0.85 NaCl). This solution was filtered through a sterile 0.22 µm Millipore membrane (SãoPaulo, Brazil). For the first evaluation, two dilutions of methylene blue were prepared: 0.01, 0.001 mg/mL. The solutions were then stored in the dark at 4°C. Aniline blue and malachite green photosensitizer solutions were prepared using similar in-

structions. Crystal violet solution was prepared by dissolving 20 g of crystal violet powder in 200 mL Ethanol. The solution was kept at room temperature at 25°C for one hour and then, 800 mL of sterile distilled water was added to the solution. The solution was stored at 37°C for 24 hours. Twelve grams of ammonium oxalate were mixed with 1200 mL of sterile distilled water. Finally the oxalate solution was added to the crystal violet dye solution and stored for a while so that the final solution looked completely transparent.

Determining theoptimum dye concentration

Of the *C. albicans* standard suspension, 300 µl was added to sterile 24-well cell culture plates (Costar Corning; NY, US). Three micro liters of the diluted methylene blue, in concentration of 0.01 and 0.001, was added to the wells. The final concentration of the dye in the wells equaled to 0.01 mg/mL in half of the samples and 0.001 mg/mL in the other half. Samples were then mixed in an orbital shaker (Solab; Piracicaba, Brazil) for 5 min in the dark.The assessed groups were: Laser plus Dye(L+D+), Dye(L-D+), Laser (L+D-) and No Laser, No Dye (L-D-).A gallium-aluminum-arsenic diode laser (Azor, Russia) was used for irradiation, with the output power of 25mW and wavelength of 660 nm which is the optimum wavelength for absorbance of most dyes. The beam area of the laser was 0.78 cm^2. In the first evaluation, the duration of laser irradiation was set at 5 minutes. Irradiation was performed under aseptic conditions under a laminar flow hood in the dark, and the plates were covered with a black screen with orifices (1 cm diameter for adaptation to laser beam).To determine colony-forming unit (CFU) counts, samples were diluted to 1% in sterile distilled water and cultured on sabouraud dextrose agar media and incubated at 35±2 °C for 24 hours. All experiments were performed in triplicate.

Determining the optimum irradiation duration

Using the optimum concentration of methylene blue dye, which was obtained from the previous evaluation, photosensitization effect on *C. albicans* and *C. dubliniensis* were assessed for irradiation duration of 5, 10, 20, and 30 minutes in similar experimental groups: L+D+, L-D+(positive control), L+D-(positive control) and L-D- (negative control). Energy densities produced by the laser were 9.6, 19.2, 38.4 and 76.8 j/cm^2 in the irradiation duration of 5, 10, 20, and 30 minutes for each sample. CFU counts were then calculated as described above.

Standard suspension assessment

Based on the optimum irradiation duration which was determined in the previous evaluation, photosensitization effect of four dyes**;** methylene blue, aniline blue, malachite green (malachite green oxalate, Merck, Germany) and crystal violet (Merck, Germany) were evaluated against *C. albicans* and *C. dubliniensis*. The study groups were L-D+ and L+D+. The rest of the procedures were similar to the first evaluation.

Biofilm formation containment

The standard strain of *c.albicans* was cultured on sabouraud dextrose agar and incubated at 35±2°C for 18 hours. Using a sterile inoculation loop, colonies were then transferred intosabaroud-2%-dextrose-broth medium (Merck, Germany) and placed in an orbital shaker for 18 hours at room temperature. Suspension was centrifuged at 1,300×g for 10 min, and the residues were discarded. Then, the yeast cells were washed twice with phosphate-buffered saline (PBS, pH 7.0) and the cell densities were adjusted to 0.5 McFarland standards at 530 nm using a spectrophotometric method in RPMI 1640 media (Sigma-Aldrich, Taiwan) buffered with morpholinopropane sulfonic acid (MOPS) (Sigma; Aldrich, Taiwan).

Of *C. albicans* suspension, 300 µl was added to 24-cell culture microtiter plate (Costar Corning, NY, USA) and the plate was incubated for 48 hours at 35º C. After incubation, methylene blue dye with concentration of 0.01% was added to the wells. After 10 minutesof incubating the plates at room temperature in darkness, the laser was irradiated for 10 minutes. The experimented samples were quadruple in each study group. The wells were washed twice with PBS. 300 µl combination of XTT-Menadione was then added to them. The wells were incubated for 3 hours at 37 ºC in the dark. Finally, the wells content were transferred to another plate and their spectral absorbance in the wavelength of 570 nmwas evaluated by amicro plate reader (Polar star omega, Germany).

XTT preparation

XTT (Sigma-Aldrich) was prepared in PBS and then the solution was filtered, sterilized through a 0.22µm pore size filter and stored at −70 ◦C. Before usage, an aliquot of stock XTT was diluted in PBS and the elec-

tron-coupling agent Menadione (10mM prepared in acetone; Sigma) was added after its dilution with PBS in 1/10 ratio.

Statistical analysis

The data was imported to SPSS Software and assessed by statistical tests of analysis of variance (ANOVA) and Tukey test. (α= 0.05)

Results

Concentration of photosensitizer (methylene blue)

Inthisexperiment, different concentrations of methylene blue (0.1, 0.01 and 0.001) were found, to significantly decrease CFU in comparison to thecontrol (L-D-) groupsin a dose dependent manner (p<0.05).

Duration of laser irradiation

As shown in the figures 1 and 2, the percentage of reduction of CFU/ml of *C. albicans* in different laser irradiation times including 5, 10, 20, and 30 min in the presence of methylene blue dye were 31%, 60%, 68% and 73%, respectively. As shown in Figure 1, laser irradiation alone (L+D-) and combined with photosensitizer (L+D+), led to a significant reduction in CFU in comparison to the control groups, regardless of duration of laser irradiation (p< 0.05).

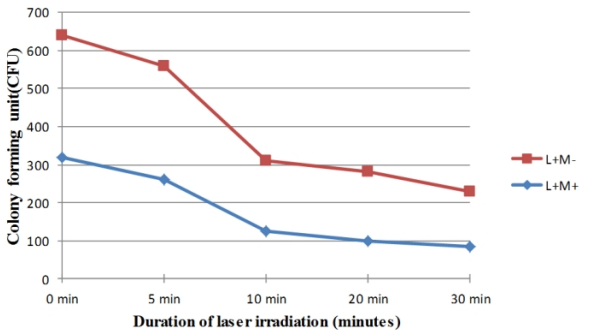

Figure 1: The effect of laser, alone and combined with methylene Blue dye on CFU of *C. albicans* in four different time of laser irradiation.

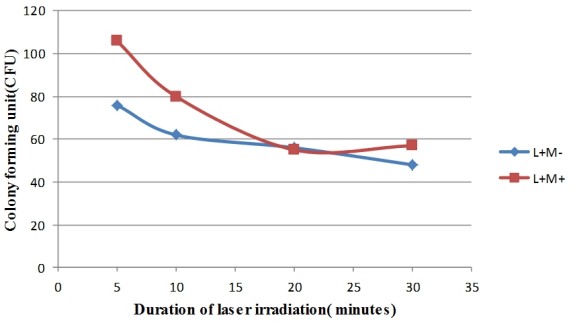

Figure 2: The effect of laser, alone and combined with methylene blue dye, on CFU of *C. dubliniensis* in four different time of laser irradiation.

Type of photosensitizer

As shown in Figures 3a and 3b, all of four photosensitizers resulted in a significant decrease in CFU in comparison to the control groups (p<0.05). However, these dyes were not statistically different in CFU reduction with each other (p>0.05). Regarding aniline blue dye, there was not a significant difference between the photosensitizer alone and in combination with laser irradiation. When it is used alone, crystal violet led to a significant reduction in CFU in comparison to malachite green. Among the examined photosensitizers, crystal violet (alone and in combination with laser) yielded the highest effectin reduction of CFU, although these differences were not statistically significant in comparison to other dyes.

Biofilm containment

The absorbance of XTT assay on *C. albicans*' biofilm treated groups including L-D-, L+D+, L+D- and L-D+ were 0.068, 0.049, 0.059 and 0.067, respectively. In this regard, methylene blue dyealone did not significantly destroy *C. albicans* biofilm. However, in L+D-andL+D+ groups, biofilm was significantly destroyed more than that of the control group (L-D-).

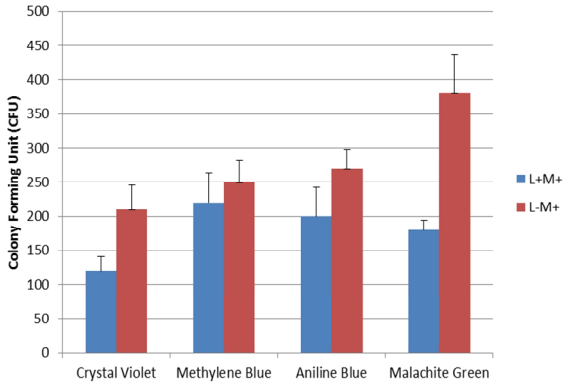

Figure 3a: The effect of four different photosensitizers, alone and combined with 10 minutes of laser irradiation, on CFU of *C. albicans*.

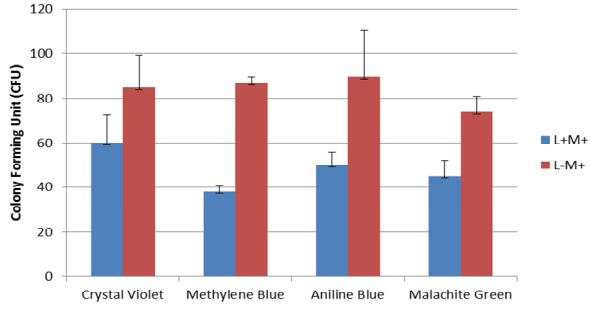

Figure 3b: The effect of four different photosensitizers, alone and combined with 10 minutes of laser irradiation, on CFU of *C. dubliniensis*.

Discussion

In the first experiment, three concentrations of methylene blue dye were evaluated. Various dilutions were studied in different literature. [29, 33] Pasyechnikova and colleagues found that, the concentration of 0.05% of methylene blue to have the most growth inhibitory effect on *C. albicans*. [29] In several studies, the growth inhibition effect of the dye was reported to be dose-dependent. [29, 33] However, they have not agreed on acommon concentration as the optimum. Besides, an attempt to reach anagreement on the common optimum concentration of the dye might be difficult because of the diversity of *Candida* species studied and different laboratory variables such as incubation duration, time of irradiation, and amount of energy applied in different experiments. Hence, in our experiments, the minimum inhibitory concentration of PS and irradiation time was determined in order to lessen the probable adverse effects of the dye and irradiation as well as obtaining a favorable result. A significant reduction in CFU by 0.01% concentration of methylene blue dye was found and this dilution was utilized in the next stages of this study.Using the minimum concentration of this dye with significant antifungal activity in PDT, might reduce the adverse effects of methylene blue including discoloration, headache, drowsiness and hypertension.

With regards to the second experiment, even though, no significant differences were found in CFU of the candidaspeciesbetween different times of laser irradiation, but the CFU was reduced about 20% by increasing the laser irradiation time from 5 min to 10 min. As shown in figure 1, no noticeable differences were found between the CFU of candida in irradiation times of 10, 20 and 30 min. Hence, we used 10 min laser irradiation as the optimal time for the other treatments. Souza and Rodrigo assessed the photodynamic therapyby methylene blue, toluidine blue and malachite green and reported the optimum duration of laser irradiation to be 8 minutes [30] which is close to the 10-minute duration, used in our study. Similar to our results, time-dependent effect of photosensitization was also reported by other researchers. [30, 34]

Despite the study of Wilson *et al.*, [31] that reported the lack of effectiveness of laser irradiation alone on fungal cell viability, in our study laser irradiation decreased the CFU after 10 min to about 50 percent and its effect was enhanced apparently with PS.

Among the four photosensitizers that were examined in our study, crystal violet and aniline blue were applied in a few studies. [31] Crystal violet dye with chemical formulation of C_{25} $H_{30}Cl$ N_3 has been used for its antiseptic or anti-helminthes properties. [35] In Wilson *et al.* study, crystal violet was applied as a photosensitizer and shown to have comparable effects to methylene blue and toluidine blue. [31] This is in agreement with our results which demonstrated that in the laser irradiation duration of 10 minutes, crystal violet caused the most reduction in CFU in compare with other dyes. Nevertheless, no significant differences in reduction of CFU were found between this dye as a photosensitizer and the other three dyes. Malachite green is an organic dye with known antimicrobial properties used in aqua culture. Moreover; malachite green can also be used as a PS in PDT. [30] Similar to the previous studies, [30, 39-40] combination of malachite green and laser decline the CFU of the tested candida species considerably. Aniline blue, also known as water blue, is a biologic dye used in differential staining. [36] To the best of our knowledge, there is no previous study on evaluating the effects of aniline blue as a photosensitizer against *C. albicans*. The photosensitizing effect of aniline blue was comparable with the other dyes and resulted in a significant reduction of CFU in comparison to the controlgroupsin bothwith or without laser irradiation.

Biofilm is composed of a complex matrix of microorganisms. [37] It has been shown previously [38] that biofilm are resistant to chemical and antimicrobial agents. As predicted, methylene blue was not statistically effective in killing the *Candida* biofilm when it was used alone. Although, laser irradiation was significantly effective in killing the formed biofilm, its combination with photosensitizer resulted in increasing this effect. This is similar to the study of Souzaand Rodrigo [30] which reported the effect of laser with photosensitizer in killing of biofilm.

Conclusion

Methylene blue dye with the concentration of 0.01 can be effective as a photosensitizer in growth inhibition of *C. albicans*. Among the examined photosensitizers,

methylene blue and crystal violet had the best inhibitory effects against growth of *C. dubliniensis* and *c. albicans*, respectively. Moreover, photosensitization successfully killed *Candida* cells in biofilm format. Altogether, as the laser doses used in this study are similar to those used in clinic, photodynamic therapy might be used in daily practice as an effective procedure to treat *Candida* associated mucocutaneous diseases such as oral candidiasis and killing biofilm in the infected surfaces such as dentures.

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Conflict of Interest

The authors of this manuscript certify no financial or other competing interest regarding this article.

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