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ORIGINAL ARTICLE

Repeated applications of photodynamic therapy on *Candida glabrata* biofilms formed in acrylic resin polymerized

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Abstract Previous studies have been suggested that photodynamic therapy (PDT) can be used as an adjuvant treatment for denture stomatitis. In this study, we evaluated the effects of multiple sessions of PDT on *Candida glabrata* biofilms in specimens of polymerized acrylic resin formed after 5 days. Subsequently, four applications of PDT were performed on biofilms in 24-h intervals (days 6–9). Also, we evaluated two types of PDT, including application of laser and methylene blue or light-emitting diode (LED) and erythrosine. The control groups were treated with physiological solution. The effects of PDT on biofilm were evaluated after the first and fourth application of PDT. The biofilm analysis was performed by counting the colony-forming units. The results showed that between the days 6 and 9, the biofilms not treated by PDT had an increase of 5.53 to 6.05 log ($p = 0.0271$). Regarding the treatments, after one application of PDT, the biofilms decreased from 5.53 to 0.89 log. When it was done four applications, the microbial reduction ranged from 6.05 log to 0.11 log. We observed that one application of PDT with laser or LED caused a reduction of 3.36 and 4.64 compared to the control groups, respectively ($p = 0.1708$). When it was done four applications of PDT, the reductions achieved were 1.57 for laser and 5.94 for LED ($p = 0.0001$). It was concluded that repeated applications of PDT on *C. glabrata* biofilms showed higher antimicrobial activity compared to single application. PDT mediated by LED and erythrosine was more efficient than the PDT mediated by laser and methylene blue.

Keywords *Candida glabrata* · Denture stomatitis · Photodynamic therapy · Laser · LED · Photosensitizers

Introduction

Denture stomatitis (DS) is a common disorder affecting denture wearers and is characterized by inflammation and erythema on the regions of the oral mucosa that are covered by the denture [1, 2]. This condition is often associates with an infection process with fungus of *Candida* genus. This disease has higher incidence in the elderly and presents multifactorial etiology, different clinical patterns, and poor symptomatology [3]. The spongy denture tissue surface, full of nutritive substances, is an ideal incubator for *Candida* species [4, 5]. *Candida* spp. is a commensal yeast that colonizes the oral cavity of humans, being isolated in approximately 53% of healthy individuals [6]. In denture wearers, the prevalence of *Candida* increases until 100%, which can be explained by the fact that dentures decrease the flow of oxygen and saliva to the underlying tissue that favors yeast overgrowth [5, 7].

Although 90% of DS cases are caused by *Candida albicans*, the non-*albicans* species, such as *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, and *Candida dubliniensis*, can also

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contribute to the pathogenesis of the disease [2, 8, 9]. Recent studies suggested that *C. glabrata* has been associated with fungemia and its incidence is higher in adults than in children [10, 11]. This specie represents a growing concern in clinical settings in which it causes mucosal infections and is related to around 15% of all *Candida*-related systemic bloodstream infections [12]. Therefore, the use of dental prosthesis can be a reservoir for *C. glabrata* and may contribute to the development of a systemic infection in immunocompromised patients.

The conventional treatment for DS is composed of conventional antifungals, such as fluconazole and micafungin. DS caused by *C. glabrata* are more difficult to treat because this specie is intrinsically less active to azoles than *C. albicans* [13]. For example, the epidemiological cutoff value for fluconazole, which indicates the minimum inhibitory concentration value identifying the upper limit of the wild-type population, is 32 μg ml^{-1} in *C. glabrata* while it is only 0.5 μg ml^{-1} for *C. albicans* [14]. With the reduction in susceptibility of *C. glabrata* to antifungal agents and higher incidence of DS in the elderly people, it has resulted in an interest in the development of new therapeutic strategies, such as photodynamic therapy [15].

As an alternative to conventional antifungal therapy, photodynamic therapy (PDT) is a promising modality due to its effectiveness against a broad range of species of microorganisms. In this context, a photosensitizing agent is activated by a light source laser or light-emitting diode at a specific wavelength in the presence of oxygen, resulting in the production of reactive oxygen species (ROS) and free radicals. These ROS disrupt the *Candida* cytoplasmic membrane and cause an increase in cellular permeability and subsequent damage to intracellular targets [16, 17].

The main photosensitizers used in antifungal PDT are phenothiazine (methylene blue), phthalocyanines, and porphyrins associated with lasers and other non-coherent light sources [18–20]. Methylene blue and laser-mediated PDT has been shown to reduce *Candida* cells in vitro and the epithelial alterations related to oral candidiasis in rats [21–23]. In the PDT against *Candida* spp. using LEDs, phenothiazines, Photogem photosensitizers, and Xanthene dyes (erythrosine) have been used achieving until 3 $\log_{10}$ of reduction. In addition, the erythrosine has attracted interest as a photosensitizer because it is not toxic to the host and is approved for use in dentistry to detect dental biofilms [20, 24, 25].

Since the PDT can be used as an alternative treatment for DS and most of the studies were focused on *C. albicans* [15, 19–23, 26, 27], the objective of the present study was to evaluate the antimicrobial effects of repeated applications of PDT on *C. glabrata* biofilms, formed on acrylic resin specimens thermally activated, and to compare the antimicrobial effect of PDT mediated by laser and methylene blue to therapy performed by the LED and erythrosine.

Materials and methods

Candida strain

In this study, we used a reference strain of *C. glabrata* (ATCC 90030). Culture of this strain grown on Sabouraud dextrose agar (Difco, Detroit, Illinois, USA) at 37 °C for 24 h was harvested in Sabouraud dextrose broth (Difco, Detroit, Illinois, USA). After an 16-h incubation at 37 °C, the yeasts were centrifuged at 358×g for 10 min, washed twice with phosphate-buffered saline (PBS), resuspended in sodium chloride physiological solution, and adjusted to an optical density of 0.284 at 530 nm (10^6 cells/ml) using a spectrophotometer (B582, Micronal, São Paulo, São Paulo, Brazil).

Photosensitizers

Methylene blue (Sigma-Aldrich, São Paulo, São Paulo, Brazil) at a concentration of 300 μM [22] and erythrosine (Sigma-Aldrich, São Paulo, São Paulo, Brazil) at a concentration of 400 μM [20] were used for the sensitization of *C. glabrata*. The photosensitizers solutions were prepared by dissolving the dye in physiological saline (0.85% NaCl) and filtration through a sterile 0.22-μm Millipore membrane (Merck-Millipore, Darmstadt, Germany). After filtration, the photosensitizer solution was stored in the dark.

Light sources

A gallium-aluminum-arsenide (GaAlAs) laser (Easy Laser, Clean Line, Taubaté, São Paulo, Brazil) emitting a continuous light at a wavelength of 660 nm and a green light-emitting diode (LED) (MMOptics, São Carlos, São Paulo, Brazil) emitting light at a wavelength of 532 nm were used as the light sources. The laser radiation conditions were an output power of 0.035 W, spot size area of 0.028 cm^2, fluence of 26.3 J/cm^2 (energy of 10 J and time of 285 s), and a fluence rate of 92 mW/cm^2. The LED radiation conditions were an output power of 90 mW, spot size area of 0.5 cm^2, an energy of 16.2 J, a time of 3 min, a fluence rate of 237 mW cm^2, and a fluence of 42.63 J cm^{-2}. The area irradiated in biofilms was 0.94 cm^2 at a distance of 0.05 cm for laser and 0.05 cm for LED. The optical output of the laser unit was measured before, halfway through, and after the experiment. The laser and LED parameters used in this study were based, respectively, in the studies of Souza et al. [22] and Costa et al. [20].

The temperature at the bottom of the 24-well microtiter plates (Costar Corning, New York, NY, USA) was monitored using an infrared thermometer (MX4, Raytek, Sorocaba, São Paulo, Brazil); no increases in temperature were observed after irradiation with the LED or Laser.

Confection of an acrylic substrate for biofilm formation

In order to develop this study, we prepared test specimens measuring 10 mm in diameter, in acrylic resin polymerized fabricated using polymethylmethacrylate (Vipi Cril plus, VIPI, Pirassununga, São Paulo, Brazil) that is routinely used for the production of dental prosthetics. The specimens were included in metal muffle, pressed in a hydraulic press for 2 h, and then taken to the microwave (ME18S, Electrolux, São Carlos, São Paulo, Brasil) for 15 min (power 10 W) and 5 min (power 5 W). The finishing of the specimens was done with dry sandpaper and polish with pumice using felt disc. Next, the specimens were submerged in distilled water for 48 h to release the residual monomer. The specimens were sterilized by gamma radiation Cobalt^{60} (20 kGy for 6 h) at Empresa Brasileira de Radiações (EMBRARAD, Cotia, São Paulo, Brazil).

In vitro biofilm formation

The in vitro biofilm formation was performed as described by Vilela et al. [28]. The sterilized acrylic specimens were positioned in the wells of first row of a 24-well culture plate (Costar Corning, New York, USA) containing 2 ml of Brain Heart Infusion broth (BHI, Difco, Detroit, Illinois, USA) with 5% sucrose. After that, 100 μl of standardized microbial suspension of *C. glabrata* was added in the well containing an acrylic specimen immersed in BHI. The plates were incubated at 37 °C for 5 days. *C. glabrata* was placed only once, and the BHI broth with 5% sucrose was changed every 24 h.

After the incubation time, the test samples were transferred to the wells of the next row of the culture plate, with each well containing 2 ml of sterilized saline solution. The plates were shaken for 5 min in an orbital shaker (Solab, Piracicaba, São Paulo, Brazil) to remove cells loosely attached to the test sample.

In vitro photosensitization

The formed biofilm were treated with four applications of PDT in 24 h intervals. For each application of PDT, the specimens were immersed in the photosensitizer and they were shaken for 10 min in an orbital shaker. Subsequently, the specimens were irradiated with light source in the above-described parameters. The control group was submitted to the same experimental conditions mentioned above; however, the biofilms of control group did not receive photosensitizer and irradiation, and they were treated only with saline.

Each assay was performed in aseptic conditions within a laminar flow chamber and with ambient lights turned off. A black mask with a hole matching the diameter of the wall opening minimized artefacts related to light scattering during the irradiation procedure.

After irradiation, each test sample was placed in a Falcon tube containing 10 ml of sterilized saline and homogenized for 30 s in a 50-W ultrasonic homogenizer (Sonoplus HD 2200, Bandelin Electronic, Germany) to disaggregate the cells from the biofilm as the experimental groups (Fig. 1).

From the homogenized solution (10^{-1}), decimal dilutions of the biofilm suspension for each test sample were prepared, and 100 μl aliquots of each dilution were spread on plates containing Sabouraud dextrose agar. The plates were incubated at 37 °C for 48 h. Afterwards, those plates containing from 30 to 300 colonies were used to calculate the colony-forming units (CFU) and converted into logarithm.

Statistical analysis

The results of the CFU/specimen were statistically analyzed by the program Graph Pad Prism, considering a significance level of 5%.

Results

The monitoring results of the biofilm growth not treated by PDT on days 6 to 9 demonstrated that there was an increase from 5.53 to 6.05 log of biofilm growth. Despite this difference that was only 0.52 log, there was a statistically significant difference between the observation times (Fig. 2).

Regarding to the treatments, the two types of PDT used in this study (association of laser and methylene blue or LED and erythrosine) were able to reduce the *C. glabrata* biofilm with one or four applications of PDT. When a single application of PDT was performed, the quantification of the biofilm was as follows: 5.53 ± 0.29 log for the control group, 2.17 ± 1.80 log to PDT with laser, and 0.89 ± 1 68 log for PDT with LED. In the treatment of biofilms with four applications of PDT, the microbial reductions observed were more significant, with 6.05 ± 0.27 log for the control group, 4.48 ± 0.87 log for PDT with laser, and 0.11 ± 0 31 log for PDT with LED (Fig. 3).

Analyzing only the groups treated with PDT, we can observe that the LED protocol with erythrosine was more effective in reducing biofilms than the laser and methylene blue, in both application times (one or four applications). With one application of PDT, the reduction achieved by the laser was 3.36 log and 4.64 log for the LED compared to the control group. With four applications of PDT, laser and LED achieved respectively 1.57 and 5.94 log of microbial reduction compared to the control group (Fig. 4).

Discussion

In the present study, we evaluated the antimicrobial effects of repeated applications of PDT on *C. glabrata* biofilms.

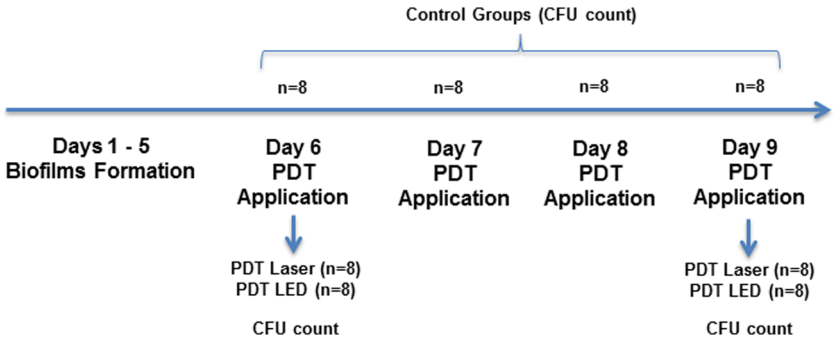

Fig. 1 Study design. *PDT*, photodynamic therapy; *LED*, light-emitting diode; *CFU*, colony-forming units

C. glabrata is often the second or third most common cause of candidiasis after *C. albicans* and its infections are difficult to treat due to its resistance to many azole antifungal agents. Consequently, *C. glabrata* infections have a high mortality rate in immunosuppressed patients [2, 29, 30]. Recently, some members of our group isolated *Candida* spp. from prosthesis-fitting surfaces of 50 individuals wearing removable maxillary prosthesis with lesions of DS and verified that *C. albicans* was the most prevalent specie followed by *C. glabrata* and *C. tropicalis*. These data indicate the importance in seeking alternative methods as PDT to control the DS caused by non-*albicans Candida* species, such as *C. glabrata* [31].

Kilic et al. [32] assessed the prevalence of DS in different attachment-retained overdenture wearers and its association with particular colonizing *Candida* species of 37 healthy individuals in Turkey. The authors found that *C. albicans* was the most common species isolated from 81.3% of patients using bar-retained overdentures and 38.1% of those using locator-retained overdentures. *C glabrata* was the second most common species, isolated from 37.5% of patients using bar-retained overdentures and 23.8% of those using locator-retained overdentures. The biofilm formation is an important factor to the development of DS in patients who wear dentures and, according Pathak et al. [33], *C. glabrata* cells in the biofilms are more metabolically active than cells from *C. albicans*, *C. krusei*, and *C. tropicalis*.

Before the study of the effects of PDT on *C. glabrata* biofilms, initially we did a study to monitor the growth of biofilms not treated by PDT. It was observed that the biofilm had good growth up to day 6 of formation and then it stabilized. This can be explained by the low growth (0.52 log) of day 6 to day 9, although there is statistical difference between days. These findings corroborate with Seneviratne et al. [34] who evaluated the growth kinetics of *Candida* biofilms by counting CFUs after 1.5, 24, 48, and 72 h of development. The authors found that the candidal biofilm appears to reach its developmental plateau between 24 and 48 h ($0.3–2.2 \times 10^8$ cells ml^{-1}) with perhaps the highest architectural stability. Then, with increased number of cells, biofilm architecture starts to stabilize by 72 h. In addition, Barros et al. [35] studied the temporal profile of biofilm formation, gene expression, and virulence analysis in *C. albicans*, and they found high expression levels of the transcriptional genes for *TEC1*, *BCR1*, and *EFG1*, which prepare the biofilm for the biomass increase, begin around 12 h of development and reduce at mature phase (48 h).

Microorganisms in biofilms have a higher resistance to antimicrobial agents and increased protection against the host immune system [15, 23, 33]. According Costa et al. [20], *Candida* in biofilms have been shown to be less susceptible to photodynamic therapy than fungi in the planktonic phase,

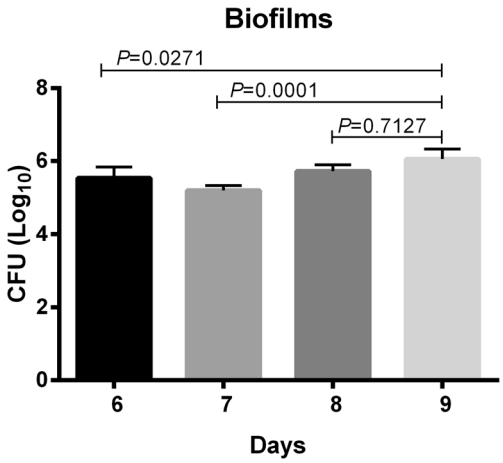

Fig. 2 Mean values and standard deviations of the CFU count (log) of *C. glabrata* in the monitoring of biofilms on days 6, 7, 8, and 9 (ANOVA and Tukey test, $p < 0.05$)

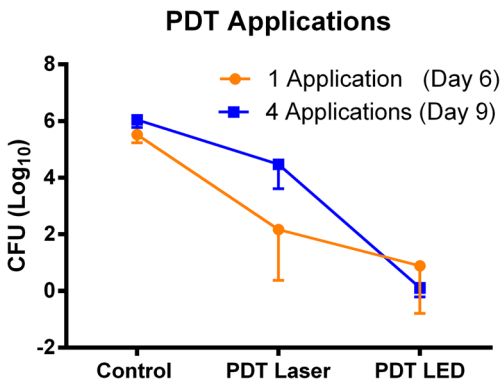

Fig. 3 Mean values and standard deviations of the CFU count (log) of *C. glabrata* biofilms in the control group, PDT with laser, and PDT with LED after one or four applications of PDT

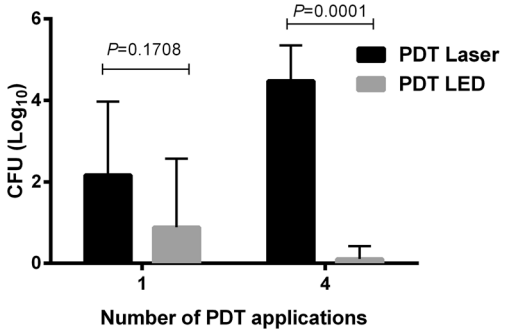

Fig. 4 Mean values and standard deviations of the CFU count (log) of *C. glabrata* biofilms of PDT groups of laser and LED after one or four applications (Student's *t* test, $p < 0.05$)

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. Regarding this effect, Schneider et al. [36] demonstrated that laser irradiation during PDT using phenothiazine chloride as photosensitizer reduced the number of live *Streptococcus mutans* cells within a layer of only 10 μm in an artificial biofilm model evaluated by confocal laser scanning microscope.

With respect to PDT, the two tested modalities (LED or LASER) were able to reduce biofilm of *C. glabrata*. According Mima et al. [26], PDT is one potential alternative for treating DS, which combines a dye and a light source. These authors evaluated in vitro the effectiveness of one application of PDT for the inactivation of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. dubliniensis*, and *C. krusei* on maxillary complete dentures. *C. tropicalis* was the most species susceptible to PDT and *C. dubliniensis* and *C. glabrata* were the least susceptible.

We found promising reductions when a single application of PDT was performed, such as 3.34 log to PDT with laser and 4.64 log for PDT with LED. These reductions of yeasts on biofilms achieved with one session of PDT by the methods employed in this work was greater than the reduction obtained by Junqueira et al. [37] and Quishida et al. [38]. Junqueira et al. [37] evaluated the PDT on seven clinical strains of *C. glabrata* by association of zinc phthalocyanine and laser on mature biofilms with mean reduction of 0.33 log. Quishida et al. [38] studied the effect of PDT with Photodithazine® and LED on biofilm of *C. glabrata* and the highest reduction in the cell viability was equivalent to 1.19 log. According to Boyce et al. [39], a minimum of 3 log steps must be achieved to state antimicrobial effect, showing that the protocol of this study can be used as an alternative method to conventional antifungal therapies.

Our reductions with four applications of PDT achieved 4.64 log for laser and 5.94 log with LED compared with the control groups, showing that multiple applications are more efficient than single session therapies. One hypothesis for this fact is that the PDT in multiple sessions weakens the surface of the biofilm becoming it more susceptible to the treatment. To the best of our knowledge, this is the first in vitro study that evaluated multiple applications of PDT mediated by laser and methylene blue on biofilms of *C. glabrata*. Similarly, Quishida et al. [40] also tested the effectiveness of three applications of PDT mediated by Photodithazine® and LED against biofilms formed by *C. glabrata* on denture base acrylic resin. The *C. glabrata* demonstrated significant differences in relation to the number of applications (one to three PDT sessions) and treatment groups, concluding that three consecutive applications of PDT were more effective for reducing the cell viability and the total biomass of biofilm [40].

In a randomized clinical trial, Mima et al. [41] analyzed the clinical and mycological efficacy of PDT for the treatment of DS and compared with topical nystatin. In the nystatin group, patients received topical treatment with nystatin four times daily for 15 days, and in the PDT group, the denture and palate of patients were sprayed with Photogem®, and illuminated by LED three times a week for 15 days. At the end of the treatment (day 15), it was verified that the number of isolates of *C. albicans*, *C. tropicalis*, and *C. glabrata* was reduced by 50, 90, and 62.5%, respectively, in the nystatin group and 50, 45.5, and 71.4%, respectively, in the PDT group compared with the baseline (day 0), demonstrating that multiple applications of PDT was so effective as topical nystatin in the treatment of DS.

Recently, Maciel et al. [27] evaluated the PDT combined with low-power laser (LPL) therapy in the treatment of 20 individuals with DS. The PDT group was submitted to one session of methylene blue-mediated PDT plus two sessions of LPL twice a week for 15 days. Forty percent of the patients achieved clinical and microbiological resolution of DS after PDT followed by LPL and 70% of the patients reported a significant reduction in symptoms (burning sensation and pain associated with DS). The success of these clinical studies using multiple PDT corroborate with our results and demonstrate that PDT in multiple application is more effective compared to PDT with a single application.

Regarding the protocols of PDT employed in this study, the LED and erythrosine was more efficient compared to PDT mediated with laser. In addition, the higher power density of the LED application used in this study compared with laser may have had an advantage on the LED protocol, and consequently achieving better reductions. LEDs are widely used in dental clinics as bleaching tools; moreover, they have shown potent activity in PDT, reduced weight and cost compared to laser, and greater flexibility in treatment irradiation time and easy operation [42, 43]. According to Mima et al. [26], the susceptibility of species of *Candida* to PDT may vary according to the type, concentration, and time of incubation of photosensitizer and light source used such as laser or LED. The divergences of reductions between our study and results

reported in the literature might be due to the lack of predefined parameters for the use of PDT, a fact that impairs a reliable comparison between the results obtained in different studies.

Based on the methodology employed in this study and the outcomes obtained, it may be concluded that repeated applications of PDT on biofilms of *C. glabrata* had significant reduction in the biofilm compared to single session. The two protocols of PDT used in this study had antimicrobial effect on the biofilm, although the protocol mediated by LED and erythrosine was more efficient than the laser and methylene blue.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Not applicable.

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