Photodynamic therapy on a biofilm monospecie of *candida albicans*: an *in vitro* study

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Summary

Antimicrobial photodynamic therapy (aPDT) is a medical treatment based on the use of a light source at a specific wavelength that activates a photosensitive molecule causing the formation of oxidizing agents that provokes the death of the target cells. The aim of this study was to evaluate the activity of aPDT against biofilms of *Candida albicans*. Biofilms of *C.albicans* were subjected to aPDT using a diode GaAlAs (λ:635nm) and a toluidin blue solution (0.1mg/ml) as photosensitizer. Three different protocols (1 minute, fluence 24J/cm²; 2 minutes, fluence 48J/cm² and 3 minutes, fluence 72J/cm²) were tested. To evaluate the number of Candida cells in biofilm before and after treatment, Bio Timer Assay was used. Candida biofilms treated with aPDT showed significant reductions of the microbial population as compared to the control groups. aPDT showed good antifungal properties *in vitro*. Clinical trials are needed to test its efficacy *in vivo* and to propose it as an adjuvant or alternative therapy to conventional medical treatment.

Introduction

The incidence of fungal infections has been increasing since the 1980s, especially for systemic forms in immunocompromised and/or hospitalized populations with other systemic diseases. (1) This increase is partially due to medical interventions, resulting in immunosuppression that leaves patients at risk for developing fungal infections (e.g., patients undergoing bone marrow transplants, blood transfusions, immunosuppressive therapy, invasive surgery, use of broad-spectrum antibiotics, anti-cancer chemotherapy, and AIDS patients). Moreover, fungal pathogens have many virulence features, such as the ability to switch themselves among different morphological states, biofilm formation and resistance to antifungal drugs (2, 3).

Candida albicans, a polymorphic microorganism, has become one of the most common agents of nosocomial infection in immunocompromised patients and those undergoing long-term treatment with antibiotics or other immunosuppressive therapies. In these patients, *C. albicans* invades the deeper tissues and can cause life-threatening systemic infections.

Candidemia occurs with an infection rate of 8-10 out of 100,000 people a year and is associated with a mortality rate of 30-50% (4-6). However, the available epidemiological data are rather heterogeneous because of the clinical features of infections and the lack of official records or statistics.

Candidiasis and oral candidiasis are opportunistic infections of the skin and oral cavity. They are common in old people, especially denture wearers, children and immunocompromised patients. *C. albicans* is the causal agent in 50% of oral mycosis cases, although in recent years, a high incidence of non-albicans species (*C. tropicalis*, *C. krusei*, *C. glabrata*, *C. parapsilosis* and *C. dublinensis*) has been documented (7, 8).

Candidiasis is the most common intra-oral infection in AIDS patients, and it is caused by an overgrowth of *Candida* species. In fact, 84% of patients with HIV infection develops candidiasis (9). Patients with candidiasis often report other disorders such as burning and taste alteration, as well as pain, dysphagia, nausea, vomit and diarrhea at times. Such symptoms can disrupt feeding, worsening quality of life. Treatment with topical or oral antifungal agents, such as nystatin, amphotericin B or fluconazole can achieve only

transient responses during treatment, generally for 15 days (10). Recurrences are very common, considering the multifactorial etiology of candidiasis, and widespread use of fungicides has resulted in the development of resistant species, especially *C. albicans*.

In addition, antifungal agents can reduce the effectiveness of antiretroviral drugs, and this reduction is a serious problem considering the systemic diseases that often affect these patients (11).

Antimicrobial photodynamic therapy (aPDT) is a medical treatment based on the use of a light source at a specific wavelength that actives a specific photosensitive substance, which reacts in the presence of oxygen causing the formation of oxidizing agents, such as singlet oxygen and free radicals. Therefore, PDT causes the death of the target cells through membrane lysis and protein inactivation.

The use of aPDT for inactivating microorganisms was first demonstrated more than 100 years ago by Oscar Raab when he reported the lethal effect of acrinide hydrochloride and visible light on *Paramecia caudatum* (12). However, it is only in recent times that aPDT has been studied as a medical treatment.

Photodynamic therapy has been approved in many countries for oncological clinical treatment, especially in head and neck tumors, Several studies have shown the antimicrobial properties of aPDT against Gram positive and Gram negative bacteria, fungi and viruses (13, 14); therefore, it may be considered an effective alternative to the use of antimicrobial agents.

Despite the promising advantages that aPDT has, such as the lack of selection of resistant strains, the absence of mutagenic effects, and the almost total absence of side effects and drugs interactions in topical applications (15), a standardized protocol for the application of such therapy in patients being treated for superficial fungal infection is lacking.

The aim of this work was to test, *in vitro*, the efficacy of aPDT against *C. albicans* biofilm using a diode laser as a light source and toluidine blue (TB) as a photo-activating agent.

Materials and methods

Biofilm production

In this study, *Candida albicans* strain ATCC 24433 was used. To obtain *C. albicans* biofilm, the fungus was plated on Brain Heart Infusion (BHI) agar medium and incubated at 37°C for 24 h. After incubation, 10 isolated colonies were picked from the plate with a sterile loop and placed into 10 ml of BHI broth medium. After 24 h of incubation at 37°C, the optical density (λ 600 nm) of the culture was adjusted to obtain a microbial suspension of approximately 1×10^5 CFU/ml. A volume of 0.1 ml was used to inoculate a sterile 96-well plate. The 96-well plate was incubated in a humid atmosphere at 37°C for 72 h to obtain a biofilm adherent to the walls of the wells.

Before proceeding to aPDT, the wells were washed three times with phosphate buffer saline (PBS) to remove the non-filmy adherent fungus.

Photodynamic treatment

The aPDT was performed using a prototype diode laser device GaAlAsat with a wavelength (λ) of 635 nm (Doctor Smile, Brendola, Italy), using a power of 0.1 mW, in continuous wave mode, with an optical fiber of 300 µm.

Irradiations were performed maintaining the fiber at the distance of 11 mm from the bottom of the wells using the fiber sheath that blocked the fiber itself in a perforated cover plate. The irradiated area corresponded to about 50 mm².

When required, the wells containing *C. albicans* biofilm were treated using the laser light for 1, 2 or 3 minutes. In these conditions, keeping constant the other parameters, three different energies of 12, 24 and 36 J, respectively, and a fluence of 24, 48 and 72 J/cm², respectively, were obtained.

Toluidine blue (TB) (Sigma-Aldrich Co. St. Louis MO-USA) was used as the photo-activating substance. A total of 50 µl of 0.1 mg/ml TB solution was added to the wells containing *C. albicans* biofilm. After 5 minutes, the TB solution was removed, and then, the laser irradiation was performed. The wells were divided into 8 groups and treated as follows:

Group 1: biofilm subjected to laser irradiation with TB for 1 minute (L1+P+)

Group 2: biofilm subjected to laser irradiation with TB for 2 minutes (L2+P+)

Group 3: biofilm subjected to laser irradiation with TB for 3 minutes (L3+P+)

Group 4: biofilm subjected to laser irradiation for 1 minute in absence of TB (L1+P-)

Group 5: biofilm subjected to laser irradiation for 2 minutes in absence of TB (L2+P-)

Group 6: biofilm subjected to laser irradiation for 3 minutes in absence of TB (L3+P-)

Group 7: biofilm exposed to TB in the absence of laser irradiation (L-P+)

Group 8: biofilm not subjected to any treatment (L-P).

After the treatment, the wells were washed once with PBS to remove the dead fungal cells.

Bio Timer Assay

BTA is an indirect method that measures the concentration of microbes in biofilm (16-21). BTA employs BioTimer medium with red phenol (BT-PR medium) prepared as follows: BHI 37 g/L, glucose 5 g/L phenol red 25 mg/L and distilled water to 1000 ml. After sterilization at 121°C for 15 minutes, the pH was checked and adjusted to 7.2±0.1. The final medium appeared clear and red. BTA measures the microbial metabolism: the time required for color switching of the phenol red indicator in BT-PR medium (red-to-yellow) (Fig. 1), due to fungal metabolism, is correlated to the initial fungal concentration. Therefore, the time required for a color switch determined the number of fungi present in a sample at Time 0 through a correla-
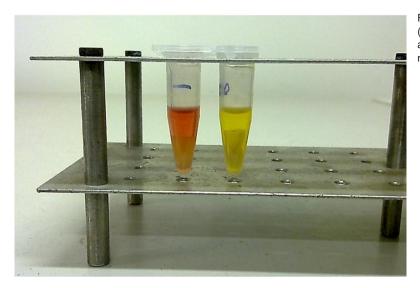
Figure 1. Reagent with phenol red (BT-PR): initial color (red on the left) and after the "switching" (yellow on the right).
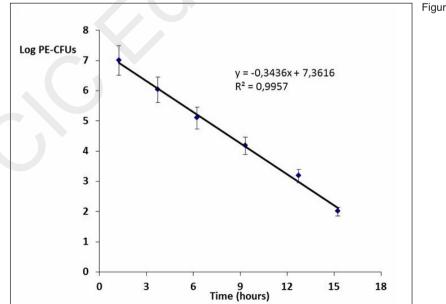
tion line. To draw the correlation line specific for *Candida* spp., 0.02 ml of BHI-overnight broth cultures were mixed with 0.18 ml of BT-PR medium. Serial two-fold dilutions in 0.1 ml of BT-PR medium were performed in 96-well plates (BD, Italy) and simultaneously counted using the colony forming unit (CFU) method. The time required for color switching of inoculated 96-well plates was recorded using Tecan Sunrise, a fully automatized spectrophotometer apparatus. The apparatus allowed the incubation at 37°C of the 96-well plates and the recording of the ODs (λ 570 and 450 nm) of each well every 15 minutes for 24 hours. The time for a color switch was plotted versus the $\log_{10}$ of CFUs to calculate the correlation line that relates the time for a color switch with the number of fungi. The correlation line was described by the

equation: $t = y = -0.3436x + 7.3616$ with $r^2 = 0.9957$ (Fig. 2).
To evaluate the number of *C. albicans* cells in the biofilm, the colonized wells were supplemented with BT-PR medium. The time required for a color switch in each well was recorded using the Tecan apparatus and it was used to determine the number of viable fungi by the correlation line. As the correlation line was constructed on planktonic cultures, the number of *Candida* in the biofilm is defined as the planktonic-equivalent CFUs (PE-CFUs).

Statistics
All experiments were repeated at least three times to obtain the mean value and standard deviation. Statistical analysis was performed using Student's T test,
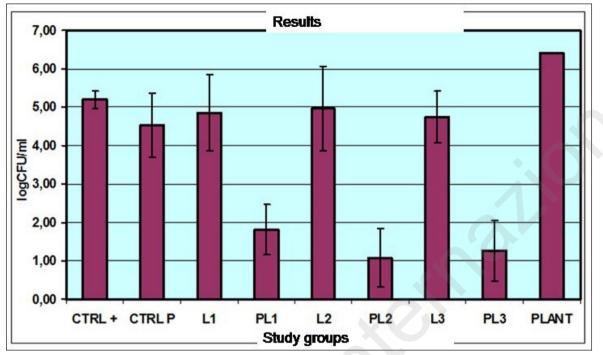

Figure 2. Correlation curve.

Figure 3. Overall results for the group in terms of logCFU/ml with relative DS.

comparing the groups by their average values, and P (probability) values lower than 0.05 (P≤0.05) were considered significant. The correlation line was obtained using linear regression analysis, and the linear correlation coefficient was calculated with the equation: $r = (n\Sigma xy - \Sigma x\Sigma y)/\{sqrt[n\Sigma x2 - (\Sigma x)2][n\Sigma y2 - (\Sigma y)2]\}$.

Results

Results are expressed as the mean value ± standard deviations of the CFU log obtained from at least three independent experiments (Fig. 3).
Laser treatments without photoactivator did not efficiently kill the *C. albicans* biofilm population, and the reduction values (of approximately 24%) were not significant according to the length of the treatment (1, 2, or 3 minutes). Similarly, the treatment with the photoactivator alone did not decrease significantly the number of CFUs.
By contrast, the aPDT treatments reduced the *C. albicans* biofilm population to different extents, depending on the time of treatment. Comparing values of the population before and after treatment, aPTD significantly reduced the number of *C. candida* cells already after 1 minute of laser irradiation. Reductions ranging from 65 to 79% were recovered, and the highest rate of reduction was observed after 2 and 3 minutes of treatment (Fig. 4). However, the differences in values of *Candida* reduction between the different times of treatments were not significant.

Discussion

C. albicans is a saprophytic fungus that is present in the oral cavity of many people (>80%), and it is responsible for superficial infections of the mucous membranes (90%) and systemic infection (44-62% of cases) (22). *C. albicans* can be found in both planktonic and biofilm lifestyles.
Increasing resistance by fungi *versus* the various conventional medical therapies, the appearance of new emerging pathogens and the antagonism of drugs in patients are the reasons that push the scientific community to look for alternative therapies.
Currently, most studies assessing the performance of anti-*Candida* laser light at low fluence (LLLT) employed cultures of fungus in the planktonic state.
The main objective of this study was to evaluate the effects of three protocols of laser irradiation against *C. albicans* grown in biofilm, and precisely, the three protocols were as follows: low level laser irradiation in association with the Toluidine Blu (TB) as photo activator (aPDT protocol), low level laser irradiation in the absence of TB (L), and only TB in the absence of any laser irradiation. TB was chosen according to Souza et al. (2010) (24), which demonstrated that employing a diode laser (Ga-Al-As) 660 nm, 0.035W with fluence of 15.8, 26.3 and 39.5 J/cm2 with 171, 285 and 428 seconds of irradiation, respectively, had the highest efficacy against planktonic *C. albicans* when using TB as a photoactivator.
In our study, a Bio Timer Assay (BTA) was used for the first time to counts of *C. albicans* biofilms. The
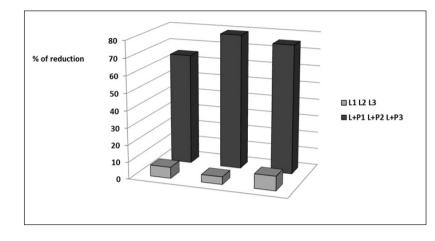
Figure 4. Percent reductions of *Candida albicans* ATCC 24433 population in biofilm after laser treatment in the absence or in the presence toluidine blue as photoactivator. *C.albicans* biofilm was treated for 1, 2, or 3 minutes with laser in the absence (L1, L2, L3) or in the presence of photoactivator toluidine blue (LP1, LP2, LP3).

method, already used for counting *Staphylococcus*, *Streptococcus* and *Enterococcus* in biofilms (19-21), is the only method that is able to count biofilm samples without any manipulation of the samples (7). For this purpose, a correlation line showing the time of color switches of the BTA medium and the number of planktonic *C. albican* cells was constructed, and the correlation line was described by a linear equation.

The results of the present study showed that laser treatment without a photoactivator was inefficient in significantly reducing the *Candida* biofilm independently of the length of treatment. These results agreed with Wilson and Mia (1993) (23), which showed that the He-Neon laser at a wavelength of 632.8 nm and 66.36 J/cm^2 of fluence in the absence of the photoactivator was unable to kill planktonic *C. albicans*. In contrast, the combined use of laser and photoactivator (i.e., methylene blue, crystal violet and thionine) reduced planktonic *C. albicans* by approximately 77%. Similar results were obtained against planktonic *C. tropicalis,* and *C. stellatoidea* whose killing rate was 65 and 63%, respectively. Similar results were reported by Queiroga et al. (2011) (25); testing the effects of irradiation with a diode laser 640 nm on planktonic *Candida* ssp using 3 different fluences, i.e., 60, 120 and 180 J/cm^2 in the presence of methylene blue, showed that the higher reduction of *Candida* was obtained at a fluence of 180 J/cm^2.

When laser treatment was carried out in the presence of the TB photoactivator, significant reductions in the *C. albicans* biofilm were observed. The duration of the treatment did not influence the killing rate, and no significant differences were recorded after 1, 2 or 3 minutes of treatment.

The efficacy of laser treatment in the presence of the photoactivator confirmed the efficacy of LLLT in combination with photosensitizing agents against planktonic *Candida*. However, Authors are focused, nowadays, on the evaluation the killing rate of laser treatment against *Candida* biofilm (26). Our results are in agreement with Pereira et al. (2010) (27). The Authors tested the effects of diode laser 660 nm at a

power of 100 mw for 98 seconds with a fluence of 350 J/cm^2 alone or in combination with methylene blue as a photoactivator on single, double and triple biofilms formed by *C. albicans*, *Sthaphylococcus aureus* and *Streptococcus mutans*. The study demonstrated a significant reduction in all species of microorganisms after the application of laser in combination with photosensitizer; in particular, monospecies biofilms were more susceptible compared to multispecies of biofilms.

However, it should be underscored that the fluence employed in the abovementioned studies on both planktonic and biofilm *Candida* were from 5.5- to 30-fold higher than the lower fluence used in the present study (24, 48 and 72 J/cm^2). In fact, our results showed that a significant reduction can be achieved even when using 24 J/cm^2 fluence and that the increase in the fluence was not able to raise the killing rate of *Candida* biofilm. The explanation for this phenomenon could be the different photoactivator concentrations, the intrinsic photoactivator properties, or even the responsiveness of the specific strain of *C. albicans*. Another possible explanation for this phenomenon could be offered assuming the peak of maximum effectiveness was reached in a time of approximately 60 seconds.

According to previous works on a murine model of oral candida infection (1, 28-30) and on the treatment of HIV positive patients with aPDT (10), this study confirmed the efficacy of photodynamic therapy against *C. albicans*.

The observation that the killing rate did not change with increases in fluence is important in the case of laser application on patients for the clinical treatment of *C. albicans* oral infections. In fact, the previous studies are applicable for clinical use, especially for the excessively long times of irradiation that are not reproducible in patients. Moreover, even if the side effects of laser irradiation were not described, putative cell damage cannot be excluded due to the duration of laser applications. Consequently, the possibility of using a true LLLT by decreasing the putative side effects may increase the compliance of the patients.

In this study, we verified the framework that emerged in the literature on the effectiveness of photodynamic protocols implemented as antifungal treatments.

Here, we showed the efficacy of aPDT in significantly reducing the fungal population of *C. albicans* ATCC 24433 grown in biofilm using a diode laser l 635 nm as a light source, already at the fluence of 24 J/cm^2.

Conclusions

Even if more studies are needed to optimize the anti-*Candida* protocol, this study suggests a possible use of aPDT in "*in vivo* infection". For this purpose, it will be necessary to carry out clinical trials that could show the real evidence for fungicidal effects of aPDT to propose this procedure as an alternative or adjuvant to traditional drug therapies currently in use for the treatment of oral infections.

Conflict of interest

The Authors declare that they have no conflict of interest.

Acknowledgements

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