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Photodynamic inactivation of *Candida* ssp. on denture stomatitis. A clinical trial involving palatal mucosa and prosthesis disinfection

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ABSTRACT

Denture stomatitis (DS) is the most common oral fungal infection in denture wearers. Photodynamic inactivation (PDI) has been showing to be an effective technique in vivo against fungi, including fungal infections in the oral cavity. The disinfection of both oral mucosa and denture may represent a real advantage in terms of fungus control. This clinical study was designed to explore methylene blue (MB)-mediated PDI on oral mucosa and prosthesis of patients with DS. Subjects with DS were divided into two groups. One group received treatment based on the use of oral miconazole gel 2% (MIC). The other group received treatment by PDI using MB at 450 μg/mL and a diode laser (λ = 660 nm) with 100 mW and fluence of 28 J/cm^2. Clinical outcome was evaluated regarding the degree of oral mucosa erythema and microbiological reduction of *Candida* spp. located in both palatal mucosa and prosthesis. Our results showed that PDI was significantly more effective than MIC in ameliorating inflammation after 15 days. Following 30 days, no statistically significant differences were observed between groups. Regarding the fungal burden, although the MIC group has presented more pronounced inactivation than PDI for both mucosa and prosthesis, no statistically significant differences were detected between them. This clinical study suggests that PDI can reduce fungal load and decrease the inflammation degree in patients with *Candida*-associated denture stomatitis.

1. Introduction

Candida-associated denture stomatitis (DS), also known as chronic atrophic candidiasis, is the most common oral fungal infection in individuals with dentures [1,2]. The etiology of DS is multifactorial and its occurrence is associated with poor adaptation of the removable denture (complete or partial), non-removal of it for long periods (e.g. during the night) and its inadequate hygiene. However, the presence of *Candida* spp. biofilm on the prosthesis is considered the most important factor for the establishment of DS [3].

Usually, DS is an infection that can be easily treated with careful cleaning of the dentures for biofilm control, removal of the prosthesis during sleep and topical or systemic antifungal drugs as polyenes and azoles [4]. In fact, miconazole (MIC) has been suggested as an effective agent for the treatment of *Candida*-DS due to its local action [5] and a recent study reported that MIC was the most effective antifungal drug, eradicating more than 98% of Candida isolates [6].

Most of the individuals affected by DS are elderly, immunosuppressed or underprivileged and often do not have access to antifungals or conditions to use the correct cleaning procedures [7]. In addition, it is known that infectious agents as bacteria, fungi, viruses, and parasites are risk factors for cancer and the role of chronic *Candida* ssp. infection in the development of some types of oral carcinoma has been investigated [8].

Candida albicans is the most common species of the *Candida* genus and it is responsible for the majority of oral candidiasis, although other species as *C. krusei*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. dubliniensis* and *C. guilliermondii* may also be involved in DS infection [9,10]. Due to the emerging fungal resistance that has been reported against the commonly used antifungal drugs and the prevalence of this infection [11], an alternative approach to treat DS is highly desirable.

Photodynamic inactivation (PDI) is a technique that has proved to be

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effective against fungi, including *Candida* spp. resistant to drugs [12], without causing damage to healthy tissues [13]. In this therapy, the cell death occurs when the photosensitizer absorbs the energy of the light source and transfers charge to environmental molecules or energy to oxygen. This leads to the production of highly reactive oxygen species, which, in turn, kill the microorganism through oxidative stress [14,15].

Recently, a review concluded that the clinical effectiveness of PDI as a potent therapeutic approach for oral fungal infections requires further studies. In fact, different light parameters and different photosensitizer types and concentrations are reported. This situation makes difficult to reach a consensus over the protocols to be used to effectively eradicate *Candida* spp. from oral cavity [16]. Particularly for DS, a few clinical trials are reported and, in those studies, only patient signal and symptoms were evaluated [17–20]. However, it has also been showed that PDI is an effective method for disinfecting dentures [21], diminishing the need for different antimicrobial agents that could increase the cost of the therapy and the microbial resistance selection. It is important to highlight that the minimum inhibitory concentration of fluconazole for *C. albicans* decreased following sublethal MB-mediated PDI [22] and, therefore, PDI could also be used as an adjuvant therapy decreasing the amount of antifungal needed for microbial eradication.

In this work, our purpose was to identify the most prevalent *Candida* spp. associated with DS in underprivileged patients from Tocantins state, located in the central zone of Brazil, an area that has characteristics of the Amazon Basin, and semi-open pastures, known as cerrado. Besides, we aimed to evaluate the effects of methylene blue (MB)-mediated PDI on both oral cavity and dentures, and compare it with the public service pharmacological protocol used in the region.

2. Material and methods

2.1. Ethical approval

This study was approved by the Ethics Committee for Research in Tropical Medicine Foundation of Tocantins (Brazil) and all participants received and signed written informed consents. Participants were given the right to withdraw from the study at any time without dental care or legal rights being affected.

2.2. Recruitment and eligibility criteria

Individuals aged between 40 and 65 years, users of removable upper complete dentures with a clinical diagnosis of DS characterized by complaints of burning sensation on the mucosa, loss of filiform papillae, and varying degrees of erythema, were selected among those who sought treatment at the ITPAC College of Dentistry in Araguaína city, state of Tocantins, Brazil. Exclusion criteria included non-detection of *Candida* spp. in the prosthesis, diagnosis of diabetes or acquired immune deficiency syndrome (AIDS), cancer, pregnancy, history of antimicrobial use in the past two months, illness during treatment (flu and common cold) and nonattendance in more than one clinical session during the therapy.

2.3. Candida spp. sampling

The occurrence of *Candida* spp. was determined by microbiological culture of the material obtained from the palatal mucosa and the prosthesis [23,24]. The swabs were incubated at 37 °C for 24 h for colony counting and species identification. This procedure was performed before starting treatment and 48 h after its end. For the presumptive identification of species, primary culture medium CHROMagar Candida® was employed and color, texture, and morphology were evaluated [25,26]. Additionally, the colonies were stained by Gram method. In samples that developed a greenish coloration, the test of growth at 45 °C in Sabouraud dextrose agar was carried out for differentiation between *C. albicans* and *C. dubliniensis* [25].

2.4. PDI and MIC treatments

To evaluate the antifungal effect of PDI on *Candida* spp. in palatal mucosa and dentures, the subjects were randomly assigned into two groups. Firstly, the prostheses were washed with water and placed over absorbent paper.

PDI Group - The surface of the prosthesis in contact with the palatal mucosa was measured using a graph paper. The individuals with DS had the prosthesis and palatal mucosa stained for ten minutes (pre-irradiation time) with a solution of MB at a concentration of 450 μg/mL applied using a cotton swab [27]. Then, prosthesis and mucosa were irradiated by a GaAlAs diode laser emitting at λ = 660 nm, 100 mW power (DMC, São Carlos, Brazil), continuous mode, by scanning the entire prosthesis and palatal mucosa. The energy density was standardized at 28 J/cm^2 and the exposure time was calculated according to equation $D = \frac{P \times t}{A}$, where *D* is the energy density (dose, J/cm^2), P is the laser output power (W), t is the exposure time (s) and A is the irradiated area (cm^2), which varied for each patient. Thus, we used t = 280 s per cm^2 according to the palatal mucosa and prosthesis area. The PDI was applied twice a week, with an interval of at least 48 h among the sessions during four weeks. All procedures above mentioned were performed in the dental clinic.

MIC Group - Subjects were instructed to perform the treatment based on the antifungal agent miconazole oral gel 2%. The drug was applied on the inner surface of the denture and the entire palatal surface covered by the prosthesis three times a day during a month. The denture and the palatal surface were dried before the use of MIC. The patients were also instructed to leave the dentures in a glass of water overnight. The subjects received guidance in the dental clinic, but performed the procedures at home. They were clinically evaluated every 48 h.

2.5. Clinical and microbiological evaluation

To quantify the clinical response of oral mucosa to the treatments, the degree of erythema was classified according to the index proposed by Budtz-Jorgensen et al. [28], which comprises four levels: 0 – no inflammation, 1 – mild inflammation, 2 – moderate inflammation and 3 – severe inflammation. This classification was made before, 15 and 30 days after treatment for both groups by two calibrated dentists.

The evaluation of the microbiological response was made by the method proposed by Olsen [29]. The colony forming units (CFU) recovered from both mucosa and prosthesis were counted and the result was expressed in degrees of density: 0 – no growth, 1 – growth from 1 to 9 CFU; 2 – growth from 10 to 24 CFU, 3 – growth from 25 to 100 CFU, 4 – growth greater than 100 CFU, 5 – confluent growth. This evaluation was carried out before and 30 days after treatment.

2.6. Statistical analysis

The Shapiro-Wilk test was used to verify the normal distribution of data. The Friedman test was used to compare groups regarding the degree of erythema. For microbiological analysis of mucosa and prosthesis, we used the Wilcoxon test for comparison within the groups, and the Fisher's exact test for comparison between groups. Statistically significant differences were established at $p < 0.05$.

3. Results

Sixty-one patients fulfilled the inclusion criteria and thirty-six individuals completed this study. Thus, PDI and MIC groups had 18 subjects with mean age of 58.1 ± 6 and 54.7 ± 7 yrs, respectively ($p = 0.08$). Regarding gender, each group had 17 women and one man. As smokers were not excluded, MIC had one while PDI group had three smokers.

Table 1
Prevalence of species of *Candida* genus into denture and mucosa of the subjects enrolled in this study.

Specie	Denture occurrence	Mucosa occurrence
C. albicans	50 (53.8%)	32 (59.2%)
C. glabrata	29 (31.2%)	13 (24.1%)
C. tropicalis	6 (6.4%)	5 (9.3%)
Candida sp.	5 (5.4%)	3 (5.6%)
C. krusei	2 (2.1%)	1 (1.8%)
C. dubliniensis	1 (1.1%)	0 (0%)
Total	93 (100%)	54 (100%)

3.1. Occurrence of Candida spp

The most prevalent species identified before treatments in the denture and in the mucosa were *C. albicans* (53.8% and 59.2%, respectively) *C. glabrata* (31.2% and 24.1%, respectively) and *C. tropicalis* (6.4% and 9.3%, respectively) (Table 1). Colonization by more than one species of *Candida* was observed in 31 cases for prosthesis and in 15 cases for mucosa. Mixed infection by *C. albicans* and *C. glabrata* was the most prevalent for both denture and mucosa (27.9% and 13.1%, respectively, Table 2)

3.2. Clinical evaluation of erythema

On the first clinical evaluation, the patients presented a mean score of 2.6 and 2.4 (PDI and MIC groups, respectively) with no statistically significant differences detected ($p > 0.05$). Fifteen days after treatments, only PDI group significantly reduced the degree of erythema ($p < 0.001$, Fig. 1). In fact, PDI group showed a significant reduction of about 62% (score mean value 2.6 → 1) compared to MIC group, in which the degree of erythema dropped about 33% (score mean value 2.4 → 1.6). Following 30 days, both treatments significantly diminished the mucosa inflammation in about 94% (final score mean value 0.78 and 0.72 for PDI and MIC groups, respectively, $p < 0.001$) with no statistically significant difference between treatments.

3.3. Microbiological evaluation

From 18 subjects who received pharmacological treatment with MIC, 4 did not present fungal growth in samples from the palatal mucosa, but all of them showed growth of colonies of *Candida* spp. in the cultures of the prosthesis before starting treatment. Thirty days after treatment, all patients diminished the fungal load in both mucosa and denture when compared to initial evaluation. In addition, 15 and 10 individuals reduced the fungal burden to zero in mucosa and denture, respectively ($p < 0.0001$) (Fig. 2A).

From 18 subjects who received PDI, 5 did not show fungal growth in samples from the palatal mucosa before treatment. The other thirteen individuals presented positive fungal growth before therapy and, among them, 9 reduced to zero the level of CFU in mucosa after treatment ($p < 0.05$). Regarding the prosthesis, all patients who received PDI showed growth of colonies before treatment. Among them, 6 reduced to zero the fungal load at the end of treatment ($p < 0.001$) (Fig. 2B).

Fig. 3 exhibits the overall mean score of *Candida* spp. growth in both mucosa and denture for MIC and PDI groups. Before treatments, no statistically significant differences were observed between MIC and PDI groups for palatal mucosa (1.89 and 1.94, respectively), and prosthesis (3.78 for both groups) ($p > 0.05$). Thirty-days after treatments, MIC and PDI groups showed a significant reduction of *Candida* spp. growth in mucosa (91% and 43%, respectively) and in prosthesis (76% and 54%, respectively) ($p < 0.01$). Between groups, no statistically significant differences were noticed ($p > 0.05$).

Table 2
Predominant *Candida* spp. in simple and mixed infections in mucosa and dentures of the patients enrolled in this work. NG: Samples with no growth.

Species	Denture occurrence	Mucosa occurrence
C. albicans	22 (36%)	16 (26.3%)
C. glabrata	4 (6.6%)	3 (4.9%)
C. albicans + *C. glabrata*	17 (27.9%)	8 (13.1%)
C. albicans + *C. tropicalis*	4 (6.6%)	2 (3.3%)
C. albicans + *Candida* sp.	3 (4.9%)	2 (3.3%)
C. albicans + *C. krusei*	1 (1.6%)	1 (1.6%)
C. glabrata + *C. dubliniensis*	1 (1.6%)	0 (0%)
C. glabrata + *C. tropicalis*	2 (3.3%)	0 (0%)
C. albicans + *C. glabrata* + *Candida* sp.	2 (3.3%)	0 (0%)
C. albicans + *C. glabrata* + *C.krusei*	1 (1.6%)	0 (0%)
C. albicans + *C. glabrata* + *C. tropicalis*	0 (0%)	2 (3.3%)
C. albicans + *C. tropicalis* + *Candida* sp.	0 (0%)	1 (1.6%)
NG	4 (6.6%)	26 (42.6%)
Total	61 (100%)	61 (100%)

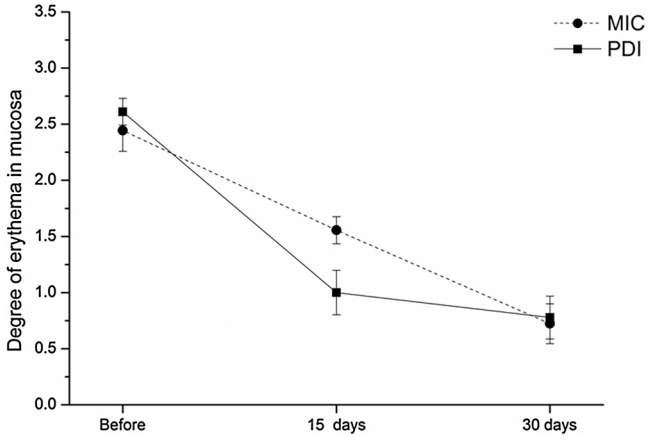

Fig. 1. Means ± standard error of the degree of erythema in the mucosa for individuals of MIC and PDI groups.

4. Discussion

In this work, we identified the most prevalent *Candida* spp. in patients who sought treatment in a public oral health care facility from a central area of Brazil and evaluated the effects of MB-mediated PDI compared to MIC treatment on patients with diagnosis of *Candida*-associated DS.

Firstly, we verified that the prevalence of *Candida* ssp. found in our study does not differ from that reported in literature: *C. albicans, C. glabrata* and *C. tropicalis* were the most prevalent species for simple infections [1–3]. We also verified that for mixed infections, *C. albicans* and *C. glabrata* were the most prevalent ones as described by other authors [30,31]. On the hand, we did not observe any sample from the mucosa with confluent growth while dentures of 16 patients exhibited confluent growth. Apparently, the presence of *Candida* spp. in the mucosa is transitory and the colonization of the denture is more important for the establishment of infection. This finding ratifies that the principal site of colonization by *Candida* spp. in DS-patients is the denture [24].

Secondly, we noticed that PDI was as effective as MIC in reducing fungal burden in prosthesis and oral cavity 30 days after treatment. Remarkably, PDI was able to reduce mucosa inflammation faster than MIC. In fact, PDI promoted significant decrease of the erythema degree 15 days after the initiation of treatment. It is important to point out that, before treatment, all patients showed mild to severe inflammation, which may impair the quality of daily life.

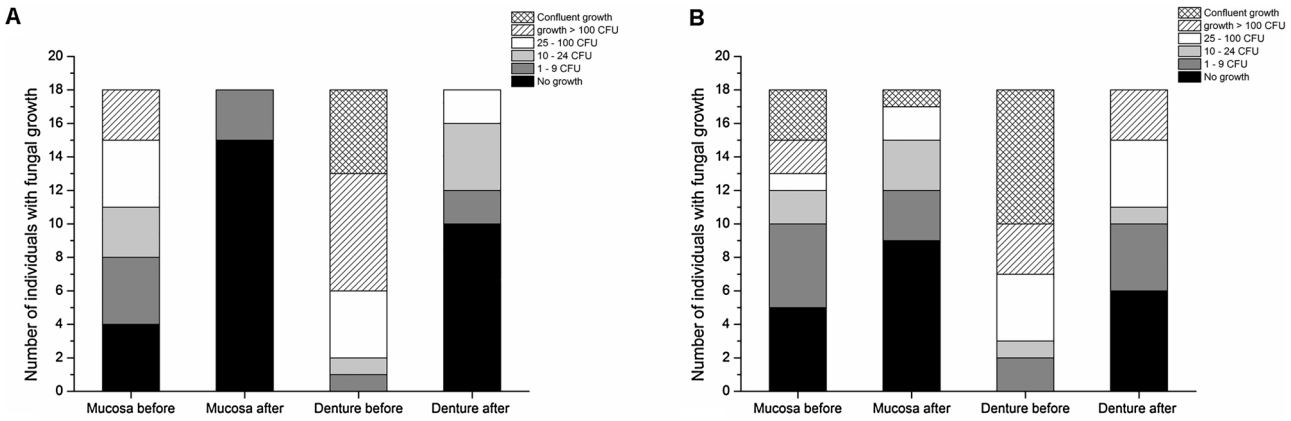

Fig. 2. Number of individuals per group with *Candida* spp. growth in mucosa and denture submitted to MIC (A) and PDI (B) according to Olsen scale before and 30 days following treatments.

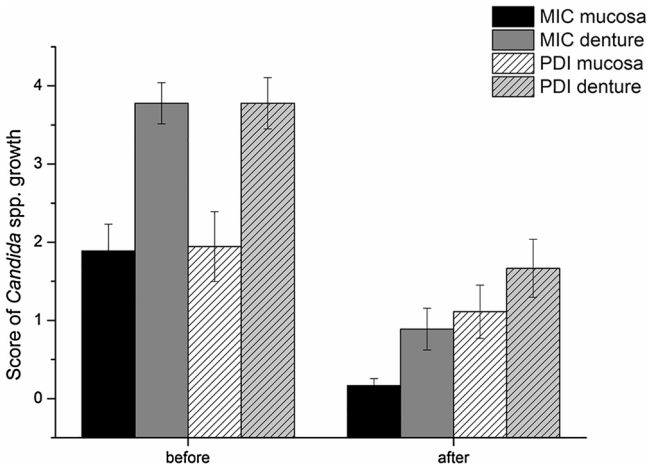

Fig. 3. Mean score ± standard error of *Candida* spp. growth in mucosa and denture submitted to MIC and PDI before and 30 days following treatments.

The clinical effectiveness of PDI for the treatment of DS has already been reported in literature. Mima et al. used 500 μg/mL of Photogem®, a porphyrin-based photosensitizer, and after 30 min of pre-irradiation time, the denture and the palate were irradiated by a blue light emitting-diode at $\lambda = 455$ nm delivering 37.5 and 122 J/cm^2, respectively, three times a week for 15 days. In that study, PDI was as effective as nystatin [18]. In contrast, Maciel and coworkers used 100 μg/mL of MB, pre-irradiation time of 5 min and the lesion area was illuminated by a red laser emitting at $\lambda = 660$ nm delivering 1 J/cm^2 in a single session. Their results showed that MIC promoted better outcome compared to PDI [19].

While conventional antifungal agents have well established clinical protocol, PDI is still in early clinical development. In this work, we chose MB since it is a good cost- benefit photosensitizer frequently associated to red light in PDI for oral infections [32–34]. It is worth noting that we used a higher MB concentration and a higher light fluence than Maciel et al., since our parameters were based on Scwingel's work [27]. Also, we performed more than one session and irradiated both oral cavity and denture as reported by Mima et al. [18]. Another interesting feature to be emphasized is that we used the same fluence for denture and mucosa. We assumed that the employment of the same parameter is an easier way to simplify PDI dosimetry. Furthermore, the denture covers the palatal mucosa and, therefore, both areas should be quite similar. We showed in this study that the same parameter can be used with success in the two sites.

An important remark is that smokers were not excluded in our trial. In fact, PDI group had 3 smokers while MIC group had one due to randomization of the study. We decided to maintain them since smokers are very prone to oral candidiasis. In fact, tobacco use influences *Candida* infection [35] and impairs tissue repair [36]. Those 4 patients presented mixed infection, i. e., more than one *Candida* species in mucosa and denture. Interestingly, inflammation decreased more quickly in patients from PDI group, while the reduction of fungal load was more pronounced in patients from MIC group. Thus, we hypothesize that the effectiveness of PDI to inactivate fungi may be compromised in patients with habitual use of tobacco. In fact, a recent study showed that PDI promoted oral fungal inactivation among cigarette smokers and non-smokers, but at 3-months follow up, the fungal load was significantly higher in smokers compared to non-smokers [37]. Tobacco use is an important variable to be addressed regarding fungal infection and the fact that PDI ameliorates the inflammation even with the continuous use of tobacco deserves deeper investigation.

In summary, we conclude that MB-mediated PDI in more than one session could be applied in patients with *Candida*-associated denture stomatitis. PDI was able to reduce mucosa inflammation before MIC and this is an important issue to improve the quality of daily life and avoid the use of anti-inflammatory medications. PDI was also able to reduce fungal burden in oral mucosa and prosthesis as MIC. Our study reinforces the use of PDI in dental clinics and encourages further clinical studies to enhance the compliance and the outcomes of the therapy.

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