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Photodynamic inactivation of virulence factors of *Candida* strains isolated from patients with denture stomatitis

Cristiane Aparecida Pereira *, Nádia Domingues, Michelle Peneluppi Silva, Anna Carolina Borges Pereira Costa, Juliana Campos Junqueira, Antonio Olavo Cardoso Jorge

UNESP, Univ Estadual Paulista, Institute of Science and Technology, School of Dentistry, Department of Biosciences and Oral Diagnosis, Francisco José Longo 777, São Dimas, São José dos Campos, CEP: 12245-000 SP, Brazil

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ABSTRACT

Candida species are major microorganisms isolated in denture stomatitis (DS), an inflammatory process of the mucosa underlying removable dental prostheses, and express a variety of virulence factors that can increase their pathogenicity. The potential of Photodynamic inactivation (PDI) in planktonic culture, biofilms and virulence factors of *Candida* strains was evaluated. A total of 48 clinical *Candida* isolates from individuals wearing removable maxillary prostheses with DS were included in the study. The effects of erythrosine (ER, 200 μM) and a green LED (λ 532 ± 10 nm, 237 mW/cm^2 and 42.63 J/cm^2) in a planktonic culture were evaluated. The effect of the addition of ER at a concentration of 400 μM together with a green LED was evaluated in biofilms. The virulence factors of all of the *Candida* strains were evaluated before and after the PDI process in cells derived from biofilm and planktonic assays. All of the *Candida* species were susceptible to ER and green LED. However, the biofilm structures were more resistant to PDI than the planktonic cultures. PDI also promoted slight reductions in most of the virulence factors of *C. albicans* and some of the *Candida tropicalis* strains. These results suggest that the addition of PDI is effective for reducing yeasts and may also reduce the virulence of certain *Candida* species and decrease their pathogenicity.

1. Introduction

Yeasts of the genus *Candida* are the most common cause of fungus infection of the oral cavity. Additionally, *Candida*-associated denture stomatitis (DS), an inflammatory process of the mucosa underlying a removable dental prosthesis, is the most frequent manifestation of oral candidiasis among denture wearers [1].

The colonization and infection by yeasts of the *Candida* genus are mediated by the formation of a biofilm, which is composed of a heterogeneous mixture of blastoconidia, pseudohyphae and hyphae embedded in extracellular polymeric substances that exhibit different phenotypic characteristics compared with planktonic *Candida*. The extracellular matrix is composed of polysaccharides, proteins, hexosamine, uronic acid, and DNA to promote biofilm adhesion and formation, protect the cells from phagocytosis, maintain the integrity of the biofilm and limit the diffusion of substances [2]. The close proximity of the biofilm and extrapolymeric matrix to the oral mucosa induces localized innate inflammatory responses, accounting for erythema and other clinical symptoms of DS [3]. Biofilm formation is a fundamental characteristic of *Candida* in the oral cavity, and its capacity to attach to dentures has been shown to maintain the retention of and provide protection to these strains within the oral environment [4].

Candidal biofilms are clinically problematic because they are intrinsically resistant to antimicrobial agents [5]. In addition, other virulence characteristics of *Candida albicans*, such as adhesion (adhesion to mucosal cells), dimorphism (ability to convert a single-celled yeast form to a filamentous form), and the secretion of hydrolytic extracellular enzymes (which are utilized in the process of host tissue invasion and nutrient liberation), makes *C. albicans* a pathogen that causes a broad spectrum of infections in different host sites [6]. Following *C. albicans*, *Candida glabrata* and *Candida tropicalis* are also the most frequently isolated species in DS [7]. *C. tropicalis* has been identified as the most prevalent pathogenic yeast species of the *Candida*-non-*albicans* group [8]. *C. glabrata* is often the second or third most common cause of candidiasis after *C. albicans*. Another species is *Candida dubliniensis*, which is closely related to *C. albicans* in evolutionary terms, and both have characteristics of commensalism and opportunistic infection [9].

Previous studies have evaluated the effects of different concentrations of antimicrobials on biofilms and have demonstrated that an increase in antifungal resistance is associated with progression through several stages of biofilm formation [10,11]. The reduction in the susceptibility of *Candida* spp. strains to antifungal agents due to an increase in

* Corresponding author.
E-mail addresses: cricabio@gmail.com (C.A. Pereira), nadiamfe@yahoo.com.br (N. Domingues), mipeneluppi@ig.com.br (M.P. Silva), carol_biolog@yahoo.com.br (A.C.B.P. Costa), juliana@fosjc.unesp.br (J.C. Junqueira), olavojorge@fosjc.unesp.br (A.O.C. Jorge).

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the survival of immunosuppressed patients and the overuse of antimicrobial drugs has resulted in an increased interest in the development of new therapeutic strategies [12].

Photodynamic inactivation (PDI) is the interaction between light and certain photoactive compounds, known as photosensitizers (PSs), which are used to inactivate cell functions [13]. When a PS is irradiated with light of an appropriate wavelength and at a certain level, the molecule becomes excited and consequently experiences a series of molecular energy transfers. These energy transfers lead to the production of cytotoxic products, including singlet oxygen and free radicals [14]. These products are capable of damaging essential components of the cells or modifying metabolic activities in an irreversible manner, which may result in cell death [15]. The main PSs used in antifungal PDI are phenothiazine dyes, phthalocyanines, and porphyrins associated with lasers and other non-coherent light sources. Erythrosine (ER) has attracted interest as a PS because it is not toxic to the host and has already been tested experimentally against oral biofilms [16]. Light-emitting diodes (LEDs) have been described as a promising light source for use in place of lasers because they are characterized by wider emission bands, are smaller in size, weigh less, cost less, exhibit greater flexibility in terms of the irradiation time that can be used in treatment and are easy to operate [17].

In this study, we examined the effect of PDI using exogenous PS, ER and a green LED in planktonic culture, biofilms, and virulence factors of *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. tropicalis* strains.

2. Materials and methods

2.1. Microorganisms

A total of 48 clinical *Candida* isolates (20 of *C. albicans*, 12 of *C. glabrata*, 12 of *C. tropicalis*, and 4 of *C. dubliniensis*) from individuals wearing removable maxillary prostheses with DS were included in the study. Samples from each individual were collected in a previous study [18] from the hard palate mucosa and the prosthesis-fitting surface by swabs and from mouth rinses in phosphate-buffered saline (PBS, 0.1 M, pH 7.2) for 1 min. The strains were collected and identified using phenotypic and biochemical tests (API®, bioMérieux, Marcy-I'Etoile, France). The existence of *C. dubliniensis* among the isolates was analyzed through multiplex polymerase chain reaction (PCR) assays. This study was approved by the local ethics committee (protocol number n 012/2010-PH/CEP) and was undertaken with written informed consent provided by each subject.

2.2. PS and Irradiation Source

ER (Sigma Aldrich) was used for the sensitization of yeasts. The PS solution was prepared by dissolving the powdered dye in PBS, filtered through a sterile 0.22-μm filter and stored in the dark before use. The absorption spectrum (400–800 nm) of the ER solution (1.0 mM in PBS) was verified with a spectrophotometer (Cary 50 Bio, Varian Inc., Palo Alto, CA, USA) coupled to a microcomputer. ER at concentrations of 200 μM and 400 μM was used for the sensitization of planktonic cultures and biofilms, respectively.

A green light-emitting diode (LED) (MMOptics, São Carlos, SP, Brazil) was used as the light source with a wavelength of 532 ± 10 nm, an output power of 90 mW, 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 the planktonic cultures and biofilms was 0.38 cm^2. The temperature at the bottom of the 96-well microtiter plates was monitored using an infrared thermometer (MX4, Raytek, Sorocaba, SP, Brazil), and no increases in temperature were observed after irradiation with the LED.

The irradiation of planktonic cultures and biofilms was performed in the dark under aseptic conditions in a laminar flow hood. During irradiation, the plates were covered with a black matte screen with an orifice that was the same size as the wells.

2.3. PDI in Planktonic Cultures

Candida strains were seeded onto Sabouraud dextrose agar (SDA) and incubated at 37 °C for 24 h. The microorganisms were then harvested in Sabouraud dextrose broth (SDB) and incubated at 37 °C for 16 h. The microbial growth in the broth was centrifuged at 358 ×*g* for 10 min and washed twice with PBS. The sediments were resuspended in PBS. Standardized suspensions of each strain were then prepared at a concentration of 10^6 cells/ml with an optical density (OD) of 0.284 in PBS using a spectrophotometer (B582, Micronal, São Paulo, SP, Brazil) set to 530 nm.

The assays were divided into four experimental groups for each *Candida* species: treatment with ER at a concentration of 200 μM and LED irradiation (P+L+, n = 10); treatment with ER at a concentration of 200 μM only (P+L−, n = 10); LED irradiation (P−L+, n = 10); and control group, treated with PBS only (P−L−, n = 10).

A 0.1-ml aliquot of the standardized suspension of each strain was added to each well of a 96-well flat-bottom microtiter plate. The P+L+ and P+L− assay groups received 0.1 ml of each concentration of the ER solution, whereas the P−L+ and P−L− assay groups received 0.1 ml of PBS. The plate was then shaken for 5 min (pre-irradiation) in an orbital shaker (Solab, Piracicaba, SP, Brazil). The wells containing the P+L+ and P−L+ assay groups were irradiated according to the protocol described. After irradiation, yeast suspensions were serially diluted in PBS to yield dilutions of 10^{-1} to 10^{-5} of the original concentration, and aliquots of 0.1 ml seeded in duplicate onto SDA plates were incubated at 37 °C for 48 h. The yeast colonies were counted, and the number of colony-forming units (CFU/ml) was determined.

2.4. PDI Studies on Biofilms

For biofilm growth, we used the Calgary Biofilm Device system (CBD — Innovotech Inc., Calgary, Canada) according to the methodology proposed by Parahitiyama et al. [19] with some modifications. This system consists of a two-part reaction vessel. The top component forms a lid with 96 identical pegs and a base formed of a microtiter plate.

Cultures of *Candida* strains that were grown on SDA at 37 °C for 18 h were harvested in yeast nitrogen base (YNB) supplemented with 50 mM glucose. After 18 h of incubation at 37 °C, the yeasts were centrifuged at 358 ×*g* for 10 min and washed twice with PBS. The pellets were resuspended in broth used for the production of biofilms [20]. The broth contains 20 g of trypticase soy broth, 2 g of NaCl, 3 g of K_2HPO_4, 2 g of KH_2PO_4, 1 g of K_2CO_3, 120 mg of $MgSO_4$, 15 mg of $MnSO_4$, and 50 g of $C_6H_8O_7$ dissolved in 1000 ml of distilled water and sterilized by autoclaving at 121 °C for 15 min. The numbers of viable cells in each suspension were counted using a spectrophotometer (B582, Micronal, São Paulo, Brazil). The optical density and wavelength used were 0.381 and 530 nm (10^7 cells/ml), respectively.

A 200-μl aliquot of each suspension was pipetted into each well of the base of the microtiter plate, and the lid with pegs was placed. The plate was incubated for 48 h at 37 °C in a 3D shaker (GyroTwister™ 3-D Shaker, Labnet, NJ, USA). The broth was changed after 24 h. The plates with biofilms formed by *Candida* species were then washed with 250 μl of PBS to remove the loosely adhered cells.

The biofilm formed by each strain was immersed in 250 μl of a solution of 400 μM ER for 5 min (pre-irradiation time) in an orbital shaker. Subsequently, the suspended plates were irradiated according to the protocol described (P+L+, n = 10). The control group, which was treated with PBS in the absence of light (P−L−, n = 10), was also evaluated.

After the treatments, the biofilm cells were dispersed from the pegs into Falcon tubes containing 10 ml of PBS and homogenized for 30 s