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ORIGINAL ARTICLE

Photodynamic inactivation in the expression of the *Candida albicans* genes ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1 in biofilms

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Abstract
The objective of this study was to evaluate the effects of photodynamic inactivation (PDI) on *Candida albicans* biofilms, evaluating its effects on gene expression of ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1 by yeast. Three samples of *C. albicans* were used in this study: a clinical sample from a patient with HIV (39S), a clinical sample from a patient with denture stomatitis lesion (Ca30), and a standard strain ATCC 18804. The quantification of gene expression was related to the production of those genes in the samples referred above using quantitative polymerase chain reaction (qPCR) assay in real time. The photosensitizer methylene blue at 300 uM and erythrosine at 400 uM, sensitized with low-power laser (visible red, 660 nm) and green LED (532 nm), respectively, were used for PDI. Four groups of each sample and PDI protocol were evaluated: (a) P+L+: sensitization with the photosensitizer and irradiation with light, (b) P+L−: only treatment with the photosensitizer, (c) P−L+: only irradiation with light, and (d) P−L−: without sensitization with the dye and absence of light. The results were analyzed by *t* test, with a significance level of 5%. The photodynamic inactivation was able to reduce the expression of all genes for both treatments, laser and LED. The fold-decrease for the genes ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1 were 0.73, 0.39, 0.77, 0.71, 0.67, and 0.60 for laser, respectively, and 0.66, 0.61, .050, 0.43, 0.54, and 0.66 for LED, respectively. It could be concluded that PDI showed a reduction in the expression of *C. albicans* genes, suggesting its virulence decrease.

Keywords Biofilms · Virulence factors · Real-time PCR · Photodynamic inactivation · *Candida albicans*

Introduction

Species of fungi belonging to *Candida* genus, being *Candida albicans* the most prevalent species in candidosis and candidemia worldwide, are one of the main causes of opportunistic infections [1, 2]. This trend has been observed over the past decade and is still the case, even in developed countries such as the USA, Denmark, Norway, and Finland [1]. *C. albicans* is a human commensal microorganism and can colonize the skin, mucosal surfaces, gastrointestinal tract, and female genital-urinary tract [3]. An important factor that contributes to the pathogenesis of candidosis is the formation of biofilm, and *C. albicans* forms biofilm on inert and biological surfaces [4].

The occurrence of denture stomatitis (DS) is also associated with the presence of *C. albicans* biofilms [5, 6]. Denture stomatitis is a common inflammatory reaction of multifactorial etiology, usually associated with *Candida* species, particularly *C. albicans*, due to its high virulence, ability to adhere and to form biofilms in tissues of the oral cavity and in total prosthesis surfaces [7].

Martins et al. [8] isolated and determined the incidence of *Candida* species in oral prosthesis. The collection was done in 66 patients and *C. albicans* was the most isolated species of microorganism (63%).

Oral candidosis is the most common fungal manifestation in AIDS patients [9]. It is reported that 84–100% of HIV-positive individuals will develop at least one episode of *Candida* spp. colonization and above 90% will develop symptomatic pseudomembranous candidosis. Candidosis is an indicator of the progression of HIV infection, revealing the patient's immunological failure,

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since specific cellular immunity plays an important role in the prevention of oral candidosis [10–12].

In a study conducted by Maheshwari et al. [13], in a total of 128 *Candida* isolates, obtained from 88 HIV-positive patients, 7 different species were identified and *C. albicans* was the most commonly isolated (50%).

With the increase of opportunistic infections caused by the *Candida* genus in immunocompromised patients and consequent emergence of conventional antifungal resistant strains, new treatment options for oral candidosis are required in order to improve the therapeutic arsenal. In order to reduce the incidence of infection by *Candida* yeast in the oral cavity, photodynamic inactivation (PDI) has been reported, which presents application as a therapeutic option for the treatment of infectious diseases, including oral candidosis [14–18].

In this study, the action of PDI in gene expression of TEC1 (transcription factor), HWP1 (cell wall protein hyphae), EFG1 (transcriptional regulator related to morphogenesis), BCR1 (regulator of biofilm formation and cell wall), CPH1 (transcriptional regulator involved in morphogenesis), and ALS3 (adhesin) in clinical samples from patients with denture stomatitis and HIV-positive patients and in a standard strain was evaluated. The importance of analyzing clinical isolates is because they may differ in virulence factors. Therefore, a detailed characterization of virulence factors of *C. albicans* is required not only for a more detailed understanding of the infection process but also for the generation of new and more effective antifungal compounds [19].

No studies associating the expression of *C. albicans* genes related to adherence, hyphae, and biofilm formation (ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1) after photodynamic inactivation were found. PDI may be an option or adjuvant treatment for antimicrobial chemotherapy, improving the treatment of oral infections, increasing efficacy and reducing their cost. In this study, the effects of photosensitization using methylene blue and low power laser and erythrosine sensitized with green LED on gene expression of *C. albicans* biofilm were evaluated.

Material and methods

Samples

Three samples of *C. albicans* were used in this study: a clinical sample from a patient with HIV (39S) of the Institute of Infectology Emílio Ribas [20], a clinical sample from a patient with denture stomatitis lesion (Ca30) of the São Paulo State University (Unesp) [21], and a standard strain ATCC 18804.

Samples were grown in a chromogenic HiCrome *Candida* medium (Himedia, Mumbai, India) and subsequently confirmed via molecular methods (PCR Multiplex). The research project for the collection of clinical samples was approved by the Ethics Committee of the Institute of Infectology Emílio Ribas, São Paulo, Brazil (274/2009) and the Ethics Committee of Institute of the São Paulo State University (Unesp), Institute of Science and Technology (012/2010-PH/CEP).

The clinical samples were selected after a screening of 60 samples, 30 from HIV-positive patients and 30 from patients with denture stomatitis lesions. After the phenotypic analysis of the capacity of biofilm and hyphae formation, the two most virulent samples of each group were selected for the PDI application. Besides that, the standard strain was also included in the experiments with PDI.

In vitro biofilm formation and photodynamic inactivation

The methods described by Seneviratne et al. [22] and Costa et al. [23] were used with some modifications for the biofilm formation. The biofilms were formed on 24-well flat-bottom plates (Costar Corning, New York, EUA). The strains stored at − 75 °C were activated on Sabouraud dextrose agar (Himedia Laboratories Pvt. Ltd., India) containing chloramphenicol (Alamar Tecno Científica Ltda.) and maintained in a constant temperature (37 °C) for 24 h. Then, a *C. albicans* inoculum was prepared in yeast nitrogen base (YNB; Himedia) broth supplemented with 100 mM glucose (diluted ten times in sterile distilled water) and maintained in an incubator for 16 h at 37 °C. After this period, the inoculum was washed twice with sterile 0.9% NaCl, and standardized suspensions prepared in YNB broth (10×) were made in a spectrophotometer containing 10^7 cells/ml (AJX-1900, Micronal, São Paulo, Brazil). For the initial adherence of the biofilms, 1 ml of the yeast suspension was added to each well, and the plates were incubated for 1.5 h at 37 °C under shaking at 75 rpm (Quimis, Diadema, Brazil). Then, the planktonic cells were gently aspirated and each well was washed twice with 1 ml of sterile 0.9% NaCl saline. After that, 1 ml of YNB broth supplemented with 100 mM of glucose (10×) was pipetted, and the plates were incubated at 37 °C under shaking at 75 rpm (Quimis, Diadema, Brazil) for 48 h. The YNB broth was changed after 24 h.

Eight experimental groups (4 for LASER and 4 for LED) were evaluated for each sample: (A) P+L+: sensitization with the dye and irradiation with light, (B) P+L−: only treatment with the photosensitizer, (C) P−L+: only irradiation with light, and (D) P−L−: without sensitization with the dye and absence of light. The groups A and B received 1 ml of the dye solution, and the groups C and D received 1 ml of sterile 0.9% NaCl saline. The plates were shaken for 5 min (pre-irradiation time) in an orbital shaker (Solab, Piracicaba, Brazil). Next, the groups A and C were irradiated according to the protocol described in the item 2.3.

Photosensitizers and light sources

Two groups were used for PDI. In the first group, the methylene blue dye (MB) (Sigma-Aldrich, Steinheim, Germany) was sensitized with red visible laser InGaAlP (Indium-Gallium-Aluminum-Phosphorous) (D.M.C. Equipamentos Ltda, São Carlos, SP, Brasil). In the second group, green light emitting diode (LED) (MMOptics, São Carlos, Brazil) was used as a light source and erythrosine (ER) (Aldrich Chemical Co., Milwaukee, WI) as a photosensitizer.

The methylene blue dye had a concentration of 300 μM and erythrosine of 400 μM. Both were prepared each time before use, by dissolving the powder in distilled water and filtered through a 0.2-μm filter membrane (Millipore, São Paulo, Brazil). After filtration, the dye solution was stored in the dark.

The InGaAlP laser emitted light at 660 nm (visible red). The wavelength of the laser corresponds to the maximum absorption of the methylene blue dye. The parameters used were measured on the Thorlabs® PM100D optical power meter equipped with a S130C (0.7 cm^2) photodiode sensor: power of 50 mW, total energy of 15 J, irradiation time of 300 s, power density of 71.42 mW/cm^2, and energy density of 21.42 J/cm^2. The laser tip was placed at a distance of 1.75 cm in each well of the 24-well plate next to a frosted black screen to prevent light scattering. The emission mode of the laser was continuous.

The green LED had a wavelength of 532 ± 10 nm. The erythrosine dye absorbs visible light in the 500–550 nm range. The parameters used were measured on the PM100D Thorlabs® optical power meter equipped with a S130C (0.7 cm^2) photodiode sensor and are exactly the same as the laser, allowing a better comparison between the two light sources. The LED tip was placed over the cap in each well of the 24-well plate next to a frosted black screen to prevent light scattering.

Irradiation of biofilms was performed under aseptic conditions in a laminar flow hood and light off.

Quantitative real-time polymerase chain reaction

After biofilm irradiation, total RNA was isolated with TRIzol reagent (Ambion, Inc., Carlsbad, CA, USA) as recommended by the manufacturer. The total RNA extracted (1 μg) was treated with DNase I (Turbo DNase Treatment and Removal Reagents - Ambion Inc., Carlsbad, CA, USA) and transcribed into complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR Kit (InvitrogenTM, Carlsbad, CA, USA), according to the protocols recommended by the manufacturer.

The primers for the genes analyzed in the present study were described and used as indicated by the authors in Table 1.

Transcribed cDNAs were amplified for the relative quantification of ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1 gene expression levels in relation to the concentration of a reference gene. In this study, four reference genes, ACT1, LSC2, RIP1, and PMA1, were tested in all experimental groups. The results were analyzed by the website http://www.leonxie.com/referencegene.phpe, which uses four different methods: Delta CT [24], BestKeeper [25], NormFinder [26], and Genorm [27].

Quantitative real-time PCR was conducted using the Platinum® SYBR® Green qPCR SuperMix-UDG Kit (Applied Biosystems, Framingham, MA, USA) in the StepOnePlus™ apparatus (Applied Biosystems, Framingham, MA, USA). The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression from the quantitative RT-qPCR experiment [28].

Analysis of results

The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in expression of the ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1 genes in the qRT-PCR experiments [28] using Graph Pad Prism 6 Program (GraphPad Software, Inc., CA, USA), *t* test, with a significance level of 5%.

Results

Efficiency evaluation of the primers used in this study

All primers used in this experiment had an efficiency between 90 and 110%. It was sought to observe in all curves, efficiency ~ 100%, slope ~− 3.32 and correlation coefficient ≥ 0.99. These data are presented in Table 2.

Selection of the reference gene for use in photodynamic inactivation using LASER with methylene blue and LED with erythrosine

The reference gene is used to normalize differences in the amount of cDNA that is placed in the PCR reaction wells and the levels of normalizing gene expression should be similar across all 3 different samples (ATCC 18804, 39S, and Ca30 clinical samples), in the 4 experimental groups (P−L−, P+L−, P−L+, and P+L+) tested. Through the website http://www.leonxie.com/referencegene.php and methods Delta CT, BestKeeper, NormFinder, and Genorm were chosen the best reference genes for the study of photodynamic inactivation using laser with methylene blue and LED with erythrosine, as described by Silver et al. [24], Pfaffl et al. [25], Andersen et al. [26], and Vandesompele et al. [27]. The genes that presented the lowest score and variation among the samples for each treatment were RIP1 and LSC2 for red laser and green LED, respectively (supplementary data).

Table 1 *Candida albicans* genes which expression were evaluated by PDI

Gene	Localization	Function	Reference
ACT1	orf19.5007	Structural integrity of the cellular cytoskeleton (reference gene)	Nailis et al. [29]
LSC2	orf19.1860	Protein present in the B-subunit cells of Succinyl CoA synthetase (reference gene)	Nailis et al. [29]
RIP1	orf19.5893	Ubiquinol-cytochrome c reductase enzyme (reference gene)	Nailis et al. [29]
PMA1	orf19.5383	Plasma membrane ATPase (reference gene)	Nailis et al. [29]
TEC1	orf19.5908	Transcription Factor	Hnisz et al. [30]
HWP1	orf19.1321	Hyphal cell wall protein	Hnisz et al. [30]
EFG1	orf19.610	Main transcriptional regulator involved in morphogenesis	Hnisz et al. [30]
BCR1	orf19.723	Regulates biofilm formation and cell wall	Finkel et al. [31]
CPH1	orf19.4433	Transcriptional factor and regulates the formation of pseudohyphia	Mait et al. [32]
ALS3	orf19.1816	Adesin-adherence to the host cell	Nailis et al. [33]

Effect of PDI using methylene blue with LASER and erythrosine with LED acting on ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1 genes

To elucidate the mechanisms involved in the treatment with PDI, the quantification of *C. albicans* genes related to adhesion, biofilm formation, and morphogenesis by qPCR (Figs. 1, 2, and 3) was evaluated. The presence of asterisks indicates statistical difference among the groups.

All analyzed genes were downregulated after PDI (group P+L+) and the groups P−L+ and P+L− did not demonstrated down or upregulation when compared with the group P−L−. The fold-decrease and *p* values for the genes ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1 are described in the Table 3.

The comparison between the groups P+L+ for LASER and LED was made and no difference statistical was found. This comparison was made using Graph Pad Prism 6, *t* test, with a significance level of 5%, comparing each gene separately (ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1) in the two protocols tested (LASER and LED).

Table 2 Slope values, correlation, and efficiency of the *C. albicans* sample ATCC 18804

Primer	Slope	Correlation (R^2)	Efficiency (%)
ACT1	− 3.32	0.991	100
LSC2	− 3.45	0.995	92.3
RIP1	− 3.32	0.998	100
PMA1	− 3.25	0.998	103
TEC1	− 3.23	0.992	103.94
HWP1	− 3.42	0.992	96
EFG1	− 3.44	0.993	95.4
BCR1	− 3.25	0.998	102.7
ALS3	− 3.33	0.999	99.64
CPH1	− 3.34	0.997	100

Discussion

C. albicans presents specific mechanisms for the development of disease that overcome host defenses and allow colonization of mucosal tissue. The expression of its virulence factors may vary depending on the type of infection, local or systemic, stage of disease, and host response [34]. It is known that the virulence of *C. albicans* is linked to its intrinsic factors, which contribute to the development of candidosis. The main virulence factors include the expression of adhesins on the surface

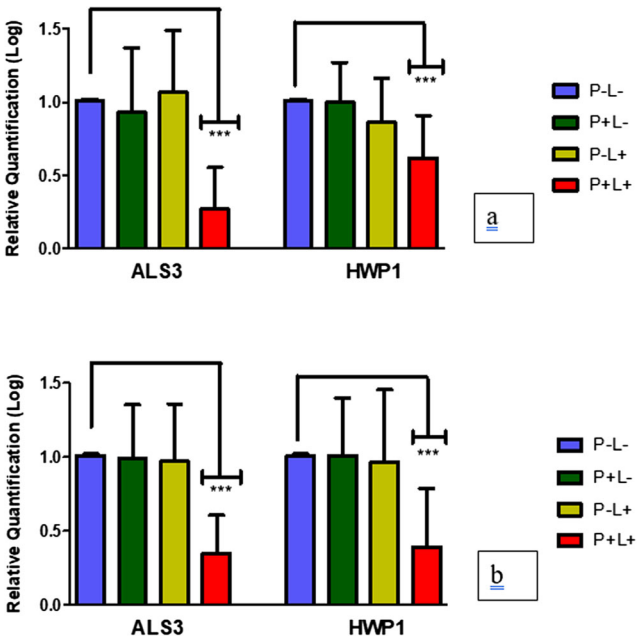

Fig. 1 Relative quantification (Log) of the expression of the adherence genes (ALS3 and HWP1) in control group (P−L−) by quantitative real-time PCR (qPCR) in relation to the groups P+L−, P−L+, and P+L+ using laser and methylene blue (Fig. 1a) and LED with erythrosine (Fig. 1b) for PDI. Values are expressed as the means and standard deviation. *T* test was used to compare gene expression. Graph Pad Prism 6 Program was used to create the artwork and the statistics. The presence of asterisks indicates $p \leq 0.05$

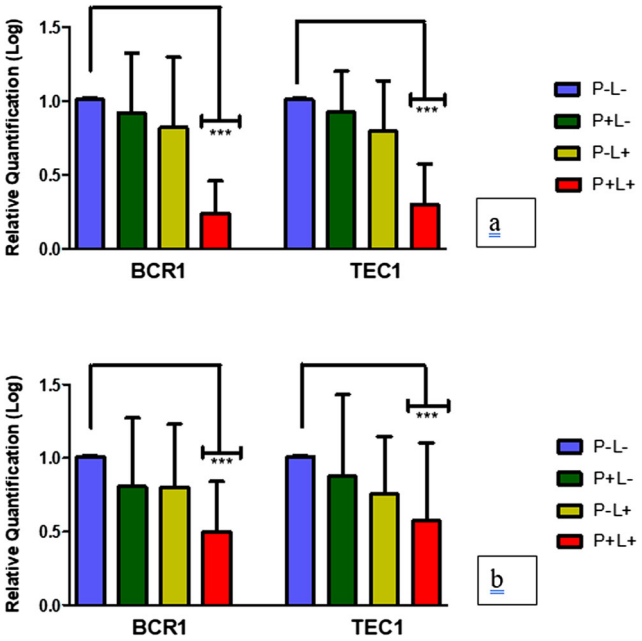

Fig. 2 Relative quantification (Log) of the expression of the biofilm formation genes (BCR1 and TEC1) in control group (P−L−) by quantitative real-time PCR (qPCR) in relation to the groups P+L−, P −L+, and P+L+ using laser and methylene blue (Fig. 2a) and LED with erythrosine (Fig. 2b) for PDI. Values are expressed as the means and standard deviation. *T* test was used to compare gene expression. Graph Pad Prism 6 Program was used to create the artwork and the statistics. The presence of asterisks indicates $p \leq 0.05$

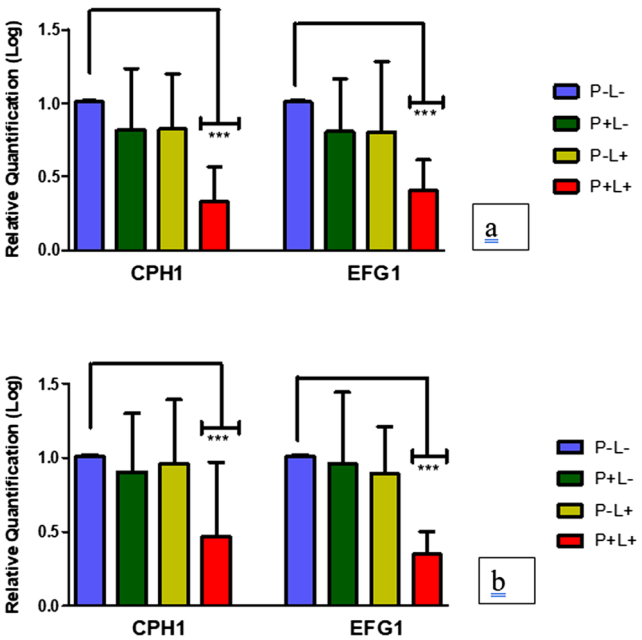

Fig. 3 Relative quantification (Log) of the expression of the genes related to morphogenesis (CPH1 and EFG1) in control group (P−L−) by quantitative real-time PCR (qPCR) in relation to the groups P+L−, P −L+, and P+L+ using laser and methylene blue (Fig. 3a) and LED with erythrosine (Fig. 3b) for PDI. Values are expressed as the means and standard deviation. *T* test was used to compare gene expression. Graph Pad Prism 6 Program was used to create the artwork and the statistics. The presence of asterisks indicates $p \leq 0.05$

Table 3 Fold-decrease and *p* values for the genes ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1 after treatment with PDI

Genes	Fold-decrease and *p* values for laser	Fold-decrease and *p* values for LED
ALS3	0.73, $p = 0.0005$	0.66, $p < 0.0001$
HWP1	0.39, $p < 0.0001$	0.61, $p = 0.0001$
BCR1	0.77, $p < 0.0001$	0.50, $p = 0.0002$
TEC1	0.71, $p < 0.0001$	0.43, $p = 0.0182$
CPH1	0.67, $p < 0.0001$	0.54, $p = 0.0031$
EFG1	0.60, $p < 0.0001$	0.66, $p < 0.0001$

of the cell, the morphological transformation capacity of yeasts to hyphae, tigmotropism (the growth in relation to mechanical stimulus), formation of biofilms, phenotypic changes in relation to the colonized site, mechanisms of interference with the host defense system, and the secretion of hydrolytic enzymes [35, 36].

The target genes chosen for this study were ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1, related to adhesion and with hyphae and biofilm formation, important virulence factors as described above. The genes EFG1 and TEC1 are transcription factors and are related to morphogenesis, which form a regulating circuit of hyphal differentiation [30]. CPH1 is a transcription factor and regulates the morphogenesis of *C. albicans* [32]. The BCR1 gene is among the major biofilm regulators [37]. Previous studies have indicated that its ALS1-3 and HWP1 target adhesins mediate cell-cell interaction in biofilms [38], but studies by Finkel et al. [31] observed that BCR1, through ALS1, also regulates cell-substrate adhesion. BCR1 target genes are believed to be induced in the development of hyphae [39]. Because of these characteristics, these genes were chosen for the investigation of their expression after PDI.

Normalization is an essential component for a reliable PCR assay because this process controls the variations in extraction yield, reverse transcription, and amplification efficiency, thus allowing comparisons between mRNA concentration in different samples. The use of reference genes as internal controls is the most common method for normalizing PCR reactions [40]. However, although the use of reference genes is accepted as the most adequate standardization [41], it is impossible to find a "universal" reference gene under all different conditions to which the cells or tissues can be submitted in various types of experiments [42, 43]. Therefore, the present study investigated which is the best normalizing gene (ACT1, LSC2, RIP1, or PMA1) for this particular experiment to analyze the gene expression of ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1 after PDI.

The website http://www.leonxie.com/referencegene.php was used to choose the most stable reference genes. This website compiled 4 different software, Delta CT, BestKeeper, NormFinder, and Genorm, according to the

following authors, respectively: Silver et al. [24]; Pfaffl et al. [25]; Andersen et al. [26], and Vandesompele et al. [27]. According to this website, the most stable reference genes for this study were RIP1 and LSC2 for treatments with red laser and green LED, respectively.

Photodynamic inactivation using methylene blue and erythrosine has been reported as a new option for reducing *Candida* yeasts. Freire et al. [18] evaluated the effects of PDI on oral candidosis in immunosuppressed mice by bioluminescence imaging. Methylene blue (MB) and new methylene blue (NMB) were combined with or without potassium iodide (KI) and red laser (660 nm) with 4 different light doses (10, 20, 40, and 60 J). The best in vitro biofilm reductions in CFU/ml were MB plus KI with 40 J (2.31 logs) and NMB without KI with 60 J (1.77 log). These conditions were chosen for in vivo treatment in an experimental model of oral candidosis in mice. After 5 days of treatment, the disease was practically eradicated, especially using MB plus KI with 40 J.

Costa et al. [44] used LED as light source and rose bengal and erythrosine as photosensitizers for PDI in planktonic cultures and biofilms of *C. albicans*. The results showed a significant reduction in planktonic cultures (1.97 log and 3.45 logs) and in biofilms (< 1 log) for PDI performed with rose bengal and erythrosine, respectively.

Because of studies like these, we wanted to investigate what happens with the expression of genes related to important virulence factors like adhesion and formation of hyphae and biofilms by *C. albicans*. Only one study was found in the literature that relates gene expression after PDI. Freire et al. [45] demonstrated that PDI using low-power gallium-aluminum-arsenide laser and methylene blue downregulated the expression of SAP5, LIP9, and PLB2 genes in some, not for all, clinical samples of *C. albicans* from HIV-positive patients. The decrease in the production of these hydrolytic enzymes should decrease the virulence of the yeasts, acting on their penetration and invasiveness in tissues. In the present study, all genes, ALS3, HWP1, BCR1, TEC1, CPH1, and EFG1, were downregulated after the treatment with PDI, with fold-decrease of 0.73, 0.39, 0.77, 0.71, 0.67, and 0.60, for laser, respectively, and 0.66, 0.61, .050, 0.43, 0.54, and 0.66, for LED, respectively, indicating that the treatment actually interfered in the gene expression.

Conclusion

Judging by the results obtained in this study, both methylene blue with laser and erythrosine with LED represent two potential antifungal strategies to treat oral candidiasis, since they downregulated the expression of important virulence factors of *C. albicans*, suggesting its virulence decrease.

Authors' contribution The manuscript in its submitted form has been read and approved by all authors. All authors have contributed significantly to this research, and are in agreement with the content of manuscript. The contributions of the authors with individuals responsibility are listed below:

Fernanda Freire: conception, design of the experiments, interpretation of data, and redaction of the manuscript

Patrícia Pimentel de Barros: conception, design of the experiments, interpretation of data, and redaction of the manuscript

Cristiane Aparecida Pereira: conception, design of the experiments, interpretation of data, and redaction of the manuscript

Juliana Campos Junqueira: conception, interpretation of data, and redaction of the manuscript

Antonio Olavo Cardoso Jorge: conception, interpretation of data, and redaction of the manuscript

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Compliance with ethical standards

Ethical approval The research project for the collection of clinical samples was approved by the Ethics Committee of the Institute of Infectology Emílio Ribas, São Paulo, Brazil (274/2009) [20] and the Ethics Committee of Institute of the São Paulo State University (Unesp), Institute of Science and Technology (012/2010-PH / CEP) [21].

Conflict of interest The authors declare that they have no conflicts of interest.

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