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ORIGINAL ARTICLE

Photodynamic inactivation of biofilms formed by *Candida* spp., *Trichosporon mucoides*, and *Kodamaea ohmeri* by cationic nanoemulsion of zinc 2,9,16,23-tetrakis (phenylthio)-29H, 31H-phthalocyanine (ZnPc)

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Abstract The biofilms formed by opportunistic yeasts serve as a persistent reservoir of infection and impair the treatment of fungal diseases. The aim of this study was to evaluate photodynamic inactivation (PDI) of biofilms formed by *Candida* spp. and the emerging pathogens *Trichosporon mucoides* and *Kodamaea ohmeri* by a cationic nanoemulsion of zinc 2,9,16,23-tetrakis(phenylthio)-29H,31H-phthalocyanine (ZnPc). Biofilms formed by yeasts after 48 h in the bottom of 96-well microtiter plates were treated with the photosensitizer (ZnPc) and a GaAlAs laser (26.3 J cm^{-2}). The biofilm cells were scraped off the well wall, homogenized, and seeded onto Sabouraud dextrose agar plates that were then incubated at 37°C for 48 h. Efficient PDI of biofilms was verified by counting colony-forming units (CFU/ml), and the data were submitted to analysis of variance and the Tukey test ($p<0.05$). All biofilms studied were susceptible to PDI with statistically significant differences. The strains of *Candida* genus were more resistant to PDI than emerging pathogens *T. mucoides* and *K. ohmeri*. A mean reduction of 0.45 log was achieved for *Candida* spp. biofilms, and a reduction of 0.85 and 0.84, were achieved for biofilms formed by *T. mucoides* and *K. ohmeri*, respectively. Therefore, PDI by treatment with nanostructured formulations cationic zinc 2,9,16,23- tetrakis (phenylthio)-29H, 31H- phthalocyanine (ZnPc) and a laser reduced the number of cells in the biofilms formed by strains of *C. albicans* and non-*Candida albicans* as well the emerging pathogens *T. mucoides* and *K. ohmeri*.

Keywords Biofilm · *Candida* spp. · *Trichosporon mucoides* · *Kodamaea ohmeri* · Photodynamic inactivation · Zinc phthalocyanine

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Introduction

Candida albicans is the most common species isolated from the oral cavity, followed by *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei*. However, other non-*Candida albicans* emerging species have arisen, such as *C. dubliniensis*, *C. lusitaniae*, *C. rugosa*, *C. norvegensis*, *C. kefy*, *C. lypolitica*, and *C. guilliermondii*, due to a growing population of immunosuppressed patients and the use of broad-spectrum antibiotics [1, 2]. Yeasts from the *Candida* genus are responsible for the majority of fungal infections in immunocompromised patients owing to the use of immunosuppressive drugs and characteristics of the individual, such as advanced age and

systemic diseases. The oral cavity is the most common site of infection [3, 4].

The treatment of diseases caused by yeast in the *Candida* genus in immunocompromised patients is problematic because the yeasts have become increasingly resistant to antifungal agents. Schelenz et al. [2] isolated 266 oral yeasts of the *Candida* genus from patients with hematological malignancies and head and neck solid tumors and found that 28.2% of the isolates were resistant and 23.7% were susceptible dose dependant (SDD) to fluconazole, itraconazole, ketoconazole, voriconazole, and caspofungin. The majority of these resistant strains were *C. glabrata* and *C. krusei*. Besides these species, *C. lusitaniae* can also develop secondary resistance to amphotericin B, and another emerging pathogen, *C. dubliniensis*, acquires secondary resistance to fluconazole more quickly than *C. albicans* [5, 6].

Candida species are able to organize into complex communities known as biofilms, which are composed of blastoconidia, pseudohyphae, and hyphae, and are embedded in a matrix of extracellular polymeric substances (EPS) that form channels and pores [7]. It is currently estimated that approximately 65% of all treated infections are associated with microbial biofilm formation on the surfaces of tissues, organs or medical devices [8]. Biofilm formation is the biggest obstacle to effective treatment of infections caused by *Candida*, because these structures exhibit different phenotypic characteristics than their planktonic counterparts, such as reduced susceptibility to antifungal agents and protection from the immune system [9].

In addition, other yeast genera have been identified from immunocompromised hosts with poor prognoses. For example, *Trichosporon* is an emerging yeast pathogen capable of causing invasive disease in patients with hematological malignancies [10, 11]. *T. asahii* is the species most often implicated in deep-seated infections, like trichosporonosis, in immunocompromised and HIV-positive patients, followed by *T. mucoides*, *T. inkin*, *T. asteroids*, *T. cutaneum*, and *T. ovoides* [11, 12]. The best therapy for invasive trichosporonosis is undefined, and little data is available on effective treatments. In the literature, there have been reports of reduced susceptibility to amphotericin B and antifungal activity of fluconazole; however, in vitro observations of fluconazole action do not have a strong correlation with good clinical outcomes [10, 13]. Furthermore, *Trichosporon* is capable of forming complex biofilms similar to *Candida* with intrinsic resistance to the antifungals amphotericin B, caspofungin, fluconazole, and voriconazole [14].

Kodamaea ohmeri, an ascosporogenous yeast and a teleomorph of *Candida guilliermondii* var. *membranaefaciens*, is an extremely uncommon human pathogenic yeast [15]. *K. ohmeri* is an environmental strain commonly used in the food industry for the fermentation of pickles, rinds, and fruit [16]. It causes invasive opportunistic infections in immunocompromised hosts and is often associated with the presence of implanted devices. It also has developed resistance to azoles and a reduced susceptibility to caspofungin [16–19]. *K. ohmeri* has already been isolated from HIV-positive patients with pseudomembranous oral candidiasis and from diabetic and hemodialyzed users of dental prostheses [20, 21].

In the future, the biggest challenge will be to choose a suitable antifungal agent that can be used to treat diseases caused by emerging pathogens. Photodynamic inactivation (PDI) has been used to successfully inactivate yeasts that are either sensitive or resistant to antifungal agents [22, 23]. PDI consists of irradiating a photosensitive, non-toxic agent with a light source of suitable wavelength in the presence of oxygen to produce reactive oxygen species (ROS) or free radicals that damage cells [24, 25]. The photosensitive agents used include hematoporphyrin derivatives, phenothiazines (toluidine blue and methylene blue), cyanines, phytotherapeutic agents, xanthenes, and phthalocyanines [26].

Phthalocyanine photosensitizers seem to be a promising alternative for PDI application in dentistry. They are characterized by far-red wavelength absorption (> 670 nm), long triplet lifetime (~1 ms), high quantum yields of singlet oxygens (> 0.2), and low dark toxicity [28–30]. The main advantage in the use of phthalocyanine as photosensitizer is the fact that this family of dyes could act by the two classical PDI mechanisms of radical production (type I) or singlet oxygen (type II) [31]. These photosensitizers are more effective in ROS generation after light activation and have less capacity to stain dental structures compared to the traditional phenothiazine molecules [27].

Phthalocyanines can be modified to have a positive or negative charge. The charge of the photosensitizer is an important factor in determining its effective photodynamic action and whether cells uptake it. Gram-negative bacteria and fungi are resistant to anionic and neutral phthalocyanines [28, 29]. In this study, the photodynamic action of a nanostructured formulation cationic zinc 2,9,16,23-tetrakis (phenylthio)-29H,31H-phthalocyanine (ZnPc) was used for inactivating biofilms of different *Candida* species, *T. mucoides*, and *K. ohmeri*.

Materials and methods

Yeasts strains

The study was approved by the Ethics Committee of the School of Dentistry of São José dos Campos, UNESP (protocol 051/2009-PH/CEP).

Thirty-nine yeast strains isolated from the oral cavity of HIV-positive patients were used, including ten *C. albicans*, seven *C. glabrata*, six *C. tropicalis*, four *C. dubliniensis*, four *C. parapsilosis*, two *C. norvegensis*, two *C. krusei*, one *C. lusitaniae*, one *C. guilliermondii*, one *T. mucoides*, and one *K. ohmeri*. These strains were obtained from the Laboratory of Microbiology and Immunology, School of Dentistry of São José dos Campos, UNESP.

Photosensitizer and light source

ZnPc (Sigma-Aldrich Chemical Co., Milwaukee, WI, USA) was used for sensitization of the yeast strains. This photosensitizer was sterilized by filtration through 0.22-μm pore membranes (MFS, Dublin, CA, USA).

ZnPc was entrapped into cationic nanoemulsions (NE) to controlled drug release and optimal biological target. NE are advanced drug-delivery systems obtained from pharmaceutical nanotechnology applied on topical and transdermical administration of active compounds. In this work, we carried out a highly stable NE that was obtained from spontaneous emulsification as described by Primo et al. [32] by a direct mixture of organic phase containing the lecithin soy/ZnPc/oily and aqueous phase containing the biopolymer/emulsifier to final drug concentration at 0.25 mg/ml. The nanomaterial was prepared under aseptic conditions being able to biological assays. Dynamic light-scattering analyzes were carried out and showed a medium particle size of 194.3 (±5.7) nm, polydispersity index (PdI) of 0.19 (±0.04) and zeta potential at +72.1 (±5.9) mV. The particle size, PdI and zeta potential were monitored until 90 days showed appropriated physical-chemistry stability according to the required to development of drug nanocarriers.

The light source used was a gallium-aluminum-arsenide (GaAlAs) laser (Easy Laser, Clean Line, Taubaté, SP, Brazil) with a wavelength of 660 nm, output power of 0.035 W, and illuminated area of 0.38 cm^2. A fluence of 26.3 J cm^{-2} (energy of 10 J and time of 285 s) and a fluence rate of 92 $mWcm^{-2}$ were used.

Photodynamic inactivation of biofilms formed by yeasts

The methodology described by Jin et al. [33] was used for biofilm growth, with some modifications. Cultures of yeasts that were grown on Sabouraud dextrose (Himedia, Mumbai, Maharashtra, India) agar at 37°C for 18 h were harvested in yeast nitrogen base (YNB, Himedia) supplemented with 100 mM glucose (Vetec, Duque de Caxias, RJ, Brazil). After an 18-h incubation at 37°C, the yeasts were centrifuged at 358×g for 10 min, washed twice with PBS, resuspended in YNB supplemented with 100 mM glucose, and adjusted to an optical density of 0.38 at 530 nm (10^7 cells/ml) using a spectrophotometer (B582, Micronal, São Paulo, SP, Brazil).

A 100-μl aliquot of each suspension was pipetted into each well of a 96-well flat-bottom microtiter plate (Costar Corning, New York, NY, USA). The plate was incubated for 1.5 h at 37°C in a shaker at 75 rpm (Quimis, Diadema, SP, Brazil) for the initial adhesion phase. After this period, the wells were washed with 100 μl of PBS to remove loosely adhered cells. A 250-μl aliquot of YNB with 100 mM glucose was then pipetted into each of the washed wells, and the plates were incubated at 37°C in a shaker at 75 rpm for 48 h. The broth was changed every 24 h. The plates containing biofilms formed by yeasts were then washed with 250 μl of PBS to remove loosely adhered cells.

The biofilm formed by each strain was immersed in 250 μl of a solution of ZnPc for 5 min (pre-irradiation time) in an orbital shaker (Solab, Piracicaba, SP, Brazil). Subsequently, the suspended plates were irradiated according to the protocol described (P+L+, $n=3$). The effects of exposure to phtalocyanine photosensitizer alone (P+L-, $n=3$), the light source alone (P-L+, $n=3$), or to neither (P-L-, $n=3$); treated with PBS in the absence of light were evaluated as well.

After the treatments, the cells in the biofilm were scraped off the well wall using a sterile toothpick and were transferred to Falcon tubes containing 10 ml of PBS. To disrupt the biofilms, the contents of the tubes were homogenized for 30 s using an ultrasonic homogenizer (Sonoplus HD 2200; Bandelin Electronic, Berlim, Brandenburg, Germany) with an output power of 50 W. The solutions in the Falcon tubes were considered to be a dilution factor of 10^{-1}. Serial dilutions were then made using each original 10^{-1} dilution, and aliquots of 0.1 ml were seeded onto Sabouraud dextrose agar plates that were then incubated at 37°C for 48 h. After the incubation period, the number of colony-forming units (CFU/ml) on each plate was determined.

Irradiation of the biofilms was performed under aseptic conditions in a laminar flow hood in the dark. During irradiation, the plates were covered with a black matte screen with an opening the same size as the wells to prevent the spread of light to neighboring wells.

Statistical analysis

For statistical analysis, the species were classified as *C. albicans*, non-*albicans Candida* species, and non-*Candida* species. The data for CFU/ml were converted to logarithmic form and subjected to analysis of variance and the Tukey test. A p value<0.05 was considered statistically significant.

The percentage of CFU/ml reduction for *C. albicans*, non-*albicans Candida* species, and non-*Candida* species was calculated, considering the groups P+L-, P-L+, and P+L+ in relation to the control group (P-L-).

Results

Table 1 shows the values of CFU/ml obtained in the P-L-, P+L-, P-L+, and P+L+ groups for the biofilms of each species studied. CFU/ml values for all species were reduced by PDI (P+L+) compared to the control group (P-L-). The greatest reductions were observed for *T. mucoides* (0.85 log) and *K. ohmeri* (0.84 log), followed by *C. tropicalis* (0.64 log), *C. guilliermondii* (0.59 log), *C. dubliniensis* (0.53 log), *C. parapsilosis* (0.51 log), *C. lusitaniae* (0.38 log), *C. norvegensis* (0.37 log), *C. krusei* (0,36 log), *C. albicans* (0.35 log), and *C. glabrata* (0.33 log).

Figures 1, 2, and 3 show the results obtained by a comparison among P-L-, P+L-, P-L+, and P+L+ groups for the strains of *C. albicans*, non-*albicans Candida* species, and non-*Candida* species. The treatments with photosensitizer (P+L-) and laser (P-L+) alone demonstrated cytotoxic effects, gauged by a reduction of CFU/ml, for all biofilms studied, but statistically significant differences were observed for *C. albicans* and non-*Candida* species in the P+L- group, and for all species in the P-L+ group, compared with the control group (P-L-). PDI (P+L+) resulted in statistically significant differences for all species in relation to the control group (P-L-).

The percentages of CFU/ml reduction for the P+L-, P-L+, P+L+ groups compared to the control group (P-L-) are shown in Fig. 4.

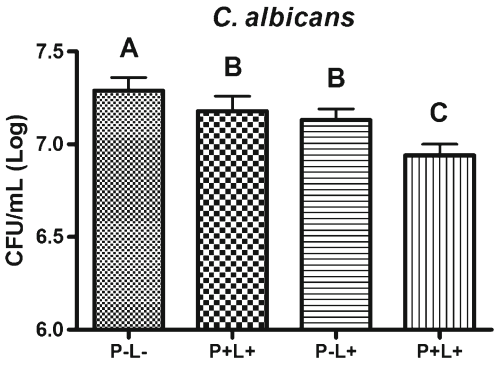

Fig. 1 Mean CFU/ml (log) and standard deviations for the strains of *C. albicans* exposed to different experimental treatments: PBS as control (P-L-); photosensitizer (P+L-); laser and PBS (P-L+); and laser and photosensitizer (P+L+). *Different letters* (A, B, and C) represent statistically significant differences among the groups (Tukey test, $p<0.05$)

Discussion

The first step in the formation of yeast biofilms is the adherence of cells to a host surface, like epithelial, endothelial or indwelling medical devices, and development of blastoconidia into distinct microcolonies, leading to the formation of a biofilm embedded in a matrix of EPS. Once established, yeasts biofilms serve as persistent reservoirs of infection and, in addition, confer greater resistance to antifungal agents and protection against host immune defenses [7, 14, 34, 35]. In this study, we evaluated the effects of photodynamic inactivation mediated by cationic nanoemulsion of zinc 2,9,16,23-tetrakis(phenylthio)-29H,31H-phthalocyanine on biofilms formed by *Candida* spp., *T. mucoides*, and *K. ohmeri.* The effects of the isolated photosensitizer (P+L-) and light source (P-L+) were evaluated as well.

Exposure to the photosensitizer in the dark (P+L-) had a cytotoxic effect on the species studied, with significant differences between the biofilms made by *C. albicans* and non-*Candida* species. The reduction relative to the control group was lower (< 0.18 log) than that found by Mantareva et al. [28]. This group treated biofilms with tetrakis-(3-methyl-pyridyloxy)- and tetrakis-(4-sulfophenoxy)-phthalocyanine zinc(II) at a concentration of 1.5 and 6 μM in the dark and observed cytotoxic effects with a reduction of less than 0.50 log for the microorganisms *C. albicans*, *Staphylococcus aureus*, and *Escherichia coli*.

Treatment with the laser alone (P-L+) in the present study also showed an antifungal effect against all biofilms with significant reduction for the species of *C. albicans,* non-

Table 1 Mean CFU/ml (log) and standard deviations for the biofilms of *C. albicans*, non-*albicans Candida* species, and non-*Candida* species submitted to different experimental treatments ($n=3$): PBS as control (P-L-); photosensitizer (P+L-); laser and PBS (P-L+); and laser and photosensitizer (P+L+)

Yeasts	Strains	P-L-	P+L-	P-L+	P+L+
C. albicans	*C. albicans* (10)	7.30±0.07	7.18±0.08	7.13±0.07	6.95±0.07
Non-*albicans Candida* species	*C. glabrata* (7)	7.68±0.12	7.50±0.21	7.54±0.19	7.35±0.24
	C. tropicalis (6)	7.35±0.07	7.26±0.08	7.08±0.03	6.71±0.14
	C. dubliniensis (4)	7.42±0.26	7.33±0.21	7.09±0.22	6.89±0.23
	C. parapsilosis (4)	7.46±0.04	7.32±0.08	7.19±0.09	6.95±0.06
	C. norvegensis (2)	7.32±0.08	7.23±0.11	7.15±0.09	6.95±0.08
	C. krusei (2)	7.39±0.04	7.30±0.02	7.14±0.01	7.03±0.01
	C. lusitaniae (1)	7.34±0.08	7.29±0.01	7.27±0.03	6.96±0.17
	C. guilliermondii (1)	7.36±0.06	7.33±0.02	7.20±0.08	6.77±0.10
Non-*Candida* species	*T. mucoides* (1)	7.44±0.08	7.31±0.01	7.10±0.04	6.59±0.03
	K. ohmeri(1)	7.36±0.10	7.26±0.03	7.18±0.09	6.52±0.05

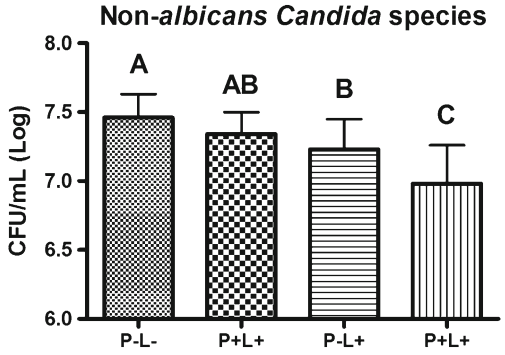

Fig. 2 Mean CFU/ml (log) and standard deviations for the strains of non-*albicans Candida* species exposed to different experimental treatments: PBS as control (P-L-); photosensitizer (P+L-); laser and PBS (P-L+); and laser and photosensitizer (P+L+). *Different letters* (A, B and C) represent significant statistically difference among the groups (Tukey test, $p<0.05$)

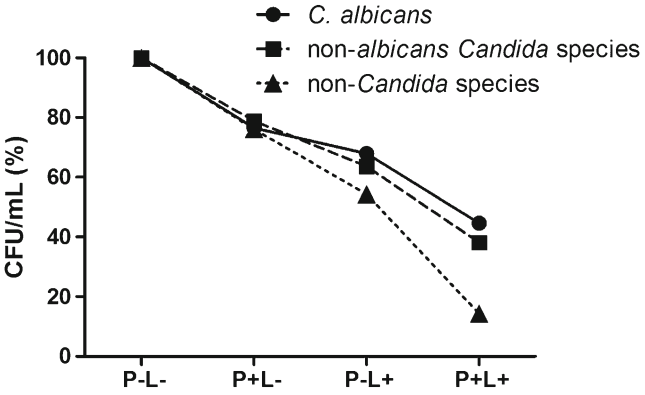

Fig. 4 Percentage of reduction, expressed as mean values (CFU/ml), in the viability of *C. albicans*, non-*albicans Candida* species, and non-*Candida* species exposed to photosensitizer (P+L-), laser light (P-L+) or both laser and photosensitizer (P+L+) relative to the control group (P-L-)

albicans Candida species, and non-*Candida* species. The susceptibility of the yeasts *C. albicans* and *C. tropicalis* to laser light has already been described in the literature, and it has been proposed that these yeasts may have endogenous photosensitizers that enable them to initiate a photodynamic reaction and subsequent cell death after exposure to radiation [36, 37].

In all species studied, CFU/ml values were reduced by photodynamic inactivation (P+L+) compared to the control group (P-L-), but the microbial reduction levels were different for the fungal species ranging from 0.33 to 0.85 log. From a microbiological point of view, a minimum of 3 log steps must be achieved to state antimicrobial effect [38]. Although the reductions obtained in this study have been less than 1 log, statistically significant differences between P+L+ and P-L- groups were observed. In addition, this reduction was achieved against biofilm growing pathogens, suggesting that PDI with cationic nanoemulsion of zinc-phthalocyanine can be a promising alternative for the dental clinic in the future.

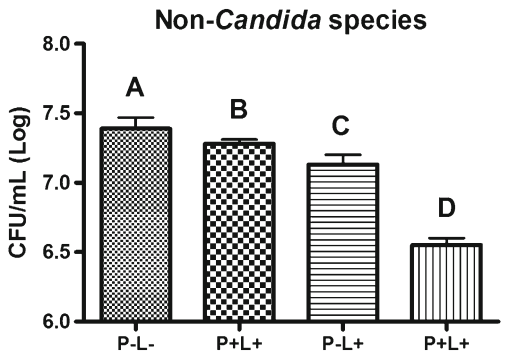

Fig. 3 Mean CFU/ml (log) and standard deviations for the strains of non-*Candida* species exposed to different experimental treatments: PBS as control (P-L-); photosensitizer (P+L-); laser and PBS (P-L+); and laser and photosensitizer (P+L+). *Different letters* (A, B, C, and D) represent significant statistically difference among the groups (Tukey test, $p<0.05$)

Among *Candida* spp., the species most resistant to photodynamic inactivation were *C. albicans*, *C. glabrata*, *C. norvegensis*, *C. krusei*, and *C. lusitaniae* (reduction < 0.5 log). The features of biofilms formed by *C. albicans*, *C. glabrata*, and *C. krusei* have been previously described [34, 35, 39, 40]. The biofilms formed by *C. albicans* are characterized by a thickly structured, spatially organized complex biofilm comprising a multitude of blastoconidia, pseudohyphae, and hyphae partially embedded in extracellular matrix [39]. *C. albicans* biofilms have profound resistance to many antifungal agents, such as fluconazole, amphotericin B, nystatin, and chlorhexidine [39, 41]. The biofilm formed by *C. glabrata* is thin, patchy, and rather compact, and consists exclusively of blastoconidia embedded within an EPS [40]. *C. krusei* form a thick multi-layer biofilm enveloped by voluminous extracellular material [34]. These last two species are known to be resistant to fluconazole and are extremely resistant to this antifungal agent when organized in a biofilm [39]. The non-*Candida albicans C. lusitaniae*, and *C. norvegensis* species have showed reduced susceptibility to antifungals and cause rare infection in human beings [5, 42]. All these characteristics could contribute to a lower susceptibility to photodynamic inactivation because the organization in biofilm seems to explain the phenotype of resistance to the treatments [43].

The other non-*albicans Candida* species, like *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis*, and *C. guilliermondii*, showed greater susceptibility to photodynamic inactivation (reduction > 0.5 log) than the other species described above. Souza et al. [36] observed the greater susceptibility to PDI mediated by methylene blue (312.6 μM) and diode laser InGaAlP (28 J cm^{-2}) for planktonic cultures of reference strains of *C. tropicalis*, *C. dubliniensis*, and *C. albicans* than *C. krusei*. The authors highlighted the difference in the susceptibility to PDI among different species, which must be further investigated.

Pereira et al. [44] evaluated the effect of PDI with methylene blue (312.6 μM) and an InGaAlP laser (350 J cm^{-2}) on biofilms formed by the reference strains *C. albicans*, *S. aureus*, *Streptococcus mutans*, which were grown on acrylic resin. PDI reduced 2.32 log_{10} of *C. albicans* biofilm and 1.00–1.90 log_{10} of *C. albicans* biofilm when associated with bacterium. The structure analysis revealed that the groups subjected to PDI exhibited less cellular aggregates. These data suggest that lethal photosensitization occurred mainly in the outermost layers of biofilms, which probably happened due to the inability of the photosensitizer to diffuse through these structures.

The emerging pathogen *T. mucoides* produces biofilms similar to *Candida* and exhibits reduced susceptibility to amphotericin B, which causes difficulties in the treatment of diseases caused by this yeast [10, 14]. *K. ohmeri* is another opportunistic pathogen that seldom causes disease but has ability to colonize central venous catheters [15, 19, 45]. These yeasts, *T. mucoides* and *K. ohmeri*, were inactivated by PDI with reduction of 0.85 log and 0.84 log, respectively. Currently, there are not any studies that evaluate the effects of PDI on *T. mucoides* and *K. ohmeri*; however, because these species exhibit higher sensibility to PDI than *Candida* spp., further studies are needed to investigate the mechanisms of PDI-mediated cytotoxicity on non-*Candida* yeasts.

The reduction of yeasts achieved by the methods employed in this work was greater than the reduction obtained by Dovigo et al. [23]. These authors evaluated PDI mediated by Photogem® irradiation by a red light-emitting diode (LED) on biofilms formed by fluconazole-resistant and reference strains of *C. albicans* and *C. glabrata* with mean reduction of 0.24 and 0.16 log, respectively. The photosensitizer used in the present work was a cationic nanostructure of zinc 2,9,16,23-tetrakis(phenylthio)-29H,31H-phthalocyanine (ZnPc) irradiation by a laser with photodynamic activity on biofilms formed by *Candida* spp., *T. mucoides*, and *K. ohmeri*. The analysis of the efficiency of cationic and anionic ZnPc irradiated by argon-pumped dye laser system (60 J cm^{-2}) on *C. albicans* planktonic culture was carried out by Mantareva et al. [28]. The cationic ZnPc was more efficient than anionic ZnPc, with total reduction of 10^6–10^8 cells/ml of *C. albicans* compared to a 1–2 log reduction of cells, respectively, which is due to the fact that cationic phthalocyanines exhibit better adhesion to the cellular membrane [29].

RLP068/Cl, a tetracationic Zn(II) phthalocyanine chloride, has been evaluated for cytotoxicity on antimicrobial-resistant and susceptible strains of *C. albicans*, *S. aureus*, and *Pseudomonas aeruginosa* to determine whether these strains develop resistance upon repeated exposure to the photosensitizer after PDI irradiated by noncoherent halogen lamp (30 J cm^{-2}) and the photosensitizer in the dark. This experiment demonstrated that 20 consecutive PDI treatments with RLP068/Cl did not result in any resistant mutants and that, in dark conditions, the minimum inhibitory concentration (MIC) of RLP068/Cl increased only for *S. aureus* strains. However, even in this case, the susceptibility of the susceptibility of these newly resistant bacteria to PDI was not affected by the increase in the MIC of RLP068/Cl [22]. This work showed that PDI exhibits a low propensity for inducing resistance in the three prevalent human pathogens tested, and it can be used to treat localized infections, such as oral and mucosal candidiasis, periodontitis, or chronic wounds of various types and origins.

Mantareva et al. [29] evaluated the photodynamic effect of two phthalocyanines, hydroxygallium(III) 2,9,16,23-tetrakis-[3-(*N*-methyl)pyridyloxy]-phthalocyanine tetraiodide (GaPc1) and hydroxygallium(III) 2,3,9,10,16,17,23,24-octakis-(3-pyridyloxy)-phthalocyanine (GaPc2), with LED (50 J cm^{-2}) in comparison to methylene blue and a water-soluble tetramethylpyridyloxy-substituted Zn(II)- phthalocyanine (ZnPcMe) on *C. albicans* biofilms grown on polymethylmethacrylate resin. Complete photodynamic inactivation of biofilms with GaPc2 and ZnPcMe was found. The susceptibility of *C. albicans* biofilms to PDI with GaPc2 and ZnPcMe suggests a potential value of the both complexes for treatment of denture-associated infections.

Longo et al. [27] developed a clinical protocol involving PDI application mediated by aluminum-chloride-phthalocyanine entrapped in cationic liposomes against cariogenic bacterials. In addition, the authors evaluated the cytotoxicity of the proposed PDI protocol in eukaryotic cells. The murine fibroblast cell line and human dental pulp cell culture were used in this study. The same PDI protocol applied against bacterials did not induce significant cytotoxicity against the two eukaryotic cell lines evaluated. These results show the tolerance of these eukaryotic cells to the treatment with the PDI antimicrobial for oral infections.

In summary, photodynamic inactivation using cationic nanoemulsion of zinc 2,9,16,23-tetrakis(phenylthio)-29H, 31H-phthalocyanine (ZnPc) in conjunction with a laser reduced the number of cells in the biofilms formed by strains of *C. albicans* and non-*Candida albicans* as well the emerging pathogens *T. mucoides* and *K. ohmeri*.

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References

1. Pomarico L, Cerqueira DF, Soares RMA, Souza IPR, Castro GFBA, Socransky S, Haffajee A, Teles RP (2009) Associations among the use of highly active antiretroviral therapy, oral candidiasis, oral *Candida* species and salivary immunoglobulin A in

HIV-infected children. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 108:203–210. doi:10.1016/j.tripleo.2009.05.008
2. Schelenz S, Abdallah S, Gray G, Stubbings H, Gow I, Baker P, Hunter PR (2011) Epidemiology of oral yeast colonization and infection in patients with hematological malignancies, head neck and solid tumors. J Oral Pathol Med 40:83–89. doi:10.1111/j.1600-0714.2010.00937.x
3. Williams DW, Kuriyama T, Silva S, Malic S (2000) Lewis MAO (2011) *Candida* biofilms and oral candidosis: treatment and prevention. Periodontol 55:250–265. doi:10.1111/j.1600-0757.2009.00338.x
4. Miceli MH, Díaz JA, Lee AS (2011) Emerging opportunistic yeast infections. Lancet Infect Dis 11:142–151
5. Hawkins JL, Baddour LM (2003) *Candida lusitaniae*: infections in the era of fluconazole availability. Clin Infect Dis 36: e14–e18
6. Paugam A, Baixench MT, Viguié C (2008) An update on *Candida dubliniensis*. Med Mal Infect 38:1–7. doi:10.1016/j.medmal.2007.10.008
7. Seneviratne CJ, Jin L, Samaranayake LP (2008) Biofilm lifestyle of *Candida*: a mini review. Oral Dis 14:582–590. doi:10.1111/j.1601-0825.2007.01424.x
8. Uppuluri P, Pierce CG, López-Ribot JL (2009) *Candida albicans* biofilm formation and its clinical consequences. Future Microbiol l4:1235–1237
9. Ramage G, Mowat E, Jones B, Willians C, Lopez-Ribot J (2009) Our current understanding of fungal biofilms. Crit Rev Microbiol 35:340–355. doi:10.3109/10408410903241436
10. Paphitou NI, Ostrosky-Zeichner L, Paetznick VL, Rodriguez JR, Chen E, Rex JH (2002) In vitro antifungal susceptibility of *Trichosporon* species. Antimicrob Agents Chemother 46:1144–1146. doi:10.1128/AAC.46.4.1144-1146.2002
11. Lacasse A, Cleveland KO (2009) *Trichosporon mucoides* fungemia in a liver transplant recipient: case report and review. Transpl Infect Dis 11:155–159. doi:10.1111/j.1399-3062.2008.00355.x
12. Flemming RV, Walsh TJ, Anaissie EJ (2002) Emerging and less common fungal pathogens. Infect Dis Clin North Am 16:915–933
13. Odero RO, Lacasse A, Mazumber S, Gelfand MS, Cleveland KO (2009) *Trichosporon asahii* infection in an HIV-positive patient. AIDS 23:1027–1033. doi:10.1097/QAD.0b013e328329d068
14. Di Bonaventura G, Pompilio A, Picciani C, Iezzi M, D'Antonio D, Piccolomini R (2006) Biofilm formation by the emerging fungal pathogen *Trichosporonasahii*: development, architecture, and antifungal resistance. Antimicrob Agents Chemother 50:3269–3276. doi:10.1128/AAC.00556-06
15. Poojary A, Sapre G (2009) *Kodamaea ohmeri* infection in a neonate. Indian Pediatr 46:629–631
16. Lee JS, Shin JH, Kim MN, Jung SI, Park KH, Cho D, Kee SJ, Shin MG, Suh SP, Ryang DW (2007) *Kodamaea ohmeri* isolates from patients in a university hospital: identification, antifungal susceptibility, and pulsed-field gel electrophoresis analysis. J Clin Microbiol 45:1005–1010. doi:10.1128/JCM.02264-06
17. Yang BH, Peng MY, Hou SJ, Sun JR, Lee SY, Lu JJ (2009) Fluconazole-resistant *Kodamaea ohmeri* fungemia associated with cellulitis: case report and review of the literature. Int J Infect Dis 13:e493–e497. doi:10.1016/j.ijid.2009.02.003
18. Chiu CH, Wang YC, Shang ST, Chang FY (2010) *Kodamaea ohmeri* fungaemia successfully treated with caspofungin. Int J Antimicrob Agents 35:93–99. doi:10.1016/j.ijantimicag.2009.09.010
19. Shang ST, Lin JC, Ho SJ, Yang YS, Chang FY, Wang NC (2010) The emerging life-threatening opportunistic fungal pathogen *Kodamaea ohmeri*: optimal treatment and literature review. J Microbiol Immunol Infect 43:200–206
20. Pires-Gonçalves RH, Miranda ET, Baeza LC, Matsumoto MT, Zaia JE, Mendes-Giannini MJS (2007) Genetic relatedness of commensal strains of *Candida albicans* carried in the oral cavity of patient's dental prosthesis users in Brazil. Mycopathologia 164:255–263. doi:10.1007/s11046-007-9052-5
21. Menon T, Herrera M, Periasamy S, Palanivelu V, Sikhamani R, Wickes B (2009) Oral candidiasis caused by *Kodamaea ohmeri* in an HIV patient in Chennai, India. Mycoses 53:458–459. doi:10.1111/j.1439-0507.2009.01731.x
22. Giuliani F, Martinelli M, Cocchi A, Arbia D, Fantetti L, Roncucci G (2010) In vitro resistance selection studies of RLP068/Cl, a new Zn(II) phthalocyanine suitable for antimicrobial photodynamic therapy. Antimicrob Agents Chemother 54:637–642. doi:10.1128/AAC.00603-09
23. Dovigo LN, Pavarina AC, Mima EGO, Giampaolo ET, Vergani CE, Bagnato VS (2011) Fungicidal effect of photodynamic therapy against fluconazole-resistant *Candida albicans* and *Candida glabrata*. Mycoses 54:123–130. doi:10.1111/j.1439-0507.2009.01769.x
24. Maisch T (2007) Anti-microbial photodynamic therapy: useful in the future? Lasers Med Sci 22:83–91. doi:10.1007/s10103-006-0409-7
25. Pleatzer K, Krammer B, Berlanda J, Berr F, Kiesslich T (2009) Photophysics and photochemistry of photodynamic therapy: fundamental aspects. Lasers Med Sci 24:259–268. doi:10.1007/s10103-008-0539-1
26. Wainwright M (1998) Photodynamic antimicrobial chemotherapy (PACT). J Antimicrob Chemother 42:13–28
27. Longo JPF, Leal SC, Simioni AR, Almeida-Santos MFM, Tedesco AC, Azevedo RB (2011) Photodynamic therapy disinfection of carious tissue mediated by aluminum-chloride-phthalocyanine entrapped in cationic liposomes: an in vitro and clinical study. Lasers Med Sci. doi:10.1007/s10103-011-0962-6, In press
28. Mantareva V, Kussovski V, Angelov I, Borisova E, Avramov L, Schnurpfeil G, Wöhrle D (2007) Photodynamic therapy of water-soluble phthalocyanine zinc(II) complexes against pathogenic microorganisms. Bioorg Med Chem 15:4829–4835. doi:10.1016/j.bmc.2007.04.069
29. Mantareva V, Kussovski V, Angelov I, Wöhrle D, Dimitrov R, Popova E, Dimitrov S (2011) Non- aggregated Ga(III)- phthalocyanines in the photodynamic inactivation of planktonic and biofilm cultures of pathogenic microorganisms. Photochem Photobiol Sci 10:91–102. doi:10.1039/b9pp00154a
30. Rosseti FC, Lopes LB, Carollo ARH, Thomazini JA, Tedesco AC, Bentley MVLB (2011) A delivery system to avoid self-aggregation and to improve in vitro and in vivo skin delivery of a phthalocyanine derivative used in the photodynamic therapy. J Control Release. doi:10.1016/j.jconrel.2011.06.034, In press
31. Séguier S, Souza SLS, Sverzut ACV, Simioni AR, Primo FL, Bodineau A, Corrêa VMA, Coulomb B, Tedesco AC (2010) Impact of photodynamic therapy on inflammatory cells during human chronic periodontitis. J Photochem Photobiol B 101:348354. doi:10.1016/j.jphotobiol.2010.08.007
32. Primo FL, Bentley MVLB, Tedesco AC (2008) Photophysical studies and in vitro skin permeation/retention of foscan/nanoemulsion (NE) applicable to PDT skin cancer treatment. J Nanosci Nanotechnol 8:340–347
33. Jin Y, Samaranayake LP, Samaranayake Y, Yip HK (2004) Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. Arch Oral Biol 49:789–798. doi:10.1016/j.archralbio.2004.04.011
34. Parahitiyawa NB, Samaranayake YH, Samaranayake LP, Ye J, Tsang PWK, Cheung BPK, Yau JYY, Yeung SKW (2006) Interspecies variation in *Candida* biofilm formation studied using the Calgary biofilm device. APMIS 114:298–306
35. Silva S, Henriques M, Martins A, Oliveira R, Williams D, Azeredo J (2009) Biofilms of non- *Candida albicans Candida* species: quantification, structure and matrix composition. Med Mycol 47:681–689. doi:10.3109/13693780802549594

36. Souza SC, Junqueira JC, Balducci I, Koga-Ito CY, Munin E, Jorge AOC (2006) Photosensitization of different *Candida* species by low-power laser light. Photochem Photobiol B Biol 83:34–38. doi:10.1016/j.jphotobiol.2005.12.002
37. Souza RC, Junqueira JC, Rossoni RD, Pereira CA, Munin E, Jorge AOC (2010) Comparison of the photodynamic fungicidal efficacy of methylene blue, toluidine blue, malachite green and low-power laser irradiation alone against *Candida albicans*. Lasers Med Sci 25:385–389. doi:10.1007/s10103-009-0706-z
38. Boyce JM, Pittet D (2002) Guideline for hand hygiene in health-care settings. Recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force. 51 (RR16):1–45
39. Hawser SP, Douglas LJ (1995) Resistence of *Candida albicans* biofilms to antifungal agents in vitro. Antimicrob Agents Chemother 39:2128–2131
40. Seneviratne CJ, Silva WJ, Jin LJ, Samaranayake YH, Samaranayake LP (2009) Architectural analysis, viability assessment and growth kinetics of *Candida albicans* and *Candida glabrata* biofilms. Arch Oral Biol 54:1052–1060. doi:10.1016/j.archoralbio.2009.08.002
41. Kuhn DM, George T, Chandra J, Mukherjee PK, Channoum MA (2002) Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob Agents Chemother 46:1773–1780. doi:10.1128/AAC.46.6.1773-1780.2002
42. Hood SV, Moore CB, Denning DW (1996) Isolation of *Candida norvegensis* from clinical specimens: four case reports. Clin Infect Dis 23:1185–1187
43. Blankenship JR, Mitchell AP (2006) How to build a biofilm: a fungal perspective. Curr Opin Microbiol 9:588–594. doi:10.1016/j.mib.2006.10.003
44. Pereira CA, Romeiro RL, Costa ACBP, Machado AKS, Junqueira JC, Jorge AOC (2011) Susceptibility of *Candida albicans*, *Staphylococcus aureus*, and *Streptococcus mutans* biofilms to photodynamic inactivation: an in vitro study. Lasers Med Sci 26:341–348. doi:10.1007/s10103-010-0852-3
45. Barros JD, Nascimento AMN, Araújo FJS, Braz RFS, Andrade VS, Theelen B, Boekhout T, Illnait-Zaragozi MT, Gouveia MNG, Fernandes MC, Monteiro MGL, Oliveira MTB (2009) *Kodamaea* (*Pichia*) *ohmeri* fungemia in a pediatric patient admitted in a public hospital. Med Mycol 47:775–779. doi:10.3109/13693780902980467