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 nanoeumulsion 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 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 Tr. 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 Tr. 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

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. kefyr, 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
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. dubliensis, acquires secondary resistance to fluconazole more quickly than C. albicans [5, 6].

Candida species are able to enter into complex communities known as biofilms, which are composed of blastococidia, 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. asteroides, 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 guillermondii 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, phytotoxic therapeutic 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-tetakis (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.

Photodynamic inactivation of biofilms formed by yeasts

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 phthalocyanine 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, Berlin, 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.

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 \( C. \) albicans, \( T. \) 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-methylpyridyloxy)- and tetrakis-(4-sulfophenoxy)-phthalocyanine zinc(II) at a concentration of 1.5 and 6 \( \mu \)M in the dark and observed cytotoxic effects with a reduction of less than 0.50 log for the microorganisms \( C. \) albicans, \( S. \) aureus, and \( E. \) 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>
<thead>
<tr>
<th>Yeasts</th>
<th>Strains</th>
<th>P-L-</th>
<th>P+L-</th>
<th>P-L+</th>
<th>P+L+</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C. ) albicans</td>
<td>( C. ) albicans (10)</td>
<td>7.30±0.07</td>
<td>7.18±0.08</td>
<td>7.13±0.07</td>
<td>6.95±0.07</td>
</tr>
<tr>
<td>Non-albicans</td>
<td>( C. ) glabrata (7)</td>
<td>7.68±0.12</td>
<td>7.50±0.21</td>
<td>7.54±0.19</td>
<td>7.35±0.24</td>
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<tr>
<td>( C. ) tropicalis</td>
<td>( C. ) dubliniensis (6)</td>
<td>7.35±0.07</td>
<td>7.26±0.08</td>
<td>7.08±0.03</td>
<td>6.71±0.14</td>
</tr>
<tr>
<td>( C. ) parapsilosis (4)</td>
<td>7.42±0.26</td>
<td>7.33±0.21</td>
<td>7.09±0.22</td>
<td>6.89±0.23</td>
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<tr>
<td>( C. ) norvegensis (2)</td>
<td>7.46±0.04</td>
<td>7.32±0.08</td>
<td>7.19±0.09</td>
<td>6.95±0.06</td>
<td></td>
</tr>
<tr>
<td>( C. ) krusei (2)</td>
<td>7.32±0.08</td>
<td>7.23±0.11</td>
<td>7.15±0.09</td>
<td>6.95±0.08</td>
<td></td>
</tr>
<tr>
<td>( C. ) lusitaniae (1)</td>
<td>7.39±0.04</td>
<td>7.30±0.02</td>
<td>7.14±0.01</td>
<td>7.03±0.01</td>
<td></td>
</tr>
<tr>
<td>( C. ) guilliermondii (1)</td>
<td>7.34±0.08</td>
<td>7.29±0.01</td>
<td>7.27±0.03</td>
<td>6.96±0.17</td>
<td></td>
</tr>
<tr>
<td>( T. ) mucoides (1)</td>
<td>7.36±0.06</td>
<td>7.33±0.02</td>
<td>7.20±0.08</td>
<td>6.77±0.10</td>
<td></td>
</tr>
<tr>
<td>( K. ) ohmeri(1)</td>
<td>7.36±0.10</td>
<td>7.26±0.03</td>
<td>7.18±0.09</td>
<td>6.52±0.05</td>
<td></td>
</tr>
</tbody>
</table>
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.

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 (31.26 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 log₁₀ of C. albicans biofilm and 1.00–1.90 log₁₀ 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 flucnazole-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 10⁶–10⁹ 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 polyethylene thacrylate 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 bacteria. 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 bacteria 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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