PHOTODYNAMIC PROCESS MEDIATED BY PROTOPORFIRIN IX DECREASE THE VIABILITY OF *Candida krusei*

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ABSTRACT

Candida krusei is present in infectious processes and shows an intrinsic resistance to fluconazole. The photodynamic therapy (PDT) uses a photosensitive agent and a light source to generate reactive species of oxygen. The aim of this study was to evaluate the efficacy of PDT mediated by PPIX against *Candida krusei*. The suspension of *C. krusei* (ATCC 6258) containing 10^6 CFU/ mL (Abs = 0.680, λ = 530 nm) was prepared. *C. krusei* was placed on Sabouraud dextrose agar (SDA) and incubated at 37 °C for 24 h. Was used a laser emission source with diode light (LED) (50 mW, 660 nm, 30 J/ cm^2). It was used protoporphyrin IX (PpIX) at the final concentration of 5 mM. PpIX was incubated for 30 min and irradiated; after this step, the cells were placed, and thereafter counted (results were expressed: log CFU/ mL). The tetrazolium salt (MTT) assays were performed to evaluate the mitochondrial activity. There is a significant reduction in the number of CFU Reduction/ mL when compared with other groups. The MTT also showed a reduction of mitochondrial activity, both results may be associated with effectiveness of PDT. We can conclude that PDT has fungicide effect, inhibiting the cellular growth and activates the mitochondrial damage.

KEYWORDS: Photodynamic therapy, *Candida krusei*, cell viability.

1. INTRODUCTION

Photodynamic therapy (PDT) is a therapeutically modality that uses a combination of photosensitizer agent and light source, in the oxygen/nitrogen presence, which produces reactive species, such as free radicals, causing cells damages and/ or cellular death. This sensitizer agents, are called photosensitizer. They do not exhibit toxicity alone, but could be toxic when combined with a light[1,2,3].

Photodynamic therapy has been applied to many types of cancers and has been focus of many studies for antimicrobial treatment[1,4,5,6]. Photoporfin IX (PpIX) is a sensitizer agents, derivate of haematoporfirin, a compound approved by the Food and Drug Administration for treatment of endobronchial and esophageal tumors in the U.S.A; currently, PpIX has been used for others clinical treatments[7].

Whereas its increase of fungi resistance to antibiotics, new alternatives to treatment of infectious diseases are required. Due to this, PDT treatment has been a modality that does not offer risk of resistance increase of pathogenic fungi[8]. The Candida species are frequent founded in normal human flora, but in immunocompromised patients, Candida species may cause mucocutaneous and systemic infection[7,9,10,11].

The *Candida kusei* is the specie related to infection by Candida, representing 2% of the infections[12,13]. The *C. krusei* had an intrinsic resistance to fluconazole due to this antifungal agent be the first option on antifungal therapy. Thus, the aim of this study was to evaluate the efficacy of PDT mediated by Protoporfirin IX-against *C. krusei*.

2. MATERIAL AND MÉTHODS

Candida krusei

Were used the strain *C. krusei* (ATCC 6258). The cells were grow seeded onto Sabouraud dextrose agar (SDA) (Difco, Detroit, MI, USA) and incubated for 24 h at 37 °C. After incubation, the microorganism was cultured in Brain Heart Infusion (BHI) broth (Difco) for 24 h at 37°C. To adjust the suspension concentration of 10^6 Colony Forming Unity (CFU)/ mL, the cells were quantified by spectrofotometry (λ: 530 nm, Abs: 0.680).

The cell suspension was divided in four assay tubes with 1 mL each. The tubes containing the cell suspension was named as (L-P-): representing a non treated group (n=10). (L-P+): representing a cell suspension group treated only with PPIX (n=10). (L+P-): representing a cell suspension treated only with light from LED (n=10). (L+P+) representing a cell suspension group treated with

photodynamic therapy (n=10). The letter "L" means Light and the letter "P" means photosensitizer.

PDT mediated by PPIX

PpIX was prepared by dissolving the salt in DMSO at the concentration of 500 µM. For the use in PDT the suspension were diluted 1:100 (5 µM) for the incubation with the cells. After of the dilution of PpIX in the suspension of *C. krusei* (10^6 CFU/ mL) the cells were incubated with PpIX for 30 min at 37°C. After this the cells were irradiated with a Laser Diode Emitted (LED) with wavelenght of 660 nm with a light dose of 30 J/ cm^2. The cells irradiated were putted in to SDA and incubated at 37 °C and the colony were counted and quantified.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl--2*H*-tetrazolium bromide) assay

The reduction of tetrazolium salts such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazoliu m bromide] or water-soluble tetrazolium derivatives (in the presence of membrane-permeant redox mediators, such as phenazine methosulfate) to purple formazan products is a quick and dirty indicator of the availability of reducing power in a cellular preparation[14].

After the PDT treatment, the cultured *C. krusei* were washed by centrifugation (8,000 xg for 10 min) and incubated with 20 µL MTT solution (0.5 mg/mL) at 37°C for 3 h. After this period, tubes containing MTT-cell suspension were centrifuged for 10 min at 400 × g to form cell pellet. The supernatant was discarded and 100 µL DMSO was added to the colored cell pellet. Finally, 100 µL of the purple-colored suspension was transferred to a 96 well microplate and analyzed using spectrophotometry (570 nm). DMSO was used as a blank.

Statistical analyses

The statistical analysis was performed using the Prism software program (GraphPad Inc., San Diego, CA, USA). Normality (Kolmogorov–Smirnov test) and homogeneous variance tests (Bartlett's test) were applied to all variables. Parametric tests (analysis of variance with Tukey's multiple comparison post-test) were used for cases with normal distributions and homogeneous variances. Non-parametric tests (the Kruskal–Wallis test with Dunn's multiple comparison) were used for cases with non-Gaussian distributions. Differences with p-values <0.05 were considered significant.

3. RESULTS

The L+P+ group showed cell reduction when compared to the other groups (Figure 1), suggesting that photodynamic action of reactive oxygen species induces cell death (p = 0.0133).

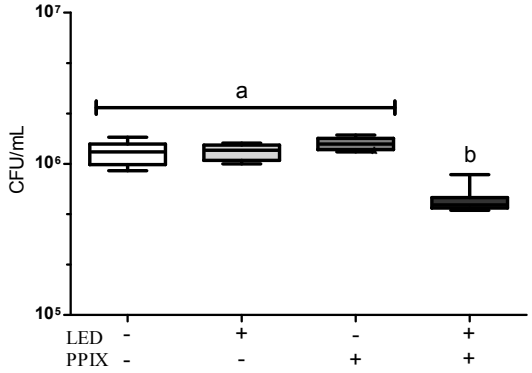

Figure 1. *Candida krusei* colony reduction in cell groups. The Y-axis represents the quantification of colony forming units (CFU) of yeast cells per milliliter (mL). In the X-axis, indicates absence (-) and/or the presence (+) of protoporphyrin IX (PpIX) and / or the Emitted Laser Diode (LED).. The letters a and b indicate statistically significant differences between them ($p < 0.05$). Nonparametric test was used for analysis of graphic.

In order to obtain another cell viability test, the MTT assay was performed. In this test it was possible to observe that PDT (L+P+) reduced the viability of the yeast, observed by decreased mithocondrial activity when compared the other groups (Figure 2).

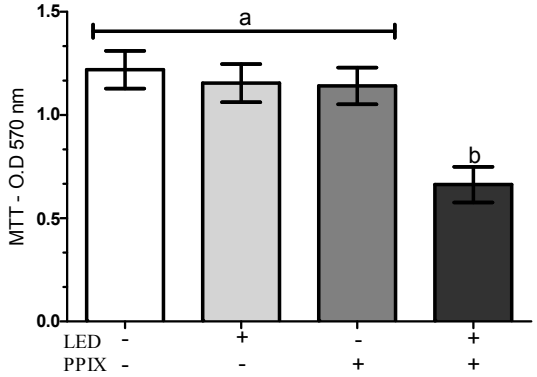

Figure 2. Values os Formazam produced after PDT treatment. The Y-axis represents the reading of the wavelength for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay (MTT). In the X-axis, indicates absence (-) and/or the presence (+) of protoporphyrin IX (PpIX) and / or the Emitted Laser Diode (LED). The letters a and b indicate statistically significant differences between them ($p < 0.05$). Parametric test were used for analysis of graphic.

4. DISCUSSION

This study evaluated in vitro a viability of *C. krusei* after photodynamic therapy mediated by PPIX treatment. As targeted, our results let us observe that, after a PDT there was an reduction of CFU/ mL, similar to study conducted by Bliss *et al.* (2004)[15], were the authors showed that there was an reduction in the CFU/mL number after PDT exposure. In our study the reduction was significant, but not like the results presented by Bliss *et al.* (2004)[15]; maybe this fact can be associated to some differences of drug or time of incubation.

Protoporfin IX belongs to porphyrin class, and even if is the same class of drug, differences on therapeutic effect is observed. Porphyrin is derivated from hematoporphyrin that is approved by the U.S. Food and Drug Administration for the treatment of endobronchial and esophageal[15].

Lambrechts (2005)[4] has performed some studies with porphyrin to understand the mechanism of action, they observed the uptake capitation of the drug by the cell and the toxicity. In our study, the PDT process does not caused a high toxicity in the cells tested (showed by only 1 log diminution cell viability). Furthermore, the photodynamic action is dose dependent (for photosensitizer and for light) and, to solve this, more PDT sections can be performed in order to achieve more effectiveness in *C. krusei* kill.

It was measured in our study that the metabolic activity is affected by this therapy. The use of MTT test showed and decrease of mitochondrial activity after PDT treatment[4], in our study also observed and reduction of mitochondrial activity of Candida species. This damage on metabolism occurs in order to generation of reactive oxygen species (ROS) inside the cell after a light exposure in association with the drug, causing an inactivation of mitochondrial enzymes and proteins, involved on energy production. In a study performed by Hilf (2007)[16], the focus of mitochondria as an target of PDT was studied. This author observed the presence of many enzymes essential to metabolism before and after the PDT treatment. In his study he could observed and reduction of Na^+K^+ ATPase, pyruvate kinase and others enzymes associated with mitochondrial activity in tumors cells of rats, testing the Photofrin II.

With these data we can observe that, the potential activity of PDT against yeast, like Candida species, have a direct influence in your metabolism even if there is no death, cells have your metabolism degraded.

5. CONCLUSION

Considering the limitations of this study, the results permits to conclude that the Photodynamic therapy presents a potential fungicide effect against *C. krusei* and some improvements on this technique are required to kill a major number of cells.

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REFERENCES

[1]. Donnelly RF, McCarron PA, Tunney MM. Antifungal photodynamic therapy. Microbiol Res. 2008; 163(1):1-12.

[2]. Zhu TC, Finlay JC. The role of photodynamic therapy (PDT) physics. Medical Physics. 2008; 35: 3127.

[3]. Dai T, *et al.* Concepts and principles of photodynamic therapy as an alternative antifungal discovery platform. Frontiers in Microbiol. 2012; 3.

[4]. Lambrechts SAG, Aalders MCG, Van Marle J. Mechanistic study of the photodynamic inactivation of *Candida albicans* by a cationic porphyrin. Antimicrobial Sgents and Chemoth. 2005; 49(5):2026-34.

[5]. Pereira Gonzales F, Maisch T. Photodynamic inactivation for controlling *Candida albicans* infections. Fungal Biol. 2012; 116(1):1-10.

[6]. Kato I T, *et al*. Antimicrobial photodynamic inactivation inhibits *Candida albicans* virulence factors and reduces in vivo pathogenicity. Antimicrobial Agents and Chemoth. 2013; 57(1):445-51.

[7]. Chabrier-Roselló Y, *et al*. Sensitivity of *Candida albicans* germ tubes and biofilms to photofrin-mediated phototoxicity. Antimicrobial Agents and Chemoth. 2005; 49(10):4288-95.

[8]. Dovigo LN, *et al.* Fungicidal effect of photodynamic therapy against fluconazole-resistant *Candida albicans* and *Candida glabrata.* Mycoses. 2011; 54(2):123-30.

[9]. Demidova TN, Hamblin MR. Photodynamic therapy targeted to pathogens. Int J of Immunopathol and Pharmacol. 2004; 17(3):245.

[10]. Carvalho GG, Felipe MP, Costa MS. The photodynamic effect of methylene blue and toluidine blue on *Candida albicans* is dependent on medium conditions. The J of Microb. 2009; 47(5):619-23.

[11]. Snell SB, Foster TH, Haidaris CG. Miconazole Induces Fungistasis and Increases Killing of *Candida albicans* Subjected to Photodynamic Therapy†. Photochem and Photobiol. 2012; 88(3):596-603.

[12]. Tortorano AM, *et al.* Candidaemia in Europe: epidemiology and resistance. Int J of Antimicrob Agents. 2006; 27(5):359-66.

[13]. Sardi J, *et al*. Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J of Medical Microbiol. 2013; 62(Pt 1):10-24.

[14]. Ishiyama M, *et al.* A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol and Pharmac Bulletin. 1996; 19(11):1518-20.

[15]. Bliss JM, *et al*. Susceptibility of Candida species to photodynamic effects of photofrin. Antimicrobial Agents and Chemoth. 2004; 48(6):2000-006.

[16]. Hilf R. Mitochondria are targets of photodynamic therapy. J of Bioenergetics and Biomembr. 2007; 39(1):85-9.