Photodiagnosis and Photodynamic Therapy 25 (2019) 421–424

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

Photodynamic Antimicrobial Chemotherapy (PACT), using Toluidine blue (TBO) inhibits both growth and dimorphism in *Paracoccidioides brasiliensis* by a mechanism involving reactive oxygen species (ROS) production

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ARTICLE INFO

Keywords:
Paracoccidioides brasiliensis
Toluidine blue
Fungus
Dimorphism
Photodynamic antimicrobial chemotherapy

ABSTRACT

Background: The thermo-dimorphic fungus Paracoccidioides brasiliensis is the pathogen of Paracoccidioidomycosis, an important public health problem in Latin American with prevalence in Brazil. Photodynamic Antimicrobial Chemotherapy (PACT) is a process that combines a photosensitizer and light, producing reactive oxygen species (ROS) that can promote damages to treated cells.
Methods: In this work was study the effect of PACT, using Toluidine blue (TBO) on both yeast and mycelial cells of P. brasiliensis.
Results: It was observed that PACT decreased P. brasiliensis yeast growth, in a dependent manner of both TBO concentrations and fluence. In the presence of TBO 0.005 mg/mL, PACT reduced P. brasiliensis yeast growth in 63, 62 and 86%, using fluences of 20, 30 and 40 J/cm^2, respectively. After PACT, ROS production increased 2.80, 4.64 and 7.90 times, in the presence of TBO 0.001, 0.002 and 0.005 mg/mL, respectively. It was observed that after PACT, the cells are predominantly in mycelia form, indicating that mycelial cells irradiated in the presence of TBO, maintained their filamentous form and absence and/or decreased presence of transition structures.
Conclusions: These results demonstrated the potential of PACT, using TBO to inhibit both yeast and mycelium development of P. brasiliensis.

1. Introduction

The thermo-dimorphic fungus Paracoccidioides brasiliensis causes Paracoccidioidomycosis (PCM), an important public health problem in Latin American, with prevalence in Brazil (Franco, 1987; Mendes et al., 2017; Shikanai-Yasuda et al., 2018; Restrepo-Moreno, 2018) [1–4]. One of the main problems of this mycosis, initially pulmonary, are the secondary mucosal lesions, which are debilitating, painful and, in some cases, generate mutilations (Shikanai-Yasuda et al., 2018; Palmeiro et al., 2005; Shikanai-Yasuda et al., 2006; Wanke and Aidê, 2009; Marques et al., 2007) [3,5–8]. The treatment is performed through the use of oral medicines anti-fungal and/or oral surgeries (Shikanai-Yasuda et al., 2018) [3]. The Photodynamic Antimicrobial Chemotherapy (PACT) has been suggested as a promising alternative and/or complementary therapy against many pathogens (Wainwright, 1998; Hamblin and Hasan, 2004; Perussi, 2007; Maisch, 2007; Calzavara-Pinton et al., 2012; Sperandio et al., 2015; Almeida et al., 2012; Baltazar et al., 2015) [9–16] and has been little explored in the treatment of individuals with PCM (Ribeiro et al., 2017; Dos Santos et al., 2017) [17,18].

1.1. Aims

The objective of this work was study the effect of Photodynamic Antimicrobial Chemotherapy using Toluidine blue as a photosensitizer on both yeast and mycelial cells of Paracoccidioides brasiliensis, isolated 18, and the possible mechanism involved on PACT action.

2. Methods

Yeast and mycelia of *P. brasiliensis* isolate 18 (Pb18) were used at a cell density of 1.0×10^6 viable cells/mL in saline solution (0.9% NaCl). Phototoxicity tests were performed on 96-well microtiter plates with *P. brasiliensis* suspensions. Toluidine blue was used as a photosensitizing

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https://doi.org/10.1016/j.pdpdt.2019.01.003
Received 31 October 2018; Received in revised form 13 December 2018; Accepted 2 January 2019
Available online 03 January 2019

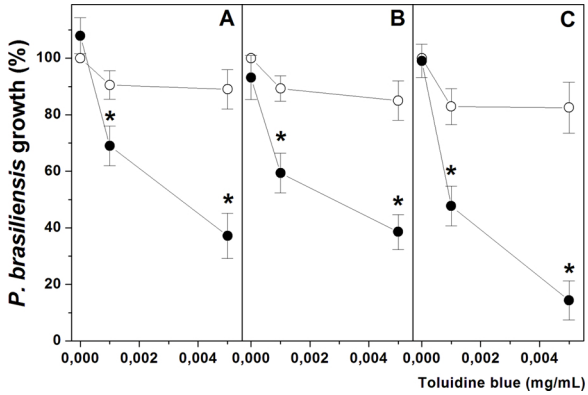

Fig. 1. Effect of different Toluidine blue concentrations on *P. brasiliensis* yeast growth, in irradiated (•) and not irradiated (o) cells. The experimental conditions are described under Materials and Methods. The cell growth was determined 48 h after PACT, using 20 (A), 30 (B) and 40 J/cm^2 (C). The data are mean ± SE (n = 8). * $p < 0.05$.

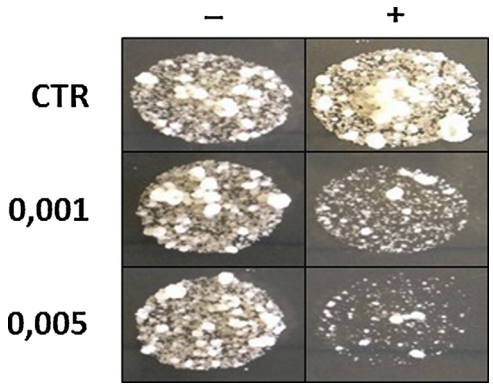

Fig. 2. Cell viability of *P. brasiliensis*. The cell viability was evaluate by inoculating suspensions of *P. brasiliensis* on Petri dish containing YPD solid medium. Cell suspensions were treated with different TBO concentrations (0.001 and 0.005 mg/mL) and irradiated. Cells incubated in sterile physiological solution alone were included as a control. After this procedure, the cells were seeded on Petri dish and the growth was evaluated after 2 days. Cells not irradiated (-) and irradiated (+) with 30 J/cm^2.

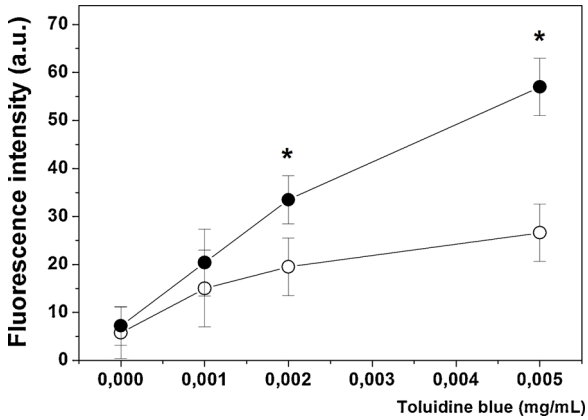

Fig. 3. Effect of PACT on ROS production in *P. brasiliensis* yeast, in irradiated (•) and not irradiated (o) cells. The experimental conditions are described under Materials and Methods. The ROS production was determined 1 h after PACT, using 40 J/cm^2. Values are expressed in arbitrary units. The data are mean ± SE (n = 6). * $p < 0.05$.

drug in the concentrations of 0.001, 0.002 and 0.005 mg/mL. As a control, were used cells in sterile physiological solution. A light emitting diode (LED) with a output power of 0.069 W and a peak

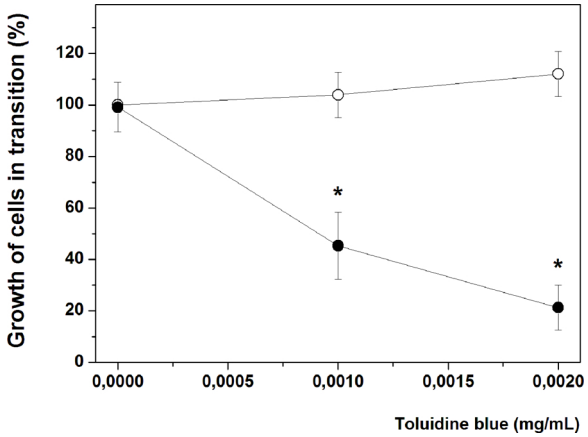

Fig. 4. Effect of PACT on *P. brasiliensis* growth during transition (M–Y) induced by alteration of temperature (from 25 to 37°C) in irradiated (•) and not irradiated (o) cells. The experimental conditions are described under Materials and Methods. The cell growth was determined 5 days after PACT, using 40 J/cm^2 (37 °C). The data are mean ± SE (n = 6). * $p < 0.05$.

wavelength of 630 nm illuminated an area of 0.38 cm^2, resulting in an fluence of 20, 30 and 40 J/cm^2. The different fluencies were obtained by irradiating the plates using different times. The experiments were performed in the dark and under aseptic conditions. Cells were incubated for 10 min before and after PACT. To determine the growth of Pb18 after PACT, mycelia and yeasts cells were incubated in medium YPD for 5 and 2 days, respectively, and the absorbance was read using the Synergy HT Multi-Detection Microplate Reader (Bio-Tek, Winooski, VT, USA), optical density of 570 nm (OD_{570}). To determine the amount of ROS produced after PACT in yeasts cells was used the method cited in Carvalho et al. [19]. The morphology of the cells submitted to mycelia to yeast transition (M–Y) was analyzed by light microscopy after PACT. Values were expressed as means ± standard deviation. Statistical differences were evaluated by analysis of variance (ANOVA) and post hoc comparison with the Tukey–Kramer test. P values of < 0.05 were considered significant.

3. Results

It was observed a significant effect of PACT in decrease *P. brasiliensis* yeast growth, in a dependent manner of both TBO concentrations and fluences used (Fig. 1). In the presence of TBO 0.001 mg/mL, it was observed a reduction in *P. brasiliensis* yeast growth of 16, 32 and 30% after PACT using fluencies of 20, 30 and 40 J/cm^2, respectively. PACT, using TBO 0.005 mg/mL promoted a reduction of 63, 62 and 86%, using fluencies of 20, 30 and 40 J/cm^2, respectively. At the same time, it was observed a great reduction in cell viability after PACT, using TBO 0.001 and 0.005 mg/mL (Fig. 2). After PACT, it was observed an increase in ROS production of 2.80, 4.64 and 7.90 times, in the presence of TBO 0.001, 0.002 and 0.005 mg/mL, respectively (Fig. 3). Still, it was observed a positive statistical correlation between the inhibition of *P. brasiliensis* yeasts growth and the increase in ROS production induced by PACT ($r = 0.965$). These results suggest that the decrease observed in *P. brasiliensis* yeasts growth could be related to ROS production induced by PACT. In addition, for the first time, it was observed a relevant reduction in the mycelia growth, after PACT in a dependent manner of TBO concentrations. It was observed an inhibition of 55 and 80% in the mycelia growth after PACT, using TBO 0.001 and 0.002 mg/mL, respectively (Fig. 4). As a well-succeeded M–Y transition of *P. brasiliensis* is determinant to the fungal pathogenicity [20], the evaluation of the effect of PACT on M–Y transition was evaluated. After PACT, the mycelia treated remained, predominantly, as mycelia, indicating that the M-Y transition was blocked after irradiation in the presence of TBO (Fig. 5).

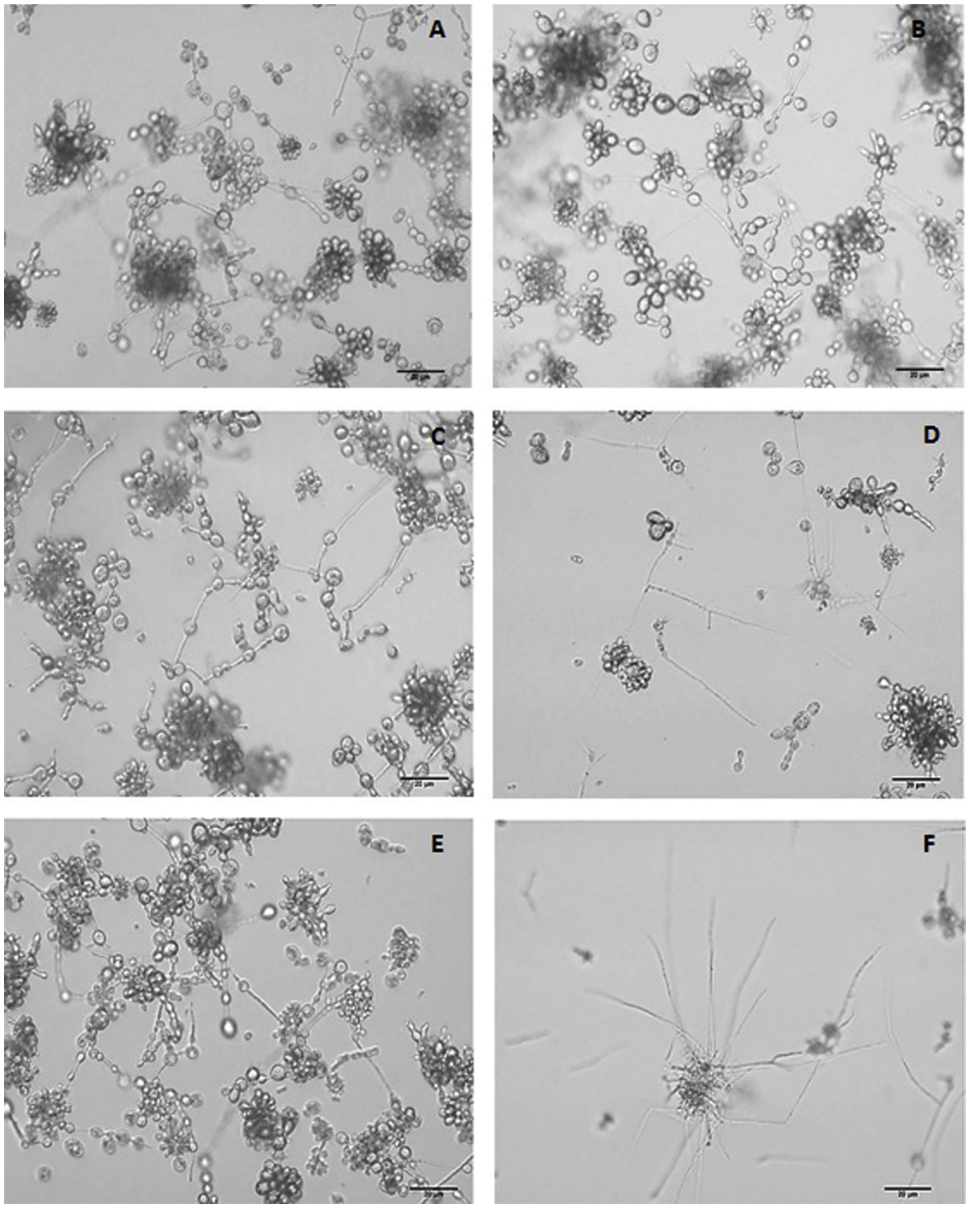

Fig. 5. Effect of PACT on the transition of *P. brasiliensis* from mycelial to yeast phase. The figure represents the morphology of the cells submitted to the transition, in not irradiated (5 A, 5C and 5E) and irradiated (5B, 5D and 5 F) cells. The cells were observed in the absence (5 A and 5B) and in the presence of either 0.001 (5C and 5D) or 0.002 mg/ml of TBO (5E and 5 F). Bar, 20 μm.

4. Conclusions

Photodynamic Antimicrobial Chemotherapy (PACT) using Toluidine blue (TBO) as a photosensitizer was able to decrease the growth of both mycelia and yeasts of Pb18, probably by a mechanism related to the production of reactive oxygen species (ROS) after PACT. Thus, these results indicate that PACT with TBO is a promising antifungal therapy, alternative and/or complementary, to treat the secondary lesions caused by *P. brasiliensis* in patients with Paracoccidioidomycosis. In parallel, the results of the effect of PACT on the thermal dimorphism of the fungus may support future researches aiming improve the knowledge about the pathogenicity mechanisms of *P. brasiliensis*.

Compliance with ethical standards

None.

Conflict of interest statement

The authors have no financial, personal, or other conflicts of interest related to this work.

Role of funding source

The authors would like to thank FAPESP and CAPES for the financial support.

Ethical approval

In this study, all experiments were performed using Cultures of *Paracoccidioides brasiliensis*, therefore there was no need for approval by local authorities.

Informed consent

We have obtained permission from all the authors, we declare that the material has not been published in whole or in part elsewhere, the paper is not currently being considered for publication elsewhere.

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). "This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001".

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