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Photodynamic Antimicrobial Chemotherapy (PACT) using methylene blue inhibits the viability of the biofilm produced by *Candida albicans*

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ABSTRACT

Background: Candida albicans is an opportunistic fungus, an etiological agent of human infections, presenting high rates of morbidity and mortality. The resistance of *C. albicans* to conventional therapies has been reported due to the extensive use of conventional antifungals. Photodynamic antimicrobial chemotherapy (PACT) is a technique that combines a visible light with a specific wavelength and a photosensitizer, producing ROS and permanent damages in the treated cells. *Methods:* In this work, the effects of PACT, using Methylene Blue (MB), as a photosensitizer, on *C. albicans* development were studied. *Results:* Significant reduction in both cell growth and biofilm formation after PACT were observed, in a dependent manner on both MB concentration and fluence. In the presence of MB 0.02 mg/mL, it was observed inhibition in biofilm formation of ˜58, 70 and 74%, using fluences of 10, 20 and 30 J/cm^2, respectively. Also, it was observed inhibition of 54, 66 and 55% in the presence of MB 0.01, 0.02 and 0.05 mg/mL, respectively in the viability of biofilm produced by *C. albicans*. The number of both yeast and filaments present in the structure of biofilm were reduced after PACT. Furthermore, PACT changed the growth kinetics of *C. albicans*. Interestingly, we demonstrated increase in the extent of lag phase and an alteration in the profile of the exponential phase after PACT. *Conclusions:* Taken together, these results indicate the potential PACT effects using MB to decrease the *C. albicans* development.

1. Introduction

Infections caused by the fungus of the *Candida* genus are common in immunocompromised hosts, as well as in healthy hosts with altered microbiota. Current studies have provided worrying data about the number of mucocutaneous and systemic infections related to this microorganism [1–8]. Systemic infections produced by some *Candida* species show significant impact on global health because it is an important cause of morbidity and mortality, which can vary from 30 to 70% of occurrence. In the USA, infections produced by *Candida albicans* are the fourth most common cause of bloodstream infections [2,7,9–16]. These opportunistic fungi cause substantial problems, especially because of their resistance to most antifungal drugs [7]. For this reason, many alternative strategies for the treatment and management of *C. albicans* infections have been reported [8,17–21].

C. albicans is well-known as a biphasic fungus, growing in both yeast and filamentous forms. *C. albicans* lives in the yeast form on the mucous membrane or on the skin surface; however, it often takes the filamentous form when it invades the tissues [22]. The biofilm produced by *C. albicans* comprises a mixed state of yeast and filamentous forms and possesses stronger pathogenicity than the yeast form. The biofilm is characterized by a complex polymeric structure, irrigated with nutrients, facilitating, thus, its colonization and virulence, besides exerting the function of barrier against drugs [13,16,23–27]. When compared to the cells adhered to biofilm surface, the planktonic cells, not adhered to the biofilm structure present less resistance to diverse antifungals, such as fluconazole and amphotericin B [17,20,28–35]. Therefore, the emergence of resistance among fungal species has led to a major research effort to find new alternative, immediate and effective therapeutic strategies.

Photodynamic Antimicrobial Chemotherapy (PACT) is a potential antimicrobial therapy that combines visible light and a photosensitizer (non-toxic), which, in the presence of molecular oxygen produces Reactive Oxygen Species (ROS), including singlet oxygen, highly

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reactive to the biological components [36–38]. ROS can promote damage to several vital components in the cell, such as DNA, proteins and lipids, resulting in cell death [39,40]. The antimicrobial effect of a variety of light-sensitive photosensitizer compounds has been demonstrated [41,42]. Furthermore, the fungicide effect of phenothiazinium salts, such as toluidine blue O (TBO) and methylene blue (MB), as photosensitizer drugs on *C. albicans* has also been reported in the literature [43–46], including. effectiveness of MB in inhibiting the *C. albicans* development [44,47–50]. In addition, it was demonstrated the inhibition of the virulence factors and the reduction of in vivo pathogenicity in *C. albicans* after antimicrobial photodynamic inactivation, using MB [51]. Recently, it was showed antimicrobial photodynamic therapy, using MB in an animal model of vulvovaginal candidiasis, reduced fungal colony-forming units [52]. In this way, the objective of this study was to study the effect of PACT, using MB, as a photosensitizer on both viability and the structure of biofilms produced by *C. albicans*.

2. Materials and methods

2.1. C. albicans and growth conditions

C. albicans was purchased from American Type Culture Collection (Manassas, VA, USA) (ATCC #1023). Cultures were seeded on Sabouraud dextrose agar (Merck, Darmstadt, Hesse, Germany) and incubated at 37 °C in an air atmosphere. A sample of the colonies was removed from the surface of the agar plate after 48 h of incubation and suspended in sterile physiological solution (0.9% NaCl, w/v). Cell density was adjusted to 10^5 and 10^6 viable cells/mL, using the Neubauer chamber in the presence of the vital dye, methylene blue (0.5 mg/mL).

2.2. C. albicans phototoxicity assay

Phototoxicity tests were performed with Candida suspensions inoculated in 96-well plate and incubated in the dark for 10 min at room temperature in the presence of different MB concentrations, depending on the type of test. The dye Methylene blue was purchased from Sigma, St. Louis, MO, USA (Dye content, ≥ 82%). Cells incubated in sterile physiological solution alone were included in all experiments, as a negative control. After, the cover of the plate was removed, and the content of the plate was irradiated with the appropriated light, at room temperature. The light source used was a LASER (Photon Lase III, DMC, São Carlos, SP, Brazil.), with an output power of 0.1 W and a peak wavelength of 660 nm. The laser beam illuminated an area of 0.38 cm^2, resulting in fluence of 10, 20, 30 or 40 J/cm^2, depending on the type of test. The different fluences were obtained by irradiating the content of the plates at different times. All experiments were performed under aseptic conditions.

2.3. Effect of PACT on C. albicans growth

C. albicans suspensions (10^6 viable cells/mL) were seeded in a 96-well plate in the presence of MB (0.005, 0.01 and 0.02 mg/mL) in a final volume of 0.3 mL. Subsequently, the medium was correctly homogenized and divided into two plates, 0.15 mL for the non-irradiated group and 0.15 mL for the irradiated group. The plates were incubated in the dark for 10 min in atmospheric air (37 °C). After this period, the 96-well plate lid was removed and the plates were irradiated (10, 20 or 30 J/cm^2), using the irradiation parameters cited in the *C. albicans* phototoxicity assay. After irradiation, the medium remained in the rest for 10 min. Then, the medium was homogenized, aliquots of 25 μL were taken and seeded in a 24-well plate containing Sabouraud dextrose broth (Merck, Darmstadt, Hesse, Germany) (2 mL). After 18 h of incubation at 37 °C, the medium was homogenized and the optical density at 570 nm (OD 570) was determined using the Synergy HT Multi-Detection Microplate Reader (Bio-Tek, Winooski, VT, USA). The optical density determined in the control group varied from 0.8 to 1.0 in all experiments. The values presented in the figure represent the percentage of growth, calculated using the control group (cells incubated in the absence of MB and not irradiated) as 100% of growth.

2.4. Effect of PACT on biofilm formation

After taking of the content of the 96-well plate to determine cell growth, aliquots of 25 μL were also taken and seeded in 96-well plate containing RPMI medium (Sigma, St. Louis, MO, USA), in a final volume of 200 μL. The plates were incubated to form biofilm for 24 h, at 37 °C. Then, the cell suspensions were aspirated, each well was washed 3 times with 200 μL PBS to remove the non-adherent cells and 200 μL PBS was added to each well. Biofilm formation was monitored by a metabolic assay based on the reduction of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt) (Molecular Probes, Eugene, OR, USA) assay. Prior to each assay, XTT solution (1 mg/mL) was thawed and mixed with a freshly prepared 0.4 mM menadione solution (a respiratory electron chain-uncoupling agent that accelerates respiration and XTT reduction; Sigma, St. Louis, MO, USA) at a volume ratio of 9:1. An aliquot of 20 μL from this mixture was added to each well. After 2 h, the reduced formazan-coloured product was measured at 490 nm (OD 490) in a Synergy HT Multi-Detection Microplate Reader. The values presented in the figure represent the percentage of biofilm formation, calculated using the control group (cells incubated in the absence of MB and not irradiated) as 100% of growth. In addition, the biofilm formed was also observed by optical microscopy to determine possible PACT-induced morphological changes, such as the shape and size of the adhered cells and the presence of filaments in the biofilm structure. The preparations were examined using a photomicroscope (Axioskop 2, Zeiss, Germany), and the images were captured with a Pixera digital camera system (Pixera Corporation, USA) attached to the photomicroscope and a microcomputer (Intel® Pentium®) using the software Adobe Photoshop version 7.0.1 (Adobe Systems, USA).

2.5. Effect of PACT on the viability of the biofilm forming at 24 h

C. albicans suspensions (10^6 viable cells/ mL) were added to a 96-well plate containing RPMI medium in the absence of MB, in a final volume of 200 μL. The plates were incubated to form biofilm for 24 h, at 37 °C. After this period, the biofilm forming at 24 h was washed 3 times with 200 μL sterile PBS to remove the non-adherent cells and, then, 100 μL of the reaction medium was added to each well. The reaction medium was prepared using different concentrations of MB (0.01, 0.02 and 0.05 mg/mL). Biofilms treated with sterile saline alone were included as a negative control. The plates were incubated in the dark for 10 min in atmospheric air, at 37 °C. After this time, the cover of the 96-well plate was removed, and the content of the plates was irradiated (40 J/cm^2) with the same irradiation parameters as described in the *C. albicans* phototoxicity assay. After irradiation, the contents of the plates remained in the rest for 10 min. Then, the wells were washed twice with 200 μL PBS in order to remove all residues from the reaction medium, and, at the end, 200 μL PBS was added to each well. Biofilm viability was determined by a metabolic assay based on the reduction of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl) -5- [(phenylamino) carbonyl] -2H-tetrazolium hydroxide (XTT). Before each assay, the XTT solution (1 mg/mL) was thawed and mixed with a freshly prepared 0.4 mM menadione solution (Sigma, MO, USA) at a 9:1 vol ratio. An aliquot of 20 μL of this mixture was added to each well and, after 2 h, the reduced color formazan product was determined at 490 nm (OD 490) in the Synergy HT Multi-Detection microplate reader. The values presented in the figures represent the percentage of the metabolism of biofilms, calculated using the control group (cells incubated in the absence of MB and not irradiated) as 100% of the metabolism of biofilms.

2.6. Effect of PACT on the growth kinetics of C. albicans

The effect of PACT on the growth kinetics of *C. albicans* was determined by a time curve. *C. albicans* suspensions (10^5 viable cells/mL) were seeded in a 96-well plate in the presence of different concentrations of MB (0.005 and 0.02 mg/mL) in a final volume of 0.3 mL. Then, the medium was homogenized and divided into two plates, 0.15 mL for the non-irradiated group and 0.15 mL for the irradiated group as described in the *C. albicans* phototoxicity assay. After irradiation, aliquots of 25 μL were taken and seeded in a 24-well plate containing Sabouraud dextrose broth (2 mL). The plates were incubated, at 37 °C and the content of plates was analyzed temporally every 1 h for 24 h. Within the flow stream, the content of plates was properly homogenized and aliquots of 100 μL were transferred to a 96-well plate, every 1 h of incubation to determine the growth kinetics. The growth was determined by measuring the optical density at 570 nm (OD 570) using the Synergy HT multi-Detection.Microplate Reader.

2.7. Statistical analysis

The data were presented as means ± standard deviation (SD) of eight independent experiments, performed at least in triplicate. The statistical technique used was one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post-hoc test for multiple comparisons. A standard significance level of $p < 0.05$ was used. The analyses were performed with GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Effect of PACT on C. albicans growth

The effect of PACT, using MB as a photosensitizer, was evaluated on *C. albicans* growth, using different fluences. It was observed a reduction in cell growth after PACT, in a dependent manner on both MB concentration and the fluence used (Fig. 1). A little inhibition was observed using 10 J/cm^2 (Fig. 1A). In this condition, it was observed inhibition of 13, 17 and 20% in the presence of MB 0.005, 0.01 and 0.02 mg/mL, respectively. Increasing the fluence used, this effect was higher. In the presence of MB 0.02 mg/mL and the fluence of 10, 20 and 30 J/cm^2 inhibited ˜20, 48 and 53%, respectively (compare Fig. 1A, B and C). The

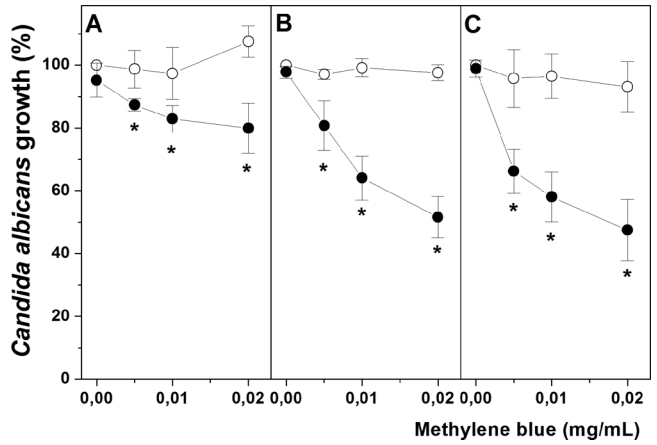

Fig. 1. Effect of different Methylene Blue concentrations on *C. albicans* growth, in irradiated (•) and not irradiated (o) cells. The experimental conditions are described under Materials and Methods. The cell growth was determined 18 h after PACT, using 10 (A), 20 (B) and 30 J/cm^2 (C). Values are expressed as a percentage of the optical density determined at 570 nm (OD_{570}) in treated cells from the optical density measured in the Control group (cells incubated in the absence of MB and not irradiated). The data are mean ± SE (n = 8). * $p < 0.05$.

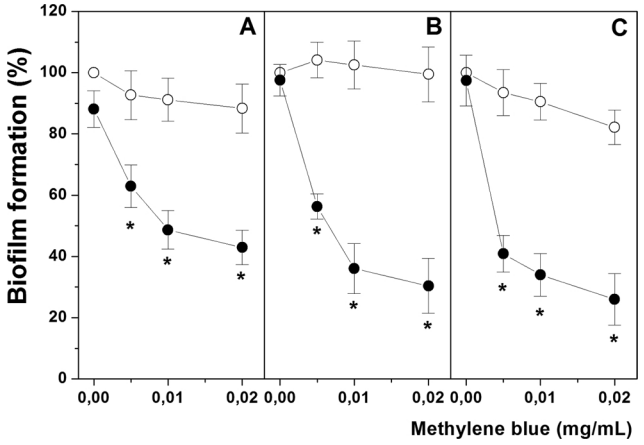

Fig. 2. Effect of different Methylene Blue concentrations on biofilm formation by *C. albicans*, in irradiated (•) and not irradiated (o) cells. The experimental conditions are described under Materials and Methods. The biofilm formation was determined 24 h after PACT, using 10 (A), 20 (B) and 30 J/cm^2 (C). Values are expressed as a percentage of the optical density determined at 490 nm (OD_{490}) in treated cells from the optical density measured in the Control group. The data are mean ± SE (n = 8). * $p < 0.05$.

effect of fluence was better observed in the presence of MB 0.005 mg/mL. In this condition, the ratio of not irradiated/irradiated cells was ˜1.13, 1.20 and 1.44 using a fluence of 10, 20 and 30 J/cm^2, respectively.

3.2. Effect of PACT on biofilm formation by C. albicans

Since the virulence and colonization, characteristics of *C. albicans* has been related to biofilm formation, we also analyzed the effect of PACT with MB using different fluences on the cell ability to form biofilms (Fig. 2). PACT was able to decrease the ability of cells to form biofilms after irradiation depending on the concentration of the photosensitizer and the fluence used. PACT using a fluence of 10 J/cm^2 inhibited ˜37, 52 and 58% of biofilm formation in the presence of MB 0.005, 0.01 and 0.02 mg/mL, respectively (Fig. 2A). The effect of PACT improved when the fluence increased. In the presence of MB 0.02 mg/mL, it was observed inhibition of ˜58, 70 and 74%; using fluences of 10, 20 and 30 J/cm^2, respectively (compare Fig. 2A, B and C). Furthermore, a positive statistical correlation was observed between the inhibition in the growth and the inhibition in the biofilm formation by *C. albicans*, after PACT ($r > 0.90$). Curiously, the effect of PACT was higher on biofilm formation when comparing to *C. albicans* growth (compare the Figs. 1 and 2). Using a fluence of 20 J/cm^2, it was observed a ratio of not irradiated/irradiated cells of ˜1.20, 1.55 and 1.89, in the presence of MB 0.005, 0.01 and 0.02 mg/mL, respectively, in *C. albicans* growth. However, using the same conditions, it was observed a ratio of not irradiated/irradiated cells of ˜1.84, 2.84 and 3.28, in the presence of MB 0.005, 0.01 and 0.02 mg/mL, respectively, in biofilm formation by *C. albicans*. This is an important feature; since *C. albicans* growth was determined using the percentage of optical density, calculated using the control group as 100% of growth. Thus, a 90% reduction in optical density represents a reduction in the number of cells of only 10 times. However, biofilm formation was determined using a percentage of the metabolism of cells present in the biofilm structure. Comparing these results, it is possible to note that the inhibitory effect of PACT was more expressive on the metabolism of cells, reducing the number of cells adhered to the plate. While the effect of PACT on the number of planktonic cells was minor. In addition, to evaluating the effect of PACT with MB on the ability of *C. albicans* to form biofilm, the morphology of biofilm produced was also observed by light microscopy (Fig. 3). It was observed a mixture of yeasts and filaments in the general structure of the biofilm, especially in the control group (biofilm formed from cells

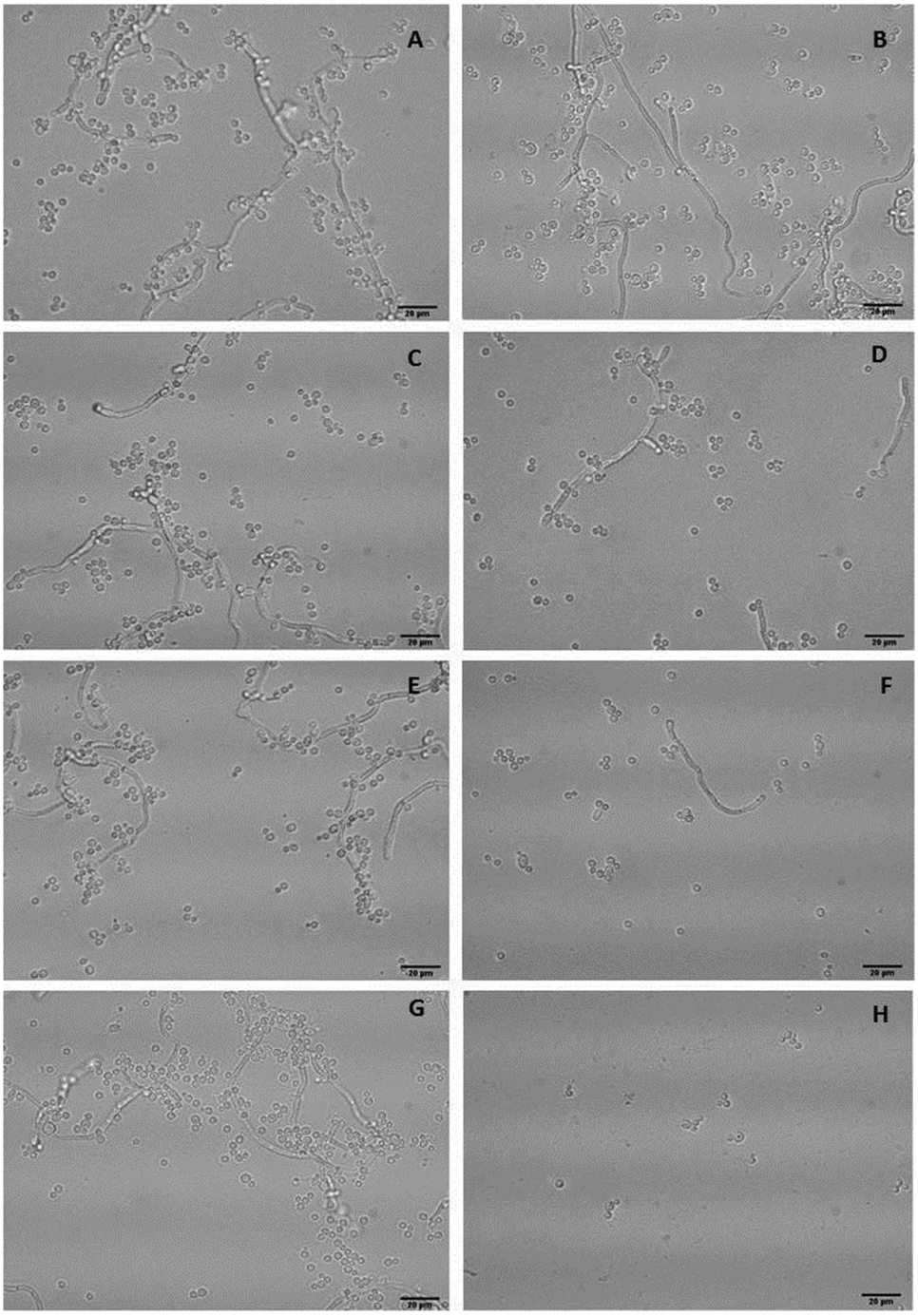

Fig. 3. Analysis of biofilm formation by *Candida albicans*. Biofilms produced by *Candida albicans* were observed in not irradiated (3 A, 3C, 3E and 3 G) and irradiated (3B, 3D, 3 F and 3 H) cells. Biofilm formation was observed in the absence (3 A and 3B) and in the presence of MB 0.005 (3C and 3D), 0.01 (3E and 3 F) or 0.02 mg/mL (3 G and 3 H). Bar, 20 μm.

incubated only with sterile physiological solution), as well as in the cells submitted only to irradiation (absence of MB) and cells incubated with MB only in the dark (absence of irradiation) (compare Figs. 3A, B, C, E and G). However, it was verified that the number of both yeasts and filaments decreased in a concentration-dependent manner of the photosensitizer in the cells submitted to PACT with MB (compare Fig. 3B, D, F and H). In fact, in cells submitted to PACT with MB at the highest concentration (0.02 mg/mL), it was observed the complete absence of filamentous form of *C. albicans*, as well as a significant decrease of its yeast form. These results demonstrated that the inhibitory effect of PACT with MB is also able to interfere with the process of transition from yeast to filamentous form, an essential stage for colonization and virulence by this fungus, characterizing its potential as an antifungal therapy.

3.3. Effect of PACT on the viability of biofilm produced by C. albicans

C. albicans biofilm is recognized for its resistance to the conventional antifungal drugs, so the effect of PACT, with MB on the biofilm viability was determined in biofilm forming at 24 h. A significant reduction in the viability of the 24-hour biofilm produced by *C. albicans* was observed in a dependent manner on MB concentration (Fig. 4). It was observed inhibition of 54, 66 and 55% in the presence of MB 0.01, 0.02 and 0.05 mg/mL, respectively in the viability of biofilm produced

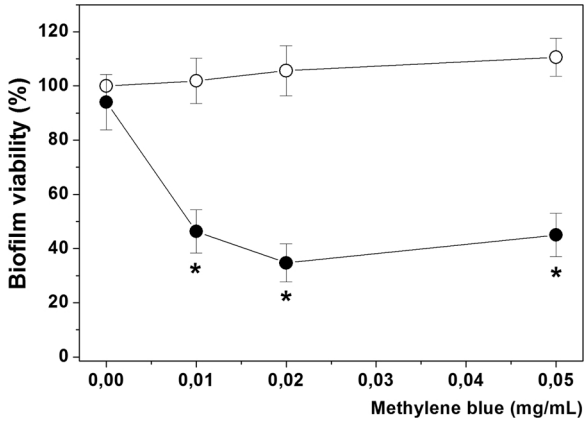

Fig. 4. Effect of PACT on the viability of biofilm produced by *Candida albicans*. The experimental conditions are described under Materials and Methods. Metabolic activity of biofilms was determined immediately after PACT, in irradiated (•) and not irradiated (o) biofilms. Values are expressed as a percentage of the metabolism of biofilms, calculated using the control group (cells not irradiated) as 100% of the metabolism of biofilms. The data are mean ± SE (n = 8). * $p < 0.05$.

by *C. albicans*. In addition, the effect of PACT on the structure of the *C. albicans* 24-hour biofilm was evaluated using light microscopy (Fig. 5). A great reduction in the number of both yeast and filaments after PACT were shown, in a dependent manner on MB concentration. The 24-hour biofilms treated with PACT presented a significant reduction in the number of cells presenting filamentous form. No morphological differences were observed in the control (Fig. 5A and B) and not irradiated groups (Fig. 5A, C, E and G). It is important to note that in the experiments presented in Figs. 4 and 5, the 24-hour biofilms were treated with PACT and, then, both the viability and morphology of the biofilms were determined. Thus, the alterations observed in the structure of biofilms happened right after PACT. These results demonstrated that PACT was able to produce alterations in the cells present in the biofilm structure, decreasing the adhesion to the plate. These results demonstrated that the inhibitory effect of PACT, with MB, is able to overcome the barrier (extracellular matrix) of the biofilm, acting on the filamentous and yeast forms of this fungus, demonstrating an antifungal potential even on the already established biofilm, the more virulent form of *C. albicans*.

3.4. Effect of PACT on the growth kinetics of C. albicans

In order to determine the effect of PACT on growth kinetics of *C. albicans*, a growth time-curve was determined after PACT, with MB (0.005 and 0.02 mg/mL). No effect of either only MB or only irradiation was observed on growth kinetics (Fig. 6). However, PACT was able to change the growth time-curve profile. It was observed a change in the extent of lag phase. Control cells presented a lag phase period of 10 h, although treated cells presented a lag phase period higher than 12 h. Cells treated with PACT using MB 0.02 mg/mL exhibited a lag phase up to 14 h, demonstrating that PACT was able to retard the *C. albicans* growth. Still, it was observed an alteration in the profile of the exponential phase. In control cells it was observed a typical *C. albicans* exponential phase, presenting linear comportment from 14 to 21 h. This result demonstrates a regular growth coefficient during this period (0.131; $R^2 = 0.985$). In addition, the growth coefficient of cells treated with MB 0.005 mg/mL (0.128; $R^2 = 0.9788$) and 0.02 mg/mL (0.1268; $R^2 = 0.992$), but non-irradiated, were similar to the control. Cells treated with PACT using MB 0.005 and 0.02 mg/mL exhibited an initial stage in the exponential phase with reduced growth coefficient (0.1096; $R^2 = 0,955$; 0.0661; $R^2 = 0.970$), observed by the reduction in the growth rate. This initial stage was observed from 14 to 19 h. However, from 19 to 23 h it was observed an increase in the growth coefficient, indicating an increase in the growth rate, comparing to the first stage of the exponential phase. After this period, the cells stay in stationary phase. Stationary phase was observed at 21 h in the control group. Cells treated with PACT using MB 0.02 mg/mL, initiated the stationary phase at 23 h. This result suggests that after PACT occur a delay in *C. albicans* growth, occurring a delay at the beginning of the exponential phase, however, after 19 h the growth rate increase, presenting a higher growth coefficient.

4. Discussion

Novel antifungal and antibiofilm drugs against these unmanageable infections are urgently needed, and PACT has emerged as a promising modality due to its effectiveness against a broad range of species of microorganisms regardless of drug resistance, including *Candida* sp infections [53–61]. In this approach, a photosensitizing agent or dye, which is activated by a light source at a specific wavelength, result in the production of ROS and free radicals, consequently, increase in cellular permeability and subsequent damage to intracellular targets of *Candida sp* [62–65]. The antimicrobial effect of MB as a photosensitizer drug on *C. albicans* has been reported by different studies [47,48,62–67]. In the present study, we showed for the first time the effects of PACT with MB on both formation and the viability of biofilms produced by *C. albicans*, which is the fundamental step for its establishment in biological tissues and resistance to different antifungal agents [13,16,27].

Several factors contribute to the colonization and virulence of *C. albicans*, including its transition from yeast cells to filamentous form, an essential process for biofilm formation on medical devices and host tissue [13,16,27]. In our results, PACT using MB inhibited significantly both cell growth and biofilm formation, suggesting its potential to reduce cell proliferation and colonization of *C. albicans*. The phenomenon of colonization happens primarily from the formation of germ tubes, which will give rise to the filamentous form of this fungus; this form presents adhesion properties that allow them to adhere to different surfaces [23]. Munin et al. [47] demonstrated the inhibition of germ tube formation after PACT, using MB. Thus, the biofilm formation reduction after PACT could be related to the inability of the yeast to form germ tube immediately after treatment. In addition, the results presented here demonstrated that the inhibitory effect on both growth and biofilm formation was higher when high fluence was used (30 J/cm^2, for instance), indicating that the effect of the metabolites produced by PACT was more effective at high fluences. Interestingly, our study demonstrated that the inhibitory effect on both cell growth and biofilm formation was similar at the two highest fluences used; suggesting that the effect of the metabolites produced on the medium by PACT has its maximum effect between 20 and 30 J/cm^2, which prevent cells transition to biofilm formation. Furthermore, the inhibition produced by PACT on biofilm formation was higher than that observed on cell growth (Compare Figs. 1 and 2). This is interesting data since biofilm formation is a key step towards the colonization and virulence of *C. albicans*. Moreover, a reduction of biofilm viability of 24 h of development after PACT suggests cellular damage even in already formed biofilms, which are considered more resistant to conventional therapy. This result corroborates the data presented by other authors. Cernakova et al. [65] reported the PACT efficacy on biofilms formed for 24 h. The same happened with the study of Rossoni et al. [62], where the author reported the effectiveness of PACT in biofilms formed for 48 h. Additionally, in this work, the cells after PACT, both in yeast and filamentous form detach from the structure of biofilm, suggesting that PACT was able to reduce the cell ability to adhere to the surface of the place. This result indicates that PACT is able to not only reduce the viability of biofilm produced but also decrease the cell ability to maintain the integrity of biofilm formation. Taken together, these data demonstrated the ability of PACT, using MB to inhibit both formation

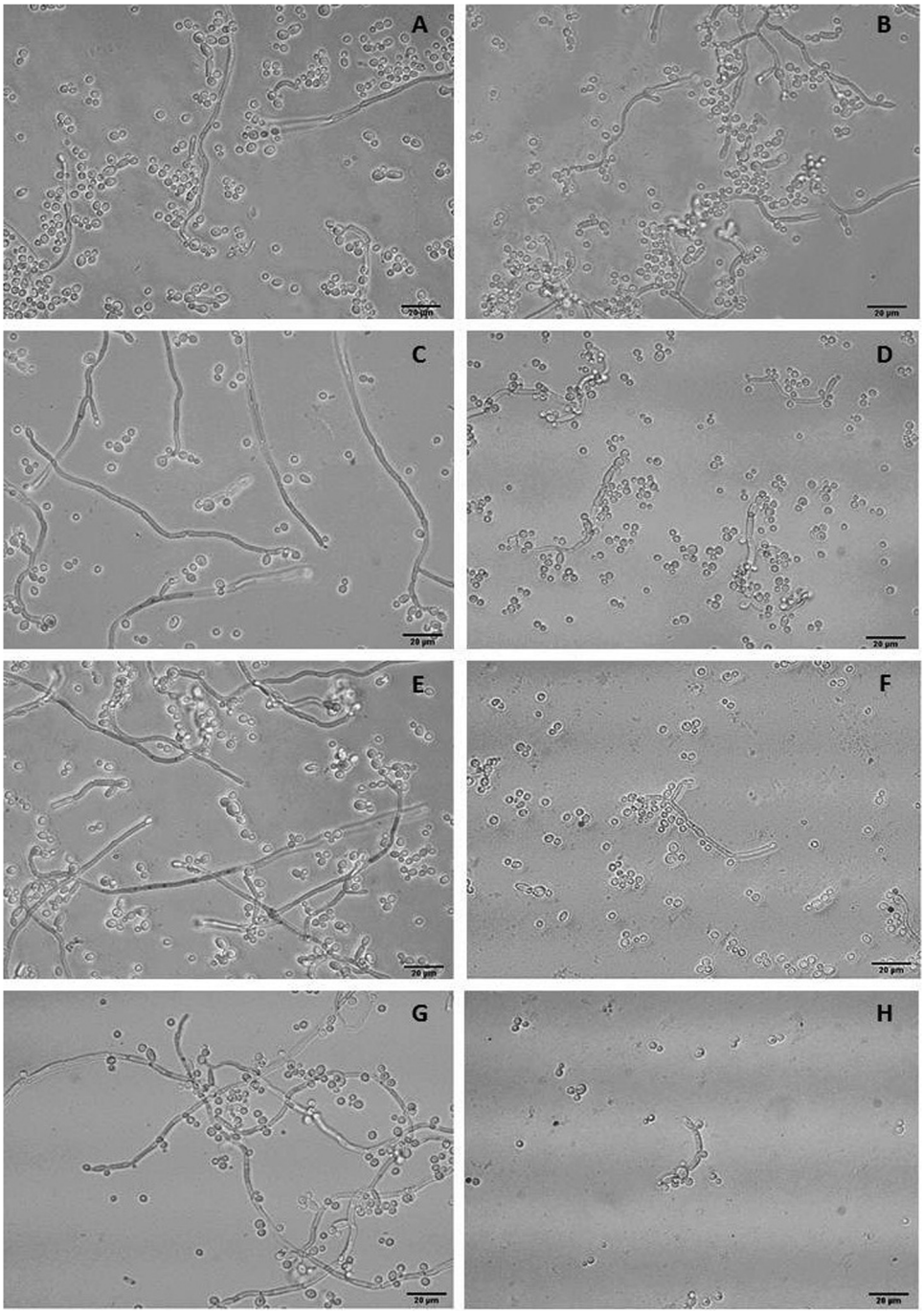

Fig. 5. Analysis of structure of biofilm forming at 24 h, immediately after PACT. Biofilms produced by *Candida albicans* were observed immediately after PACT, in not irradiated (3 A, 3C, 3E and 3 G) and irradiated (3B, 3D, 3 F and 3 H) cells. Biofilm formation was observed in the absence (3 A and 3B) and in the presence of MB 0.01 (3C and 3D), 0.02 (3E and 3 F) or 0.05 mg/ mL (3 G and 3 H). Bar, 20 μm.

and the viability of biofilm produced by *C. albicans*.

The study of growth kinetics of *C. albicans* after PACT demonstrated that PACT was able to change the growth time-curve profile. It was observed an increase in lag phase demonstrating that PACT was able to retard the *C. albicans* growth. In addition, it was observed a reduction in the growth rate in the exponential phase, after PACT. These results demonstrated that PACT was able to reduce the rate of cell proliferation. This is an important point because this fact can modify the intensity of infection produced by *C. albicans*. The effectiveness of MB as a photosensitizer in inhibiting the *C. albicans* growth has been demonstrated by different studies [44,47–49]. However, few studies relating the effect of PACT on both biofilm formation and biofilm viability are reported in the literature. Since biofilm formation is an essential step for colonization and infection produced by *C. albicans*, the development of new and efficient therapies against this fungus is crucial. Thus, the results presented here corroborate that potential of PACT, using MB as potential therapy decreasing *C. albicans* infection.

5. Conclusion

It is concluded that PACT using MB was able to inhibit both growth and biofilm formation by *C. albicans*, probably by a mechanism related to the increase of ROS production, which alter cellular permeability, producing permanent cellular damages. Therefore, PACT using MB

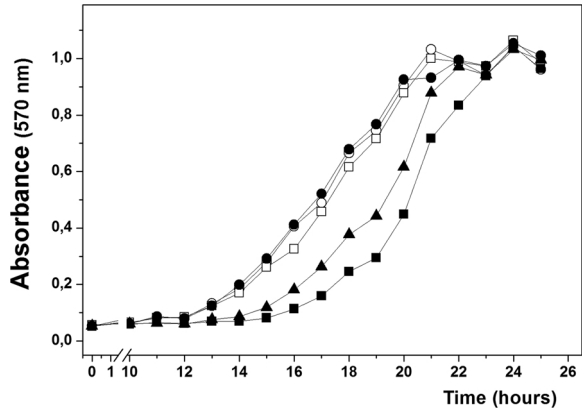

Fig. 6. Effect of PACT on the growth kinetics of *C. albicans*. The experimental conditions are described under Materials and Methods. The cell growth was determined by a time curve in *C. albicans* suspensions in Control group (cells incubated in the absence of MB and not irradiated) (o), not irradiated cells in the presence of MB 0.02 mg/mL and irradiated cells in the absence (•) or in the presence of MB 0.005 (□) and 0.02 mg/mL (■). Values are expressed in absorbance determined by measuring the optical density at 570 nm (OD 570). Data are means ± SD (n = 4). * $p < 0.05$.a.

could be considered as a potential therapy against *Candida albicans* development.

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Ethical approval

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

Conflict of interest statement

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

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.

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