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The Photodynamic Effect of Methylene Blue and Toluidine Blue on *Candida albicans* Is Dependent on Medium Conditions

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Due to the increased number of immunocompromised patients, the infections associated with the pathogen of the genus *Candida* and other fungi have increased dramatically. Photodynamic antimicrobial chemotherapy (PACT) has been presented as a potential antimicrobial therapy, in a process that combines light and a photosensitizing drug, which promotes a phototoxic response by the treated cells. In this work, we studied the effects of the different medium conditions during PACT, using either methylene blue (MB) or toluidine blue (TB) on *Candida albicans*. The inhibition of the growth produced by PACT was decreased for different pH values (6.0, 7.0, and 8.0) in a buffered medium. The phototoxic effects were observed only in the presence of saline (not buffered medium). PACT was modulated by calcium in a different manner using either MB or TB. Also when using MB both verapamil or sodium azide were able to decrease the phototoxic effects on the *C. albicans*. These results show that PACT is presented as a new and promising antifungal therapy, however, new studies are necessary to understand the mechanism by which this event occurs.

***Keywords*:** photodynamic antimicrobial chemotherapy, *C. albicans*, methylene blue, toluidine blue

Due to the increased number of immunocompromised patients, the infections associated with the pathogen of the genus *Candida* and other fungi have increased dramatically in the recent years (Gudlaugsson *et al*., 2003; Morgan *et al*., 2005). A new treatment method, known as photodynamic antimicrobial chemotherapy (PACT), has been presented as a potential antimicrobial therapy. PACT is a process which combines light and a photosensitizing drug, which promots a phototoxic response by the treated cells, usually via oxidative damage (MacDonald and Dougherty, 2001; Jori, 2006). This technique involves the production of highly cytotoxic singlet oxygen and other reactive oxygen species, which promoteing photodynamic microbial damage. The photodynamic effects on the pathogen *Candida albicans* using different photosensitizing drugs have been demonstrated by several authors (Bliss *et al*., 2004; Chabrier-Roselló *et al*., 2005; Demidova and Hamblin, 2005; Lambrechts *et al*., 2005; Cormick *et al*., 2009; So *et al*., 2009); and the fungicide effects of methylene blue as a photosensitizing drug on the *C. albicans* was recently demonstrated (Souza *et al*., 2006; Munin *et al*., 2007; Peloi *et al*., 2008). However, in spite of the growing interest in PACT, the specific mechanisms associated with this are not clear. Different authors have showed that the medium conditions during photodynamic therapy are able to change their effects on the cells. Sharma *et al*. (2005) demonstrated that the extracellular pH can modify the mode of cell death in human colon adenocarcinoma cells subjected to photodynamic treatment with chlorin p6. Böhmer and Morstyn (1985) showed that the uptake of hematoporphyrin derivative from normal and malignant cells increased when the medium pH was decreased from 7.4 to 6.0. This effect was also described by Sharma *et al*. (2004), who demonstrated that both the uptake and phototoxicity of chlorin p6 were increased as the pH of the incubation medium decreased, in human colon adenocarcinoma cells. Uzdensky *et al*. (2007) recently studied the involvement of Ca^{2+}-mediated signaling pathways in photooxidative injury of neurons and glia. Zheng *et al*. (2006) also demonstrated the increase in the intracellular Ca^{2+} concentration after Photodynamic therapy (PDT) using 5-aminolevulinic acid (ALA) on colon cancer cell line SW480. Thus, the purpose of this study was to investigate the effects of the different medium conditions during PACT, using either methylene blue (MB) or toluidine blue (TB) as photosensitizing drugs on the *Candida albicans*.

Materials and Methods

Organisms and growth conditions

Cultures of *C. albicans* strain ATCC 10231 were put on Sabouraud dextrose agar (Merck, country) and incubated in atmospheric air at 37°C. After 48 h of incubation, a sample of the colonies was removed from the surface of the agar plate and suspended in sterile physiological solution (0.85% NaCl) at a cell density of ($1\sim2\times10^5$) viable cells/ml, which was determined using the Neubauer chamber in the pres-

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ence of the vital dye, methylene blue.

Phototoxicity assay of *C. albicans* treated with either MB or TB

Candida suspensions were inoculated in 96 well plate and incubated in the dark for 5 min, at room temperature in the presence of different concentrations of photosensitizer, ranging from 0.01 to 0.1 mg/ml, in a final volume of 0.2 ml. Cells incubated only in sterile physiological solution (0.85% NaCl) were included as a control. In order to determine the consequence of different pH values on the phototoxic effects in *C. albicans*, MOPS-Tris (50 mM) was added to the medium (Saline) during incubation to adjust the pH at 6.0, 7.0 or 8.0. An unbuffered saline medium was included as a control. In the experiments with either verapamil (50 μM) or sodium azide (1 mM), *C. albicans* suspensions were incubated in the presence of these reagents during irradiation, either in the presence or in the absence of the photosensitizer. The light source used was a diode laser (Theralaser, DMC, Brazil), with output power of 0.035 W and a wavelength of 684 nm in the experiments which used MB. When TB was the photosensitizer, a diode laser with output power of 0.030 W and wavelength of 660 nm (model: laser unit, Kondortech) was used. The laser beam illuminated an area of 0.38 cm^2, resulting in an energy dosage of 28 J/cm^2. After the incubation period of 5 min, the cover of the 96 well plate was removed, and the plate was illuminated with the appropriated light at room temperature in accordance with Munin *et al*. (2007). Aliquots of 50 μl were taken before and after illumination and inoculated in a 24 well plate containing Sabouraud dextrose broth medium (Merck) (2 ml). After 18 h of incubation at 37°C, the medium was properly homogenized and the optical density at 570 nm (OD_{570}) was measured using a Synergy HT Multi-Detection Microplate Reader (Bio-Tek, USA), in order to determine the Candida growth. The experiments were performed in the dark and under sterile conditions. The values presented in the figures represent the percentage of growth, calculated using the control group as 100% of growth.

Statistical analysis

Values were expressed as Means±SD. Statistical differences were evaluated by analysis of variance (ANOVA) and post hoc comparison with the Tukey-Kramer test. P values <0.005 were considered significant.

Results

As previously reported by our group (Munin *et al*., 2007), 0.05 mg/ml is the optimal MB concentration to inhibit 80∼90% of the *C. albicans* grown after irradiation (Fig. 1A). Using TB, the concentration necessary to inhibit 80∼90% of the *C. albicans* grown was 0.1 mg/ml (Fig. 1B). This inhibition was observed in a unbuffered medium containing only *C. albicans* suspension, the photosensitizing drug and saline. When the PACT was performed a buffered medium (MOPS-Tris added to the medium to control pH values), a significant decrease in the phototoxic effects was observed, when either MB or TB (Fig. 1) were used. This effect occurred in buffered medium at pH 6.0, 7.0 or 8.0. Curiously, in a buffered medium, at pH 7.0, phototoxic effects were similar to not buffered medium (physiological solution, pH ∼7.4). Also, the phototoxic effects were not observed when saline was substituted for Sabouraud dextrose broth medium during irradiation (data not shown). These results demonstrate that when using either MB or TB as photosensitizing drugs, the phototoxicity effects are not related to the specific pH values of the medium, but to the ability of the medium to permit changes in the pH values.

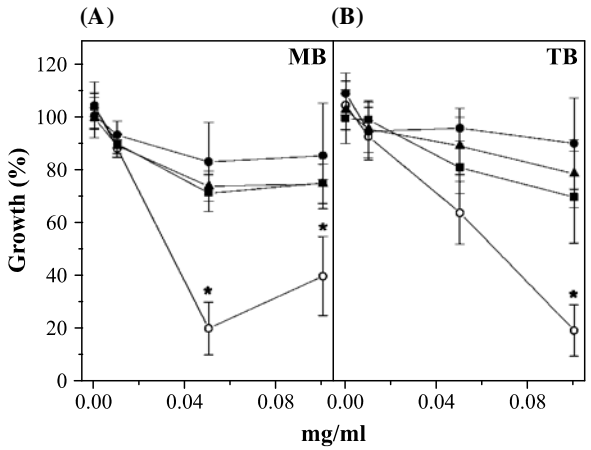

Fig. 1. Effect of different concentrations of methylene blue (A) and toluidine blue (B) on the *C. albicans* growth. The assay medium composition and experimental conditions were as described in the methods section, at pH 6,0 (●); pH 7,0 (▲); pH 8,0 (■); e saline (○). The values are expressed as a percentage of growth determined by the presence of the different photosensitizer concentrations from the total growth measured in the absence of photosensitizer. Data are Means±SD (n=10). The asterisk represents the conditions which presented statistical differences between control and experimental groups.

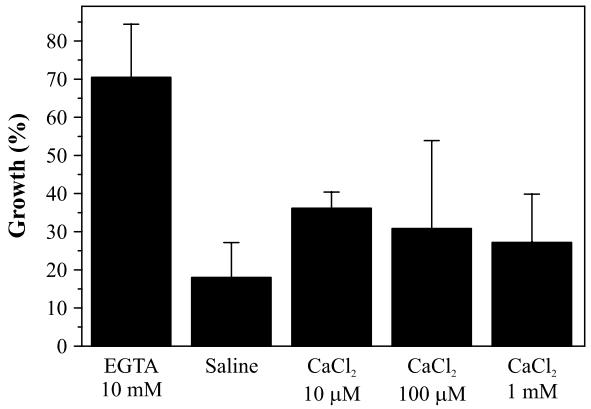

Fig. 2. Effect of different calcium concentrations on the *C. albicans* growth, using methylene blue. The assay medium composition and experimental conditions were as described in the methods section. The values are expressed as a percentage of growth determined by the presence of the MB (0.05 mg/ml) from the total growth measured in the absence of MB. Data are Means±SD (n=8).

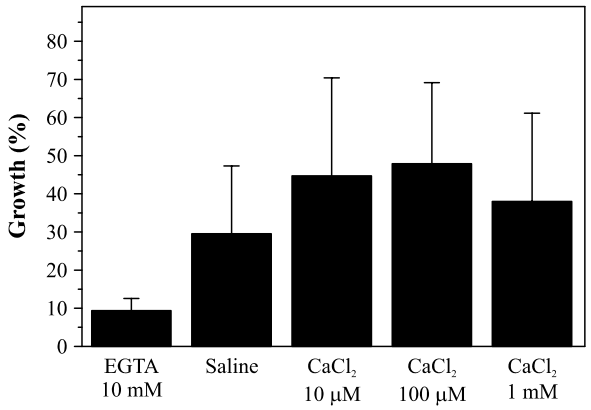

Fig. 3. Effect of different calcium concentrations on the *C. albicans* growth, using toluidine blue. The assay medium composition and experimental conditions were as described in the methods section. The values are expressed as a percentage of growth determined by the presence of the TB (0.1 mg/ml) from the total growth measured in the absence of TB. Data are Means±SD (n=8).

In order to investigate the calcium dependence on PACT, we studied the phototoxicity effects of either MB or TB on the *C. albicans* growth in the presence of different calcium concentrations. It was observed that the inhibition of the *C. albicans* growth, which was promoted by PACT, using MB, was diminished in the presence of EGTA (10 mM) (a calcium chelating agent), when compared to the control medium (only physiological solution) (Fig. 2). This result demonstrates that the MB phototoxicity effects are dependent on the extracellular calcium presence. However, using TB as a photosensitizing drug, the addition of EGTA (10 mM) during the irradiation promoted an increase in the PACT effects (Fig. 3). These results indicate that MB and TB are able to promote the inhibition of *C. albicans* growth by different mechanisms. The increase of the calcium concentrations in the medium did not significantly modify the effects

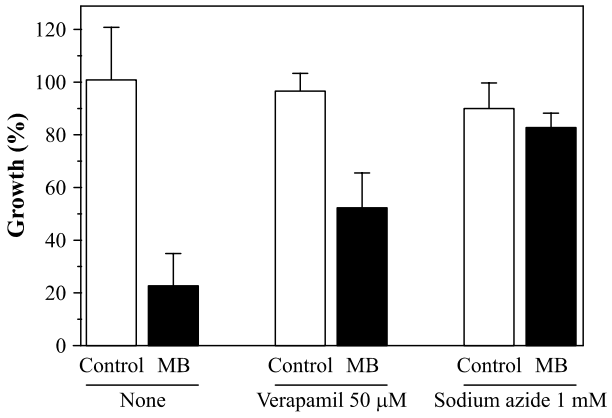

Fig. 4. Effect of either verapamil or sodium azide on PACT, using MB (0.05 mg/ml) in *C. albicans*. The assay medium composition and experimental conditions were as described in the methods section. The values are expressed as a percentage with the saline group as a control (100%). Data are Means±SD (n=6).

of PACT when either MB or TB (Fig. 2 and 3) were used. In order to study the mechanism by which PACT is able to inhibit *C. albicans* growth, the phototoxicity effects were determined in the presence of verapamil (a blocker of Ca^{2+} channels in mammalian cells) and sodium azide (a known quencher of singlet oxygen) with MB as a photosensitizing drug. The presence of verapamil (50 μM) during irradiation the inhibition of *C. albicans* growth was diminished (Fig. 4), which show the importance of the plasma membrane calcium channels to the PACT mechanism. When the PACT with MB was performed in the presence of sodium azide (1 mM) the phototoxicity effects on *C. albicans* growth were completely eliminated (Fig. 4). In addition, DMSO, a hydroxy radical scavenger, was also able to decrease the inhibition produced by PACT (with MB) on the *C. albicans* (data not shown). These results demonstrate the involvement of reactive oxygen species (ROS) on the production of phototoxicity damage in the cells.

Discussion

Several authors have shown the increase both in the number of reports relating to the resistance to all available antifungal agents (Horsburgh and Kirkpatrick, 1983; Barchiesi *et al*., 1994; Ruhnke *et al*., 2000; Pfaller *et al*., 2002; Bennett *et al*., 2004; Sanguinetti *et al*., 2005; Posteraro *et al*., 2006; Cannon *et al*., 2007) and in the prevalence of both mucocutaneous and systemic infections in immunocompromised patients by Candida (Calderone, 2002). Thus, the development of more effective antifungal therapies is crucial. A new treatment modality, known as photodynamic antimicrobial chemotherapy has been presented as a potential antimicrobial therapy (Jori, 2006). In this process, the microorganisms are irradiated with low-intensity visible light, in an appropriate wavelength, and then are treated with a photosensitizer, which produces cytotoxic reactive oxygen species and promotes photodynamic microbial damage (MacDonald and Dougherty, 2001; Jori, 2006). In recent years, many authors have demonstrated the effects of different photosensitizer drugs, such as toluidine blue (Bhatti *et al*., 1998; Tegos and Hamblin, 2006) and methylene blue (Gad, 2004; Peloi *et al*., 2008) on the microbial eradication. The use of PACT in superficial infection has been demonstrated by several authors (Hamblin *et al*., 2002; Komerik *et al*., 2003; Lambrechts *et al*., 2005), which suggest that photodynamic therapy is a promising therapy in the treatment of superficial infections. However, it is necessary to understand the mechanisms by which PACT promotes microbial eradication. Our results demonstrate that the medium is important for permiting the phototoxic effects on the cells. In a buffered medium, the phototoxic effects, with either MB or TB, dramatically decreased. These results demonstrate the ability of the medium in controlling the pH can decrease the phototoxic effects, which suggests that the PACT efficiency could be decreased in a medium which is able to control the pH. The external calcium dependence was also shown, when MB was used as a photosensitizer. The use of either EGTA or Verapamil in the medium during the irradiation significantly decreased the *C. albicans* eradication. It was observed that low concentrations of calcium are crucial to PACT efficacy, using MB.

These results are in accordance with other authors who have demonstrated that PDT is able to promote an increase in the cytosolic free calcium concentration which leads to cell death (Almeida *et al*., 2004). Curiously, when using TB, the presence of EGTA during the irradiation promoted an increase in the *C. albicans* eradication; this shows that MB and TB can produce phototoxic effects through different mechanisms. The important role of the singlet oxygen promoting cell death in the PACT mechanism has been proven (Hamblin *et al*., 2001; Smijs *et al*., 2007). Our results showed that sodium azide abolished the phototoxic effects of MB on *C. albicans*, which indicates that singlet oxygen production is crucial to the PACT event. Taken together, these results show that, although PACT is presented as a new promising antifungal therapy, new studies are necessary to understand the mechanism by which this event occurs in order to amplify its effects on microorganisms during infections.

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