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Glucose modulates antimicrobial photodynamic inactivation of *Candida albicans* in biofilms

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ABSTRACT

Candida albicans biofilm is a main cause of infections associated with medical devices such as catheters, contact lens and artificial joint prosthesis. The current treatment comprises antifungal chemotherapy that presents low success rates. Photodynamic inactivation (PDI) involves the combination of a photosensitizing compound (PS) and light to generate oxidative stress that has demonstrated effective antimicrobial activity against a broad-spectrum of pathogens, including *C. albicans.* This fungus senses glucose inducing an upregulation of membrane transporters that can facilitate PS uptake into the cell. The aim of this study was to evaluate the effects of glucose on methylene blue (MB) uptake and its influence on PDI efficiency when combined to a red LED with central wavelength at λ= 660 nm. *C. albicans* biofilms were grown on hydrogel disks. Prior to PDI assays, MB uptake tests were performed with and without glucose-sensitization. In this system, the optimum PS administration was determined as 500 μM of MB in contact with the biofilm during 30 min before irradiation. Irradiation was performed during 3, 6, 9, 12, 15 and 18 min with irradiance of 127.3 mW/cm^2. Our results showed that glucose was able to increase MB uptake in *C. albicans* cells. In addition, PDI without glucose showed a higher viability reduction until 6 min; after 9 min, glucose group demonstrated a significant decrease in cell viability when compared to glucose-free group. Taken together, our data suggest that glucose is capable to enhance MB uptake and modulate photodynamic inactivation of *C. albicans* biofilm.

1. Introduction

Infections caused by *Candida* species are still a challenge for patients either suffering from local or systemic infections. The biofilm formed by fungi, especially by the pathogen *Candida albicans,* is a main cause of infectious diseases on skin and mucosa, as well as disseminated infections related to colonization of medical devices, such as catheters [1,2]. Biofilms are defined as three-dimensional microbial communities adhered onto surfaces. Microorganisms in biofilms produce an organized extracellular polysaccharide matrix (EPM) composed by sugars and proteins that often functions as a barrier for permeation of chemical substances, such as antimicrobials and photosensitizers. More mature forms of biofilms can yet assume highly complex architectures containing pockets and water channels that form a network of metabolite exchange between different organisms. Thus, besides the available treatments for the management of candidiasis, new therapies are urgently needed to eliminate fungal biofilms.

Biofilm maturation occurs in presence of nutrient circulation that encourages further microbial growth and EPM synthesis and, therefore, biofilms continuously become more resistant to the penetration of chemicals [3,4]. Furthermore, approximately 1% of cells in biofilms are intrinsically resistant to antimicrobial agents at concentrations much higher than the minimum inhibitory concentration from average cells [5]. These cells, often referred as

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persisters, are usually located in deep biofilm regions with low nutrient concentration and remain in a vegetative state of low metabolic activity. When concentrations of carbon sources are increased, the metabolism of these cells can return to normal levels of activity [6].

Photodynamic inactivation (PDI) has been reported as an effective method to inactivate a broad spectrum of pathogens [7–9], including microorganisms that are highly resistant to conventional antimicrobials [10] and biofilms [11,12]. In fact, our group showed that up to 6-log of yeast reduction in planktonic suspension was obtained when PDI was mediated by methylene blue (MB) and red light [7]. However, the inactivation of *C. albicans* cells organized in biofilms remains as a challenge [13], where impaired photosensitizer (PS) uptake by cells could play an important role [14].

C. albicans uses glucose as a carbon source, and glucose sensing and response can upregulate expression of hexose transporters (over than 20 types) to increase glucose uptake rate [15,16]. HGT4 membrane sensor of glucose generates a signaling cascade that induces *hgt* genes encoding hexose transporters expression, and this mechanism may facilitate cellular PS uptake. Those transporters, members of the major facilitator superfamily (MFS), when inhibited by INF_{271} decrease MB uptake in *C. albicans* by 70%, resulting in less photodynamic inactivation efficiency [17].

In this study, our purpose was to develop a standardized and reproducible *C. albicans* biofilm in hydrogel disks to investigate its susceptibility to PDI mediated by MB and red light prior and after exposure to glucose solution. The role of glucose in MB uptake by cells within biofilm was investigated using two different methods: fluorescence spectroscopy and confocal microscopy. After, we explored whether glucose could enhance PDI inactivation and induce structural changes in *C. albicans* biofilms.

2. Methods

2.1. Biofilm formation

C. albicans ATCC 10231® was aerobically grown on Sabouraud dextrose agar for 24 h at 37 °C. Then, colonies were collected, placed in 50 mL falcon tube with 10 mL of yeast nitrogen base broth with 50 mM dextrose (YNB modified) and incubated at 37 °C for another 24 h [18]. The cells were washed twice in phosphate buffered saline (PBS, pH 7.4) solution and the inoculum was prepared with approximately 2×10^6 colony-forming units per mL (CFU/mL). Meanwhile, 4 mm diameter hydrogel flat disks of poly-(metyl-metacrylate) (Acuvue 2 soft contact lenses, Johnson & Johnson Vision Care) were incubated in 100 μL of fetal bovine serum during 24 h. Thereafter, the disks were individually transferred to 96-well flat bottom plates with 100 μL of inoculum and incubated for 90 min; this procedure was performed to deposit proteins onto hydrogel surface and facilitate adherence of the yeast cells. Non-adherent cells were gently washed with PBS and the wells were filled with 100 μL of modified YNB and incubated for 48 h at 37 °C to allow biofilm growth. Following 48 h of biofilm maturation, non-adherent cells were washed immediately before MB uptake and PDI experiments. To evaluate biofilm growth, samples were analyzed by low-vacuum scanning electronic microscopy (SEM, TM 3000, tabletop microscope, Hitashi, Krefeld, Germany) without further sample preparation and biofilm thickness was measured by optical coherence tomography (OCT, λ = 930 nm, FWHM = 100 nm, P = 2 mW, OCP930SR, Thorlabs, EUA). The system has a hand probe coupled to fiber optics, where biofilm samples were placed underneath for data collection. Resolution for this system is of 6.2 μm and maximum image depth of 1.6 mm (in air). Images were processed by ImageJ software, version 1.47 (National Institutes of Health, USA).

2.2. Methylene blue uptake

A stock solution of MB (Sigma-Aldrich, MO, USA) was prepared in distilled water at a concentration of 10 mM, filtered through a sterile 0.22 μm filter membrane and stored in the dark for a maximum of 4 weeks. In order to determine an appropriate MB concentration for PDI studies, the biofilms were immersed in 200 μL of MB at 100 μM, 500 μM or 1 mM for 30 min. Thereafter, the samples were gently washed with PBS to remove excess of MB and subsequently placed individually in 96-well plates. A 200-μL aliquot of 0.1 M NaOH and 1% sodium dodecyl sulfate was added to each well containing samples to dissolve the biofilm cells. After 24 h resting in orbital shaker (60 rpm) at 37 °C [19], each sample was mechanically homogenized with micropipettes, and the respective supernatant was individually included in Eppendorf tubes following 3 min of centrifugation at 2000 G. A hundred-μL of each sample supernatant was recovered and placed in a 96-well plate for MB fluorescence analysis (excitation at λ_{exc} = 532 nm and emission at λ_{em} = 690 nm) using a proper spectrophotometer (Spectramax M4, Molecular Devices, Sunnyvale, CA – USA).

The influence of glucose on MB uptake in biofilm was also evaluated. Before incubation with MB, the biofilm samples were immersed in 200 μL of 50 mM D(+) glucose (Sigma-Aldrich MO, USA) filtered solution for 90 min. After this procedure, samples were submitted to the same treatment describe above for MB: alkaline cell lysis, centrifugation, supernatant recovery, and fluorescence analysis. Absorbance spectra of MB and MB + glucose were also obtained to verify if glucose could modify any MB optical properties.

The localization and distribution of MB inside glucose-pretreated and glucose-free biofilms were evaluated by confocal scanning laser microscopy (CSLM, Leica TCS-SP8, Leica Microsystems, Wetzlar, Germany). Samples were incubated with Hoechst 33342 (5 μg/mL, 30 min) (Invitrogen, Molecular Probes, USA) and CdTe-Concanavalin-A (CdTe-(Con-A)) quantum-dots (5 μM, 30 min) [20]. In the CSLM channel 1, the fluorescence of Hoechst 33342 nuclear stain (λ_{exc} = 405 nm, λ_{em} = 461 nm (±1 nm)) was acquired, and in channel 2 the fluorescence of CdTe-(Con-A) was obtained to mark the biofilm EPM and cellular wall (λ_{exc} = 405 nm; λ_{em} = 605 nm (±1 nm)). Finally, using channel 3 we measured the fluorescence of MB (λ_{exc} = 532 nm; λ_{em} > 690 nm, long pass). All fluorescence values were normalized in respect to the fluorescence intensity at the initial read position (depth = 0 μm). Quantitative analysis of fluorescence by confocal microscopy was performed using the software LAS AF (Leica Microsystems, Germany), using a standard biofilm area (3366 μm^2) and fluorescence intensity in relation to depth of scan (Z axis).

2.3. Biofilm susceptibility to PDI

The biofilm grown for 48 h on hydrogel disks were individually immersed in 96-well plate containing 200 μL of PBS (glucose-free groups) or 200 μL of 50 mM glucose solution in PBS for 90 min (glucose groups). After this period, all samples (except for control groups) were left in 200 μL of 500 μM MB-PBS solution and pre-irradiation time was set in dark conditions for 30 min. Following, samples were gently transferred to Petri dishes to perform irradiation.

Control groups without any treatment (MB or light), groups exclusively with MB (time = 0) or light and PDI groups were analyzed. To verify effects of PDI, samples were exposed to 3, 6, 9, 12, 15 and 18 min, which resulted in radiant exposures of 23, 46, 69, 92, 115 and 138 J/cm^2, respectively. A light-emitting diode (LED) device emitting at λ = 660 ± 20 nm and 260 mW of output power, measured by a power meter (Fieldmate, PM10, Coherent, CA, USA), was placed 1.5 cm above the samples illuminating an area of 0.126 cm^2

and providing a constant irradiance of 127.3 mW/cm^2, which uniformly covered all biofilm surface. Following irradiation, biofilms on hydrogel were transferred to Eppendorf tubes with 200 μL of 1 mg/mL XTT solution in PBS for XTT reduction assay. The XTT or 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide is a tetrazolium salt used to measure mitochondrial metabolic activity. The reduction of XTT in the presence of menadione results in a production of formazan dye, and the amount of this product formed is quantified by a spectrophotometer as optical density of the solution in the wavelength of λ= 492 nm [18]. The XTT stock solution was constituted by 8 mL of PBS, 100 μL of 1 mg/mL XTT (Sigma-Aldrich, MO, USA, cat. # X4251) and 8 μL of 1 mM menadione (Sigma-Aldrich, MO, USA, cat. # M5625). Biofilm samples were included in Eppendorf tubes with 200 μL of XTT solution and incubated for 5 h in the dark, at 37 °C. After this period, the tubes were vortexed for 1 min, centrifuged at 2000 G for 5 min and the supernatant was withdrawn, placed in 96-well plates and the optical density was measured by a spectrophotometer (Spectramax M4, Molecular Devices, Sunnyvale, CA – USA at λ= 492 nm). All data values were normalized relative to control mean values and are presented as cell viability (%).

2.4. Statistical analysis

All data obtained were submitted to statistical analysis, which was performed using one-way analysis of variance (ANOVA). Mean comparisons were carried out with Tukey's post-test and the overall significance level was set at $p < 0.05$ [21]. Data are presented as mean values ± standard deviation (SD).

3. Results

3.1. Biofilm formation

We obtained reproducible biofilm cultures showing *C. albicans* cells in yeast and filamentous forms, embedded in EPM. The mean thickness obtained by OCT imaging, from three measurements of 4 samples, was 56 ± 10 μm.

3.2. Methylene blue uptake

We previously evaluated different periods of incubation with MB using 96-well flat bottom plates as substrate to form the biofilms (n = 3 per group), instead of hydrogel disks. The remaining methodology was kept the same. In this assay, we verified that incubation of biofilms with 500 μM of MB for 5 min, 10 min and 30 min, the fluorescence signal acquired were 10.3 ± 2.7 a.u. (arbitrary units), 16.6 ± 3.0 a.u. and 20.7 ± 2.0 a.u., respectively. MB uptake in biofilm was influenced by time of incubation, and significant difference was observed between 5 min and 30 min ($p < 0.05$), but not between 10 min and 30 min ($p > 0.05$). Thus, using 30 min of incubation time we evaluated different MB concentrations to propose the optimum condition for MB uptake into *C. albicans* biofilms (Fig. 1). Higher PS uptake was also reached by increasing MB concentration, as expected. Although 500 μM and 1 mM showed significantly higher uptake when compared to 50 μM, 100 μM, and 250 μM ($p < 0.05$), no statistically significant difference between them (500 μM and 1 mM) was observed ($p > 0.05$). Thus, we continued the experiments using 500 μM MB and 30 min of incubation time to verify the influence of glucose in MB accumulation into biofilms.

The sample containing glucose showed statistically significant higher fluorescence signal intensity compared to sample only exposed to MB (119.9 ± 27.7 a.u. and 81.8 ± 6.7 a. u., respectively; $p < 0.05$) indicating greater MB uptake in the biofilms pretreated with glucose (Fig. 2A). Furthermore, we obtained absorbance spectra of MB solution and MB + glucose solution (MB concentration = 50 μM) and glucose did not alter MB optical characteristics (Fig. 2B), showing no signs of MB aggregation or reduction.

To identify the localization of MB within the biofilm, we used specific fluorescent probes to label cell nucleus (Hoechst 33342)

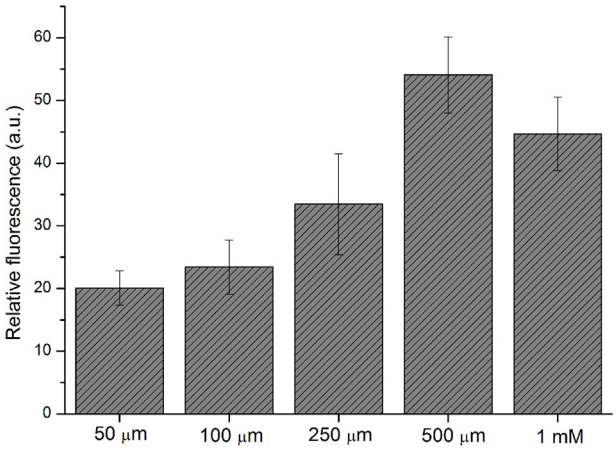

Fig. 1. Mean values ± SD of relative fluorescence obtained from *C. albicans* biofilms after 30 min exposure to different concentrations of MB. Excitation was set at λ_{exc} = 532 nm and emission at λ_{em} = 690 nm.

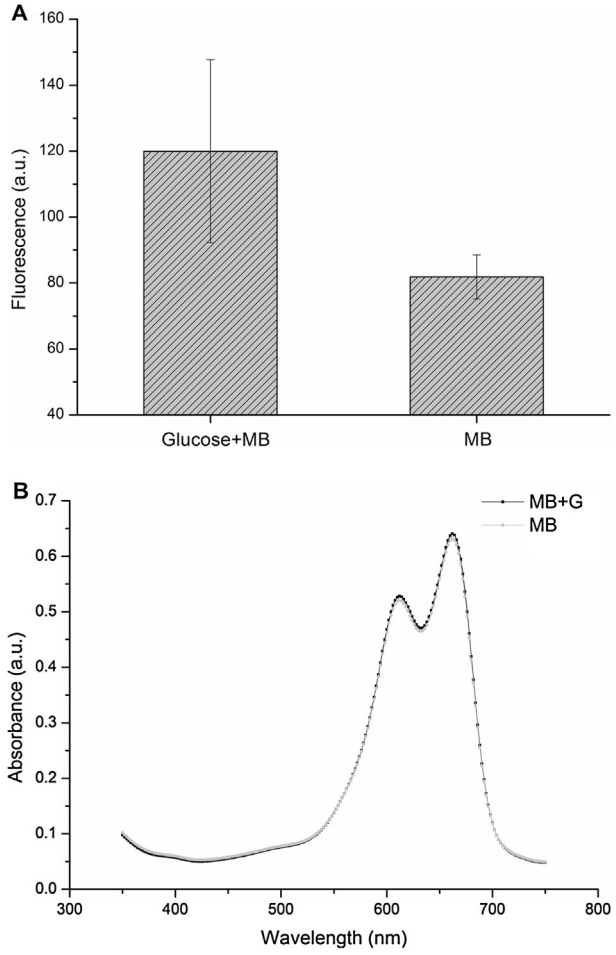

Fig. 2. Mean values ± SD of relative fluorescence of MB in *C. albicans* biofilms pretreated or not with glucose (A). Absorbance of MB (50 μM) in PBS and glucose solution (B). Excitation was set at λ_{exc} = 532 nm and emission at λ_{em} = 690 nm.

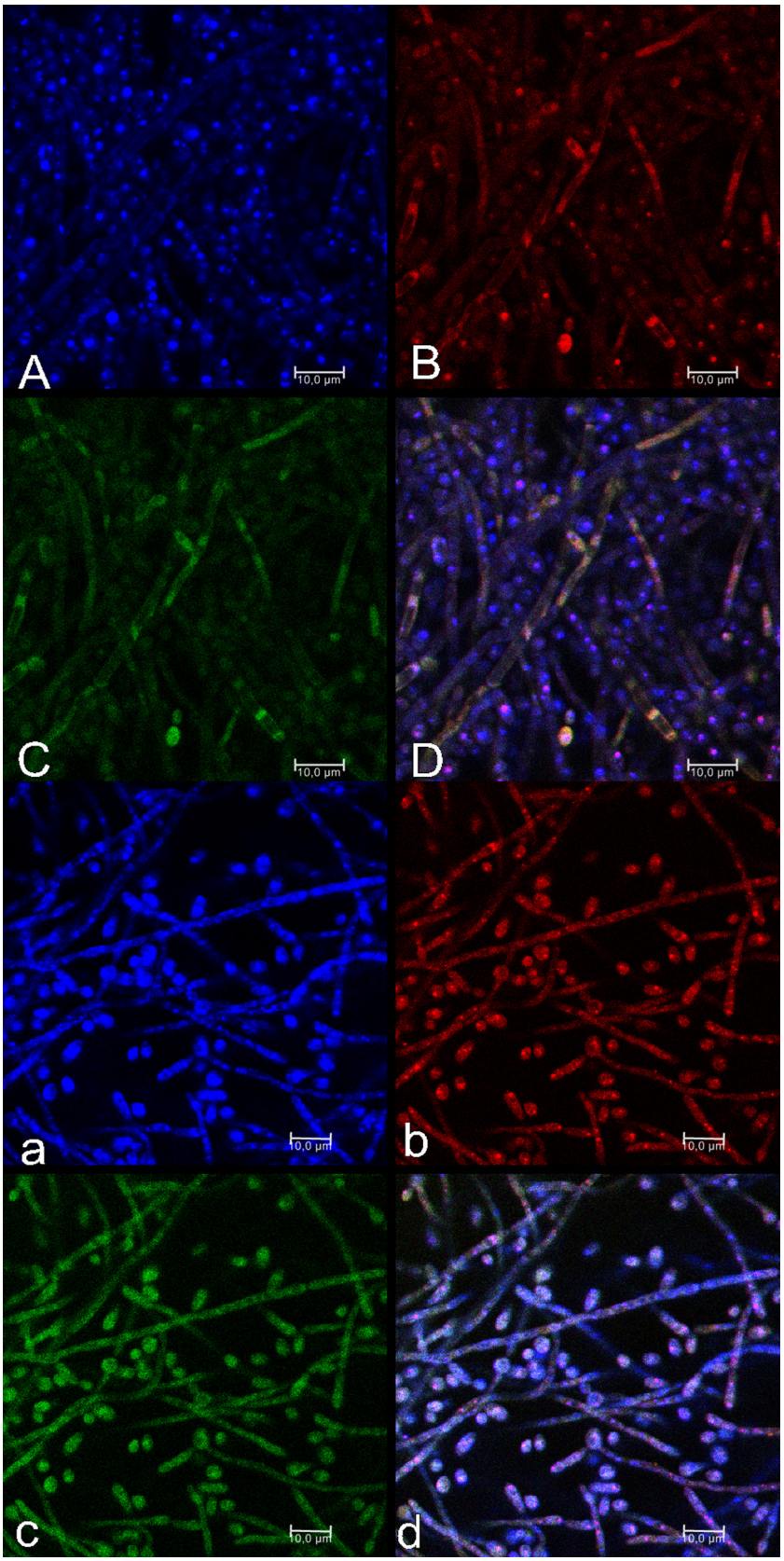

Fig. 3. Confocal fluorescence micrographs of *C. albicans* biofilms sensitized by methylene blue (500 μM MB) during 30 min without glucose (A–D) and with 50 mM glucose pretreated for 90 min (a–d). Nucleus fluorescence is shown in blue (A and a), extracellular matrix in red (B and b) and MB fluorescence in green (C and c). An overlay of the images is presented in D and d. Scale bar = 10 μm.

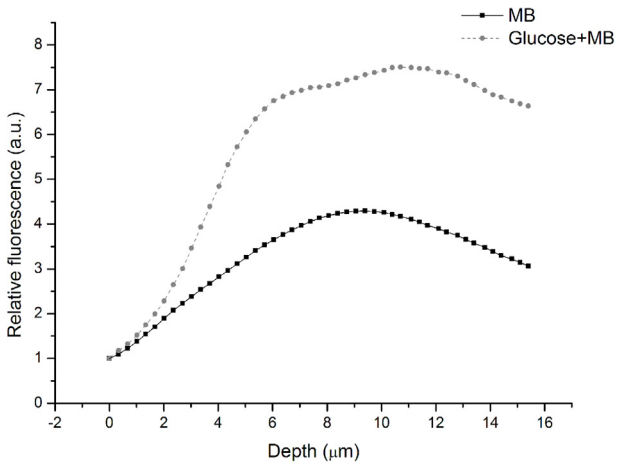

Fig. 4. Relative fluorescence of MB in *C. albicans* biofilms, pretreated or not with glucose, as a function of the biofilm depth.

and cell wall plus extracellular matrix (CdTe-(Con-A)). Fig. 3 displays the CSLM images of *C. albicans* biofilms incubated in 500 μM MB solution. Nuclei stain fluorescence are presented in the blue channel of fluorescence, cell wall and EPM are shown in red channel and MB is presented in green channel. Comparing images obtained from glucose-free and glucose-sensitized biofilms (Fig. 3, capital letters and lower cases, respectively), it is noticed the higher fluorescence signal intensity of MB in glucose-pretreated biofilms. Besides, overlaid images showed intense MB incorporation into the cell wall and EPM in both samples, but uptake in the nuclei was only observed in glucose-sensitized biofilms.

We also evaluated the distribution of MB inside the biofilm. The samples that had previous contact with glucose for 90 min presented greater permeability since higher MB uptake in deeper regions of the biofilm was observed (Fig. 4). We read up to 14 μm in biofilm depth, since deeper regions presented intense fluorescence emitted by the hydrogel in the MB acquisition channel. The highest signal intensity was detected around 9.5 μm in depth and it was about 40% higher in glucose-sensitized biofilms.

3.3. Biofilm susceptibility to PDI

Control biofilms pretreated or not with glucose showed similar formazan absorbance at λ = 492 nm (0.2953 ± 0.047 and 0.2836 ± 0.023, respectively) demonstrating that glucose for 90 min was not able to induce growth or basic metabolic alterations of *C. albicans* cells. When performing MB-mediated PDI on *C. albicans* biofilms, we observed greater reduction in cell viability by increasing the time of irradiation (Fig. 5A). However, the highest antimicrobial effect was observed after 15 min of light exposure whereas 18 min of irradiation did not enhance cell killing ($p > 0.05$). Next, we explored whether glucose might potentiate PDI with MB, since glucose increased MB accumulation by biofilms. Similar to PDI alone, PDI + glucose also decreased cell viability as irradiation time increased and no additional killing was observed after 12 min of light exposure ($p > 0.05$).

We also compared the antimicrobial effect of PDI in the presence or absence of glucose. At 6 min of irradiation, PDI with glucose promoted a less pronounced effect than PDI alone ($p < 0.05$, Fig. 5A). However, after 12 min of irradiation, the group pretreated with glucose showed a statistically significant reduction of about 85% while PDI without glucose pretreatment presented only about 70% ($p < 0.05$). After 15 and 18 min of irradiation, all groups showed similar killing effect. No significant reduction of cell viability was

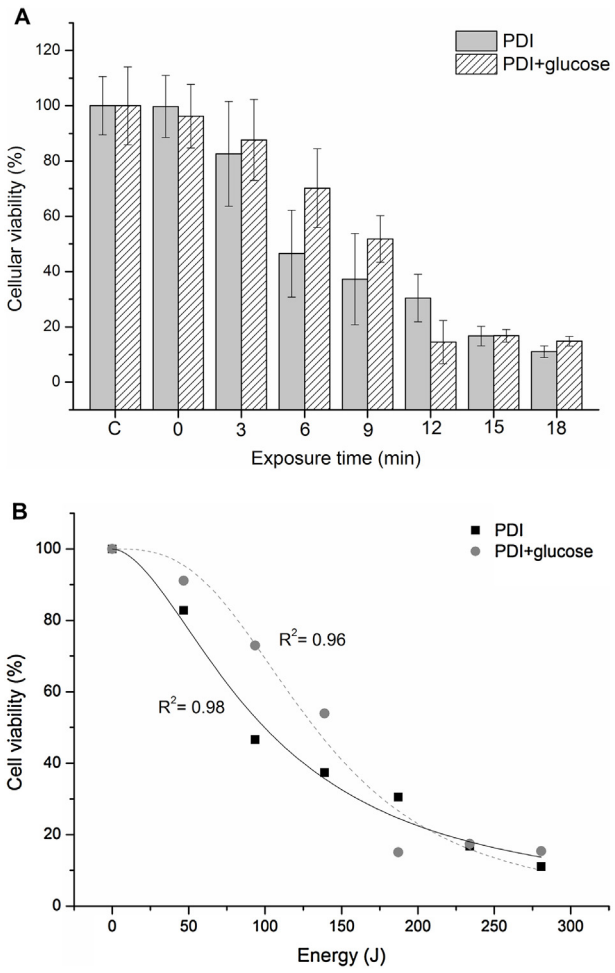

Fig. 5. Mean values ± SD of *C. albicans* biofilm viability following PDI with or whithout glucose-pretreatment. C refers to control group without MB or light exposure and 0 refers to MB group with no irradiation (A). Dose-response curve of *C. albicans* biofilms following PDI (B).

observed on biofilm treated only with light or with MB alone in the presence or absence of glucose.

To further understand the role of glucose in cell viability of *C. albicans* biofilms, we fitted a dose-response curve to correlate cellular viability and light energy (Fig. 5B). Our findings show that the dose (E) response to cell viability (CV) can be expressed by:

$$CV(E) = CV_{\min} + \frac{CV_{\max} - CV_{\min}}{1 + \left(\frac{E}{E_{50}}\right)^{k}}$$

where CV is the percentage of cell viability, CV_{max} is the maximum cell viability (set at 100%), CV_{min} is the minimum cell viability (set at 0), E is the delivered energy, E_{50} is the energy necessary to reduce viability 50% and k is the steepness of the curve. Interestingly, the energy necessary to reduce 50% cell viability is about 30% higher in PDI glucose-sensitized biofilms (132.3 J compared to 99.7 J), but for 99% of inactivation energy is 2.1 times lower (640 J and 1323.7 J, respectively).

Following the XTT assay, biofilms from before and after PDI were analyzed by SEM imaging (Fig. 6). As expected, the biofilm grew with a complex organization, including layers of yeast and hyphae morphologies (Fig. 6A and B) surrounded by EPM. Biofilms that were in contact with glucose did not show significant morphological differences compared to glucose-free biofilms. After 15 min of irradiation, we observed a biofilm disorganization in both PDI

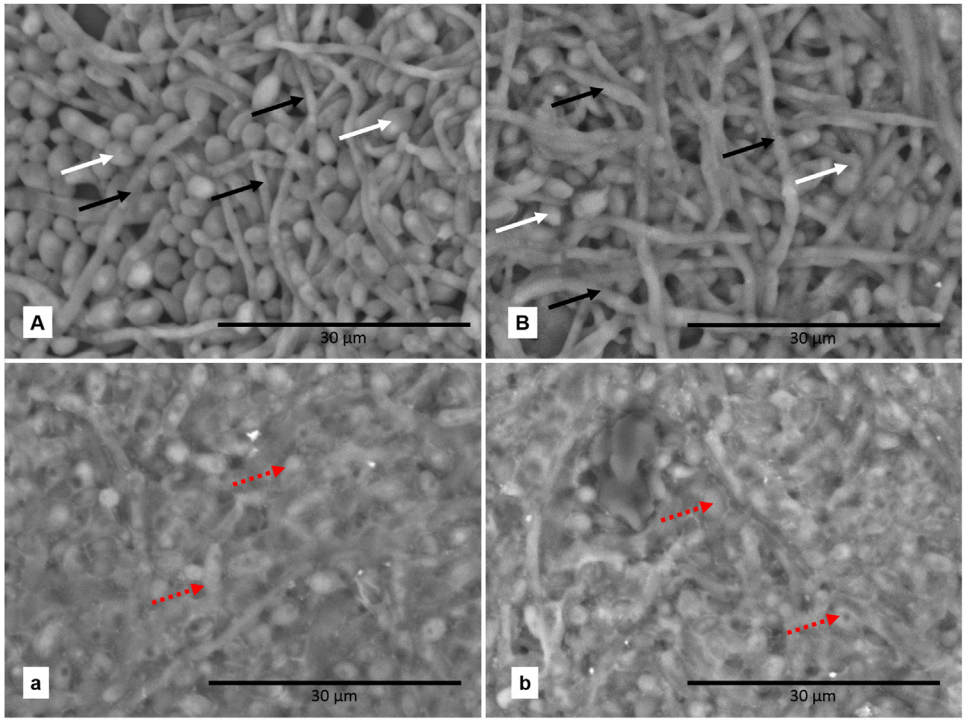

Fig. 6. Scanning electron-micrographs of *C. albicans* biofilms before (capital letters) and after (lower cases) PDI. No morphological differences were observed between glucose-free biofilms (A and a) and glucose-pretreated biofilms (B and b). White arrows point to yeast forms and black arrows point to hyphae forms. The red dotted arrows indicate the fungal cells damaged by PDI.

groups (i.e. with and without glucose pretreatment) presenting collapsed cells and loss of extracellular matrix (Fig. 6a and b).

4. Discussion

Some strategies have been proposed to enhance MB-mediated PDI on microorganisms such as the use of urea to reduce the ionic strength of the medium and avoid MB aggregation [22] and gold nanoparticles to enhance MB-mediated phototoxic effects [23]. In this study, we hypothesized whether 50 mM glucose could influence MB uptake in biofilms and, consequently, improve the antimicrobial effects of PDI. Our results showed that 50 mM glucose increased MB uptake and modulated the killing effect of PDI.

In this study, first we established a reproducible and standardized *C. albicans* biofilm model grown on hydrogel disks that also allows easy analysis via imaging techniques. Secondly, we determined the optimum pre-irradiation time that promotes the highest PS uptake by our biofilm model. Thereafter, we scrutinized different MB concentrations to select the lowest concentration possible to reach the highest uptake by the biofilm. Thus, we continued our experiments pre-incubating *C. albicans* biofilms with 500 μM MB during 30 min to verify MB uptake and susceptibility to PDI in glucose-pretreated and glucose-free biofilms.

Using fluorescence spectroscopy and confocal microscopy, we observed that glucose-sensitized biofilms presented a higher MB uptake and MB fluorescence was also detected more deeply in biofilms. A plausible explanation for these observations is that glucose enhanced MB influx into the cells by means of membrane transporters upregulation. MB uptake in *C. albicans* is regulated by MFS class of transporters, and blocking this pathway decreases MB uptake and PDI killing efficiency [17]. In addition, it has been demonstrated that *C. albicans* presents membrane receptors that senses glucose and can induce intracellular signaling and expression of MFS transporters [24]. By these means, MFS could enhance transport of hexoses, MB and other solutes by facilitated diffusion.

Once we verified that glucose increased MB uptake in biofilms, we investigated the effects on the biofilm susceptibility to PDI. We observed that after 6 min of irradiation, PDI group efficiently reduced cellular viability compared to glucose-pretreated groups. In glucose-pretreated biofilm, only after 12 min of irradiation there was a significant reduction in cellular viability compared to PDI group. We hypothesize that despite a higher uptake of MB, increased antioxidant mechanisms triggered by contact with glucose prevented the damage caused by PDI until 9 min of irradiation. Additionally, glucose can be a good hydroxyl radical scavenger that could act as an antioxidant for a limited time (i.e. until it is consumed by oxidation), even though glucose has a low reaction rate constant towards singlet oxygen ($\sim 10^4$ L mol^{-1} s^{-1}) [25]. However, these resistance mechanisms appear to have been exhausted in 12 min, when glucose pretreated biofilms presented lower survival fraction values in relation to glucose-free group. In fact, according to Huh and Kang, when *C. albicans* is exposed to glucose for one hour it maintains increased resistance against hydrogen peroxide and intracellular ROS [26] by activating AOX1a and AOX1b oxidase genes that could also protect the cells of the oxidative stress produced by PDI [26,27].

On the other hand, it has been recently showed that glucose may enhance killing efficiency of daptomycin against *Staphylococcus aureus* persister cells [28]. In this case, we could assume that glucose may have increased the metabolism of persisters to bring them back to lag phase, however, a time for the adaptive response is necessary [29]. Thus, after 12 min, glucose-sensitized *C. albicans* persister cells could also be more susceptible to PDI.

In this work, we used XTT assay to quantify *C. albicans* viability instead CFU/mL, since hyphae and yeast should not be classified equally as units [23]. Hence, we also attempted to correlate the XTT reading with CFU for *C. albicans* cells in suspension. As expected, the optical density values diminished together with reduction of CFU (data not shown). In fact, a sigmoid curve represented the relationship between these two quantification methods, showing that

XTT assay has sensitivity limit for values below 1×10^4 CFU/mL. It is important to notice that after 12 min of exposure (E > 170 J) the lack of statistically significant differences between PDI and PDI + glucose groups may be related to XTT sensitivity limitation since our dose-response curve suggests that much less energy is necessary for almost complete fungi inactivation in glucose-sensitized biofilms.

The SEM analysis of biofilms after PDI showed a disorganized biofilm with possible leakage of cytoplasmic content for both groups. These findings agree with other authors who described a disruptive action of PDI on biofilms [30]. However, glucose-pretreated biofilms did not show impressive morphological differences compared to glucose-free biofilms following PDI.

In summary, this study suggests that glucose-mediated PDI promoted enhancement of MB uptake in *C. albicans* biofilms and modulated cell inactivation kinetics. Thus, glucose could be an adjuvant to improve the PDI effects against biofilms present in topical infections or inorganic surfaces. We expect that this work offers an insight to inspire further investigations in this area.

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