The irradiation parameters investigation of photodynamic therapy on yeast cells

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ABSTRACT

It has been proposed that photodynamic therapy (PDT) can inactivate microbial cells. A range of photosensitizers and light sources were reported as well as different fluence parameters and dye concentrations. However, much more knowledge regarding to the role of fluences, irradiation time and irradiance are required for a better understanding of the photodynamic efficiency. The aims of this study were to investigate the role of light parameters on the photoinactivation of yeast cells, and compare cell survivors in different growing phases following PDT. To perform this study, a suspension (10^6cfu/mL) of *Candida albicans* ATCC-90028 was used in log and stationary-phase. Three irradiances 100mW/cm², 200mW/cm² and 300mW/cm² were compared under 3min, 6min and 9min of irradiation, resulting in fluences of 18, 36, 54, 72,108 and 162J/cm². The light source used was a laser emitting at 660nm with output power of 30, 60 and 90mW. As photosensitizer, 100μM methylene blue was used. PDT was efficient against yeast cells (6 log reduction) in log and stationary-phase. Neither photosensitizer nor light alone presented any reduction of cell viability. The increase of irradiance and time of irradiation showed a clearly improvement of cell photoinactivation. Interestingly, the same fluences in different irradiances presented dissimilar effects on cell viability. The irradiance and time of irradiation are important in PDT efficiency. Fluence per se is not the best parameter to compare photoinativation effects on yeast cells. The growing-phases presented the same susceptibility under *C. albicans* photoinactivation.

Keywords: Antimicrobial Photodynamic Chemotherapy; Antifungal; Diode laser; Methylene Blue; *Candida albicans*; Photosensitizers.

1. INTRODUCTION

Photodynamic therapy (PDT) is a phototherapy based on the utilization of substances that can photosensitize biological tissues and are capable of being activated in the presence of light. The cells that are considered therapeutical targets are stained with the photosensitizing agent and irradiated with light [1-5]. The photodynamic process rapidly generating reactive oxygen species (ROS) as for instance peroxides, hydroxyl radicals, superoxide ions and singlet oxygen, the last being implicated as the major causative agent of cellular damage in photodynamic process [6]. In addition, this technique has been shown to have effects against a range of pathogens and also against drug-resistant microorganisms [7-9].

Candidiasis is a common opportunistic infection in immunocompromised individuals (i.e., HIV infection, transplantation, corticosteroid therapy and lymphoma), causing significant problems in patient management. Thus, this yeast is capable of rapid conversion to a pathogen causing superficial and nosocomial bloodstream infections. Moreover, *C. albicans* is the most prevalent pathogen representing about 60% of all yeasts isolated in clinical samples. In addition, others *Candida sp.* has been emerging as yeast pathogens and they are demonstrating various degrees of resistance to common antifungal agents [10]. Recently, resistance of *C. albicans* against antifungal agents has begun to appear [11, 12].

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Yeast photoinactivation is a challenge on antimicrobial PDT due to the relatively lower susceptibility of these cells to oxidant agents. By the other hand, this phototherapy is a substantial hope against antifungal resistant species. The photosensitizer researches are forward, with great developments in molecules which produce a high quantum yield for singlet oxygen formation, more resonant with light source and closer interaction with microbial structure [4, 13-15]. However, more knowledge regarding to the importance of irradiation parameters are required to a better understanding of photodynamic efficiency.

The literature suggests the fluence as the most important light parameter to obtain a high eradication of microbial cells in photodynamic therapy [4, 5, 16-19]. However, the influence of others light parameters as time of irradiation and irradiance are not frequently reported. Moreover, sometimes neither wavelength is correctly presented [20]. It makes the idea that fluence leads the range of parameters to compare different microorganism photoinactivation studies.

The aims of this study were to investigate the role of light parameters in the photoinactivation of yeast cells, and to compare cell survivors after PDT in different growing phases using MB as photosensitizer.

2. MATERIALS AND METHODS

2.1 Inoculum preparation

Candida albicans ATCC 90028 was sub-cultured from vial stock onto Sabouraud dextrose agar. The incubation conditions were in air atmosphere for 12h (growing-phase) and 48h (stationary-phase) at 37°C. The estimation of the cell concentration was made by the turbidity of the *C. albicans* suspensions which were measured at 530nm in an optical spectrophotometer in the transmittance of 73±4% (~1×10^6 CFU/mL) [21]. The inoculum initial quantification was confirmed by cells growing in the control group.

2.2 Irradiation source

A GaAlAs diode laser (Photon Lase III, DMC, São Carlos, Brazil) with wavelength of 660 nm was used in this study. Yeast strains were irradiated from the top of a well microtitulation plaque and the laser beam passed through all the suspension at 0.3cm^2 spot size, which was coincident for all groups. Three irradiances A: 100 mW/cm², B: 200 mW/cm² and C: 300mW/cm² were compared at 3min, 6 min and 9min of irradiation, resulting fluences from 18J/cm² to 162J/cm² (Table 1). The coincident fluences were compared for the different irradiances.

	Wavelength λ=660nm		
Parameters	A	B	C
Spot size (cm^2)	0.3	0.3	0.3
Irradiance (mW/cm^2)	100	200	300
Output power (mW)	30	60	90
Minutes of Irradiation (Fluence- J/cm^2)	3 (18)	3 (36)	3 (54)
	6 (36)	6 (72)	6 (108)
	9 (54)	9 (108)	9 (162)

Table 1- Irradiation parameters.

2.3 Photodynamic therapy studies and colony-forming units (CFU) determination

A suspension (10^6 CFU/mL) of each strain was divided into four groups. The control group (L-PS-) was untreated by either laser (L) or photosensitizer (PS). In the laser groups, the yeast suspensions were irradiated for 9 min with a fluence of 162 J/cm^2 in the absence of the photosensitizer (L+PS-). In PDT groups, methylene blue (Sigma Ltd, Poole, UK) was added to the yeast suspensions to a final concentration of 100μM for 10 min in dark conditions [19]. Three aliquots of 200μL of the yeast suspension with PS were putted in a 96 wells plaque, and before the irradiation, an aliquot of 20μL (L-PS+) was removed from each well in dark condition to serial dilution. Subsequently, these samples were treated with laser (L+PS+), performing irradiances of 100mW/cm², 200mW/cm² and 300mW/cm² (table 1). Aliquots of 20μL were

also collected from the wells in each irradiance parameter at 3, 6 and 9 min. during the irradiation; thereafter, they were serially diluted in PBS to generate dilutions of 10^{-1} to 10^{-5} times the original concentration [22].
Ten-µL aliquots of each dilution were streaked onto a sabouraud plaque in triplicate and incubated for 24h at 37°C to allow colony formation [23].

2.4 Statistics:

The yeast colonies were counted and converted into CFU for analysis. All samples were submitted to this process and statistical analysis of the experimental data was performed using one-way analysis of variance (ANOVA) and the Tukey's test means comparison. For all tests, significance was set at $\alpha = 0.05$ [21].

3. RESULTS

Neither light nor photosensitizer alone presented any effect on yeast cell inactivation. The control groups (L-PS-) showed no significant differences of viable cells compared to laser group (L+PS-) and group with photosensitizer before irradiation (L-PS+) in both phase of *C. albicans* growth. One hundred-µM methylene blue at 10min of cell contact had no citotoxicity on these samples, as well as the laser alone did not change the number of viable yeasts (fig. 1).

The irradiance of 100mW/cm^2 presented moderate effect against the tested yeast cells and it was dependent on irradiation time; thus, it was less effective than higher irradiances. The reduction on yeast strains in the tested PDT groups was higher under the irradiance of 300mW/cm^2, which had an increased effect on yeast cells inactivation. At 3min of irradiation, a significant reduction on yeast viability was found compared to 100mW/cm^2, for both growth phases. The optimum photodynamic effect occurred at 6 min of irradiation, where no viable cells were found in any sample under 200mW/cm² and 300mW/cm² for both *C. albicans* log and stationary phases (fig. 1).

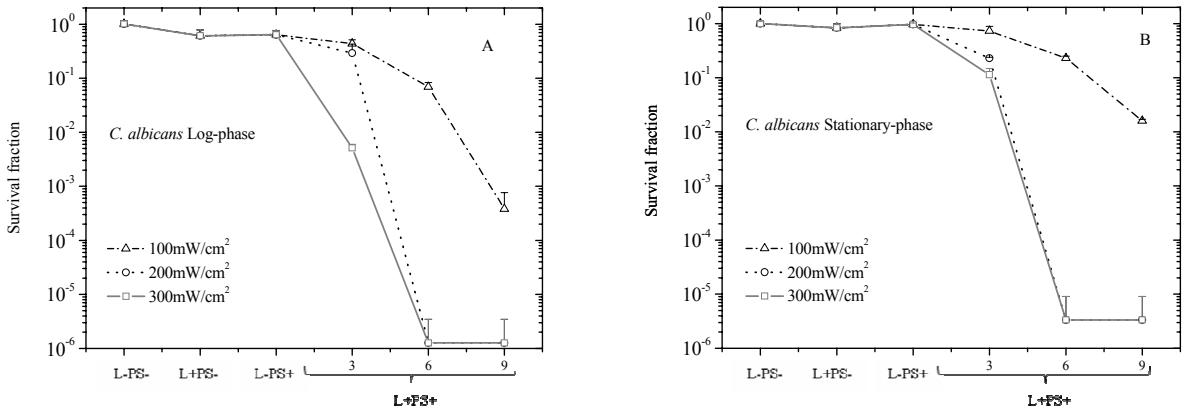

Fig. 1. Means values +SD of *Candida albicans* survival fraction comportment on A- log-phase and B- stationary-phase. Neither light nor photosensitizer alone presented any effect on yeast cell inactivation. The samples which were treated by PDT (L+PS+) presented an irradiation time-dependent inactivation following laser irradiation at 3min, 6min and 9 min. Bars are standard deviations (SD).

For the irradiance parameter, coincident fluences were observed. Three-min of irradiation (200mW/cm²) and 6 min (100mW/cm²) resulted in a fluence of 36J/cm² (tab. 1) while at 3 min of irradiation (300mW/cm²) and at 9 min (100mW/cm²) a fluence of 54J/cm² was obtained. For the fluence of 36J/cm², the stationary-phase presented no significant differences on microbial kills with respect to irradiance. However, the log-phase presented a higher inactivation at 100mW/cm² (fig. 2A) than 200mW/cm^2. By the other hand, a fluence of 54 J/cm² presented a higher inactivation on *C. albicans* at 9 min of irradiation in the lower irradiance (100mW/cm²) for both growth phases (fig. 2B).

This finding suggests that the same fluence in different irradiation times may present different results on cell inactivation.

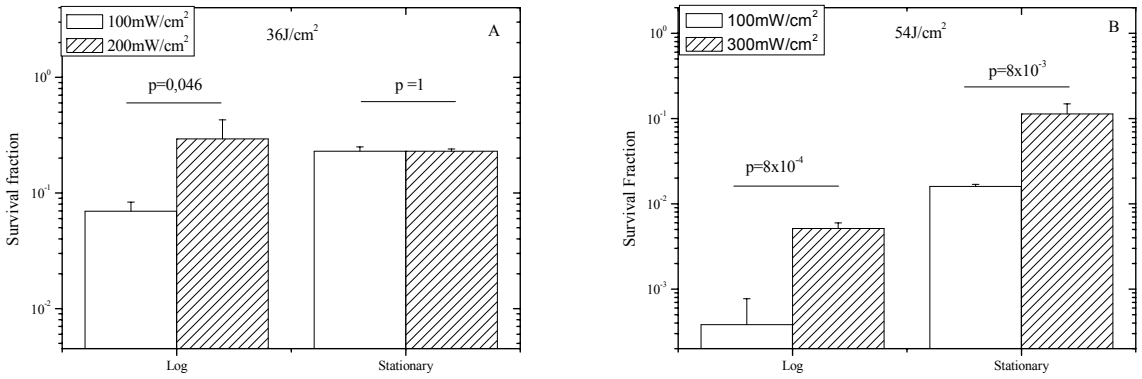

Fig. 2. Means values +SD of *Candida albicans* survival fraction under the same fluence in different exposure times presented statistically significant differences on yeast inactivation. A- 36 J/cm² and B- 54J/cm². Bars are standard deviations (SD).

4. DISCUSSION

This study showed a photoinactivation of yeast cells exposure time-dependent for the three fluences used. *C. albicans* in stationary-phase presented a lower reduction than in log-phase, and at 100mW/cm² this effect was just 2 log reduction at 9 min of irradiation. Souza et al. [18] showed that *C. albicans* demonstrates to be susceptible to photoinactivation and they obtained a reduction on this yeast following PDT. However, the fluence reported was 28J/cm², 35mW of output power for 5 min of irradiation. In our work, a higher inactivation degree was obtained by higher parameters of illumination. We found a 6 log reduction that clearly showed that this yeast can be eradicated by PDT. This result agrees with Demidova e cols.[16], that found a 7 log reduction on *C. albicans* using touluidine blue as photosensitizer. In addition, the yeast species seem to be less susceptible to photoinactivation than bacterium species.

C. albicans is the most common pathogen of yeast species and it is able to produce both superficial and systemic infection. In addition, others species are less common in clinical isolated but some of them present high antifungal resistance. These yeasts are considered emergent pathogens, causing significant management problems in immunocompromised patients [10].

One hundred-μM MB did not show any toxicity on yeast cells with 10 min of contact. It is presented in figure 1, where no significant differences were observed between the control group and photosensitizer group. The laser group also presented no significant differences on cell inactivation, and this finding agrees with Munin et al [19].

In this study, the fluences of 36J/cm² and 54J/cm² were compared using three irradiances. The correlation energy for area is the most common parameter to differ PDT groups in the literature [4, 16, 22, 24]. Interestingly, when we compared the same fluence in different parameters, it is worthy to note that the same numeric fluence (54J/cm²) presented significant different results for the different irradiances (figure 2B). The samples illuminated by 100mW/cm² presented a higher inactivation at 9 min that 300mW/cm² at 3 min However, at 36J/cm² the groups showed the same inactivation at 6min by 100mW/cm² like at 3 min. by 200mW/cm². Therefore, the time of irradiation must be observed in all comparative studies, as well as the irradiance and the output powers, which are determinant to cell inactivation.

The amount of photons passing through a microbial suspension is more efficient to inactivate cells in a longer time of irradiation [25]. Thus, an adequate time of irradiation should result in the eradication of the microorganisms treated. In

agreement, Qin et al. [25] showed that an increase in the irradiance enhances the microbial killing. The enlargement of irradiance also amplifies the microbial damage, although it seems to have an upper limit of photons to observe this effect. In fact, if the number of photons is higher in the same exposure time, consequently this best result will decrease because the dye in the suspension will not absorb all the excess of light. In addition, a higher irradiance could promote a higher photobleaching as well as photothermal effect [26].

5. CONCLUSION

It has been shown by this study that irradiance and time of irradiation play an important role in PDT efficiency. Fluence should not be used as the only parameter to compare photoinativation effects on yeast cells. The different growth phases treated by PDT presented a similar susceptibility under *C. albicans* photoinactivation.

6. ACKNOWLEDGMENTS

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