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Efficient *in vitro* photodynamic inactivation using repetitive light energy density on *Candida albicans* and *Trichophyton mentagrophytes*

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ABSTRACT

Background: We compared the effectiveness of a single irradiation *vs* repetitive irradiation of light, for *in vitro* photodynamic inactivation (PDI) of *Candida albicans* and *Trichophyton mentagrophytes,* by using methylene blue (MB) and rose bengal (RB) as photosensitizers (PS).
Methods: MB from 5 to 60 μM and RB from 0.5 to 10 μM, with energy densities from 10 to 60 J/cm^2, were tested in *C. albicans*. We further optimize the PDI by reducing the light energy density and PS concentration for the single irradiation experiments by using repetitive doses (two and three times). MB was tested in *C. albicans* and *T. mentagrophytes*, and RB was tested in *C. albicans*.
Results: MB-PDI and RB-PDI in *C. albicans* significantly reduced the number of colony-forming units per milliliter (CFU/mL) when compared to the control groups. Using a single irradiation, over 99% growth inhibition of *C. albicans* was obtained with MB at 20 μM–60 J/cm^2, and with RB at 1 μM–30 J/cm^2 and 5 μM–10 J/cm^2. With repetitive doses, similar results were obtained by reducing several times the light energy density and the PS concentration for *C. albicans* and *T. mentagrophytes*.
Conclusions: The results showed that RB was more effective than MB for *C. albicans* inactivation. In addition, it is possible to significantly reduce the amount of PS and light energy density requirements by using repetitive irradiations in both genera tested. It makes the technique less invasive and could reduce the side effects in people extremely sensitive to the PS or the light.

1. Introduction

Photodynamic inactivation (PDI) is carried out when a photosensitizer (PS) inside or very close to the cellular wall of the cells absorbs photons within its absorption band. The excited PS may decay to its long-lived triplet excited state and then by interaction with the surrounding media lead to the production of singlet oxygen and other reactive oxygen species (ROS) which may induce cell death. There are two types of ROS mechanisms production: Type I mechanism involves electron transfer reactions to form free radicals and radical ions such as superoxide anion, hydrogen peroxide and hydroxyl radicals. Type II mechanism results by energy transfer from the triplet state of the sensitizer to the ground state of triplet molecular oxygen ($^3O_2$), leading the generation of highly toxic singlet oxygen ($^1O_2$) which is believed to be the main cytotoxic agent in PDI since it exhibits several orders higher reactivity toward various biological molecules (such as lipids, proteins or nucleic acids) than other ROS [1]. In order to cause cell death, the presence of oxygen is needed [2–4] otherwise PDI may become inefficient. PDI represents an alternative to traditional treatments and potentially more efficient against several medical conditions, commonly with very few side effects for the patients. In particular, it has proven to be efficient to treat infections caused by opportunistic and resistant microorganisms to pharmacological treatments. There are many pathogens that can cause harm to humans; possibly one of the most common is the fungus *Candida* and in particular *Candida albicans*. The incidence of *Candida* infections has increased throughout the world and most pronounced among specific patient groups like hospitalized in intensive care units or immunosuppressed patients [5]. On the other

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hand, *Trichophyton mentagrophytes* is one of the etiological agents most frequently implicated in dermatophytosis or ringworm.

There are a great variety of PSs that are commonly used [6]. Among them, we can mention rose bengal (RB) from the xanthene family and methylene blue (MB) from the phenothiazine family. One quick search on the literature about the concentration of PS used in PDI experiments reveals a wide range of values. For example, the reported values of RB concentration used to treat *C. albicans* lies between 10 and 200 μM [7–11]. The same can be said about the applied light energy density which ranges from 14 to 95 J/cm^2. Likewise, the range of MB concentration varied enormously from 1 μM up to 300 μM [12–18] which makes comparison of results very hard. Traditional studies and treatments with PDI include the application of the PS and a single exposure of light to activate it. The aim of this study was to confirm the *in vitro* effect of the PDI technique using MB and RB on planktonic cells of *C. albicans* and *T. mentagrophytes,* and improve the classical process of PDI application (a single light dose) by reducing the concentration of PS and light energy density but applying repetitive exposures of low light energy density. This is particularly important to establish a method as less invasive as possible to decrease the risks of side effects caused by hypersensitivity to the PS and/or the sensation of pain in sensitive patients with serious injuries.

2. Materials and methods

2.1. Microorganisms strains and growth conditions

T. mentagrophytes and *C. albicans* strains were donated by the Department of Mycology from Benemérita Universidad Autónoma de Puebla (BUAP), México. *C. albicans* was grown in Sabouraud dextrose agar (SDA) media for 24 h, then the yeast was suspended in PBS, inoculated 1:10 in Sabouraud dextrose broth (SB) (OmniChem, México) and incubated for 12 h. *T. mentagrophytes* were grown on SDA (Omnichem, México) at 30 °C for 14 days. Conidia collection was performed with phosphate buffered saline solution (PBS), pH 7.4 (OmniChem, México). The solution obtained was filtered with a qualitative filter paper No. 2 (Whatman, United Kingdom). The concentration of conidia or yeast was calculated with a hemocytometer (Neubauer improved 0.1 mm depth; Marienfeld, Germany). The initial concentration was adjusted by diluting with PBS solution to 2–4 × 10^5 conidia-cell/ml. After adding the MB solution a final concentration of 1–2 × 10^4 conidia-cell/ml was obtained.

2.2. PS solutions

The PSs used were MB (Omnichem, México) and RB (Omnichem, México). Each one was prepared as stock solution in PBS: 200 μM for MB and 100 μM for RB. The solutions were sterilized with a syringe filter (cellulose acetate; pore size, 0.20 μm; diameter, 25 mm) (GVS Life Sciences, USA), and stored at 4 °C in the dark before use. Working MB and RB solutions were diluted with sterile PBS. For PDI evaluations in *C. albicans,* PS concentrations are within the range of reported values (see Table 1) For *T. mentagrophytes* the optimum MB concentration and light energy density for PDI were determined in a previous work [19].

2.3. Light source

The light sources were two home-made LED array devices (red light, λ = 600–650 nm, to activate MB and green light, λ = 490–540 nm, to activate RB) consisting of 12 LEDs light sources each with 30 mW of optical power. Each LED was focused in a well with an area of 0.35 cm^2 of a sterile flat-bottom polystyrene microplate (Cientifica Senna, Ciudad de Mexico, Mexico). The tested energy densities are shown in Table 1.

Table 1
PDI experimental parameters used.

SINGLE DOSE OF LIGHT		REPETITIVE DOSE OF LIGHT		
C. albicans		*C. albicans*		*T. mentagrophytes***
Red light, MB	Green light, RB	Red light, MB	Green light, RB	Red light, MB
10 J/cm^2, 20 μM 10 J/cm^2, 60 μM 30 J/cm^2, 10 μM 30 J/cm^2, 20 μM 30 J/cm^2, 60 μM 45 J/cm^2, 5 μM 45 J/cm^2, 10 μM 60 J/cm^2, 10 μM 60 J/cm^2, 20 μM*	10 J/cm^2, 0.5 μM 10 J/cm^2, 1 μM 10 J/cm^2, 5 μM* 10 J/cm^2, 10 μM 30 J/cm^2, 0.5 μM 30 J/cm^2, 1 μM* 30 J/cm^2, 5 μM 30 J/cm^2, 10 μM 45 J/cm^2, 5 μM 45 J/cm^2, 10 μM 60 J/cm^2, 5 μM 60 J/cm^2, 10 μM	5 J/cm^2, 5 μM 5 J/cm^2, 10 μM 10 J/cm^2, 5 μM 10 J/cm^2, 10 μM 20 J/cm^2, 5 μM 20 J/cm^2, 10 μM	3 J/cm^2, 0.5 μM 5 J/cm^2, 0.5 μm 10 J/cm^2, 1 μM 10 J/cm^2, 2 μM	3 J/cm^2, 20 μM 5 J/cm^2, 20 μM 10 J/cm^2, 5 μM 10 J/cm^2, 10 μM 10 J/cm^2, 20 μM 20 J/cm^2, 20 μM

* Optimal conditions (lowest PS concentration) of treatment where cells were no longer observed.

** Optimal conditions (lowest PS concentration) of treatment where cells were no longer observed (> 99% inhibition growth, 60 J/cm^2 and 20 μM MB) was reported in a previous work [19].

2.4. PDI

2.4.1. Single dose of light

The used method was similar to that reported before by Spezzia-Mazzocco et al. [19] but with some simple variations. Planktonic cells or conidia solution (50 μL at a concentration of 1–2 × 10^4 colony-forming units per milliliter (CFU/ml), and PS solution (50 μL of MB or RB in PBS, final concentrations described in Table 1) were placed in each well of the microplate and incubated for 30 min in the dark at 30 °C, after this we irradiate them with green or red light accordingly. All experiments were done in triplicate (n = 3 per group) with the corresponding controls: cells control without any treatment, light controls (green or red irradiation alone) without PS, dark toxicity controls (MB or RB alone) *i.e.* samples incubated with PS without irradiation. After experiments, the 100 microliters of each well with the planktonic cells were poured onto SDA media and incubated at 37 °C, during 48 h for *C. albicans*. CFU/ml was counted and the efficiency of the treatments was assessed.

2.4.2. Repetitive dose of light

To reduce the PS concentration and/or light energy density requirements as much as possible, we used the same procedure described above for the application of PDI, adding exposures of light with dark incubation time (DIT) of 30 min between exposures (considered from the moment of irradiation starts) (Fig. 1). From the optimal conditions (99% of growth inhibition) obtained for a single dose of light for each PS tested before, the concentration of the PS and the light energy density were further reduced using repetitive dose of light: one, two and three exposures (repetitive light energy density and PS concentrations are reported in Table 1). After treatments, planktonic cells were inoculated in SDA media and CFU/ml was counted after 48 h for *C.*

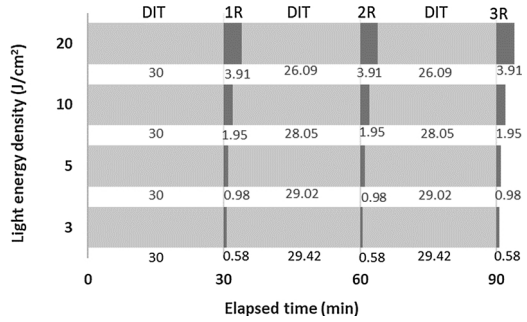

Fig. 1. Irradiation sequences employed for different treatments. The vertical axis shows the used light densities; the dark incubation time (DIT) and the irradiation time in minutes (indicated below the gray and dark bands, respectively) employed for the first (1R), second (2R) and third irradiation (3R).

albicans, and 72 h for *T. mentagrophytes*, the efficiency of the treatments was assessed. Finally, we tested shorter DIT between exposures of light (15 min, 7.5 min and 4 min, considered from the moment the irradiation starts) and the treatments were made in the same way described above (Fig. 1). The results were analyzed by analyses of variance (ANOVA) and the Tukey test. A significance level of 5% ($p < 0.05$) was considered to indicate a statistically significant difference. The analysis was achieved with the software OriginPro 9.0.

3. Results

With MB-PDI on *C. albicans,* good results were obtained in all treatments tested. However, the best results for the *in vitro* PDI with MB were obtained with a concentration of 20 μM activated with 60 J/cm^2 where yeast growth could no longer be observed (Fig. 2). For RB-PDI on *C. albicans,* the energy densities used were the same as for MB (10, 30, 45 and 60 J/cm^2) although PDI was more efficient for all the treatments, as the RB concentrations used are much less than those of MB (see Table 1 for the tested doses). From these results, we selected treatments of (1 μM–30 J/cm^2) and (5 μM–10 J/cm^2) given that no yeast growth was observed (see Fig. 2). For treatments with repetitive light irradiation we used a DIT of ~30 min between irradiations (see Fig. 1 for radiation sequences).

In general, the lower the PS concentration the higher the light energy density as expected. However, care must be taken because too high light energy density can lead to PS bleaching, which was avoided here.

3.1. Repetitive MB-PDI in C. albicans

All tested treatments showed some degree of growth inhibition. However, it is clear that the higher the concentration of PS and light energy density the greater inhibitory effect (Fig. 3A). The most effective conditions were:

- 5 μM–20 J/cm^2, where > 90% of inhibition was obtained with 3 exposures to light (3R). It means the same photons were used in comparison with a single exposure of light (1R) of 20 μM–60 J/cm^2, but one quarter less of the PS. This represents six times the inhibition obtained with 1R at 10 μM–20 J/cm^2.
- 10 μM–10 J/cm^2, where > 90% of inhibition was obtained with 3 exposures to light. It means half of photons were used in comparison with 1R of 20 μM–60 J/cm^2 but also half the PS concentration. This represents two times the inhibition obtained with 1R at 10 μM–10 J/cm^2.
- 10 μM–20 J/cm^2, where > 95% of inhibition was obtained with 2 exposures to light (2R). It means two thirds of photons were used compared with 1R of 20 μM–60 J/cm^2, but halving the PS concentration. This represents more than double the inhibition that was obtained with 1R at 10 μM–20 J/cm^2.

3.2. Repetitive RB-PDI in C. albicans

For RB, we also started from the optimal conditions where yeast growth could no longer be observed (1 μM–30 J/cm^2 and 5 μM–10 J/cm^2), as expected, the higher concentration of PS and light energy densities the greater inhibitory effect (Fig. 3B). The most effective conditions were:

- 1 μM–10 J/cm^2, where > 90% of inhibition was obtained with 2R, it means, double of photons were used in comparison with 1R of 5 μM – 10 J/cm^2, but one fifth of PS concentration. Equivalently, two thirds of the photons in comparison with 1R of 30 J/cm^2, 1 μM. This represents six times the inhibition obtained with 1R at 10 J/cm^2, 1 μM.
- 2 μM–10 J/cm^2, where > 98% of inhibition was obtained with 2R, it means two thirds of the photons were used in comparison with 1R of 5 μM–10 J/cm^2 but double of PS concentration. Equivalently, as many as twice of photons were used in comparison with 1R of 1 μM–30 J/cm^2 and the half concentration of PS.

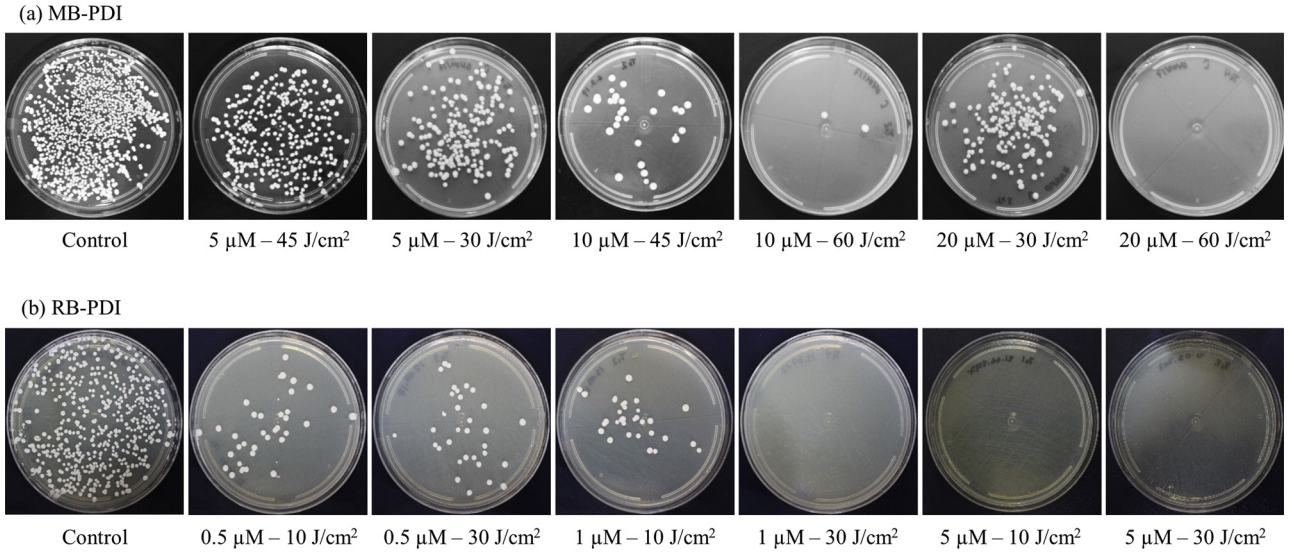

Fig. 2. Control treatments for *C. albicans* growing in SDA media 24 h after (a) MB-PDI and (b) RB-PDI.

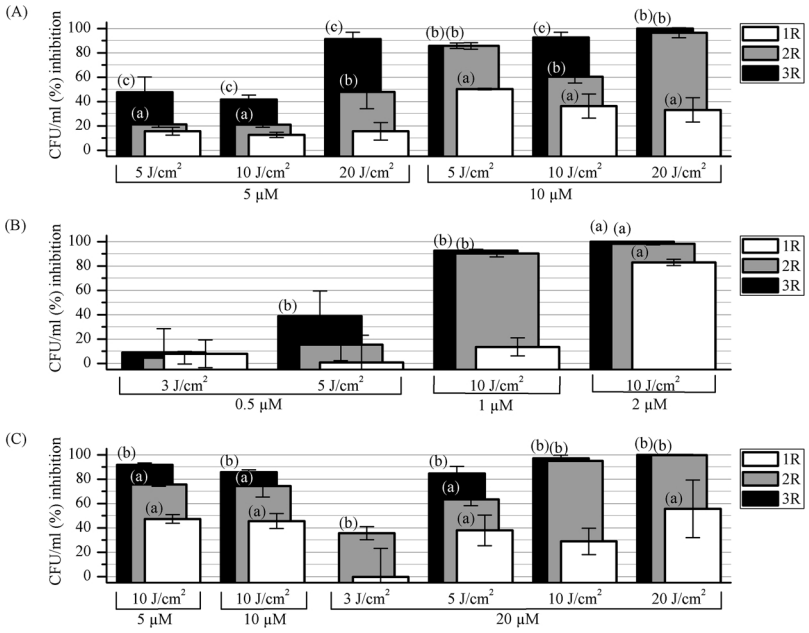

Fig. 3. PDI efficiency testing different energy light density after the first, second or third irradiation (1R, 2R and 3R, respectively) on *C. albicans* using (A) MB as PS and (B) RB as PS. (C) PDI efficiency with repetitive doses of light in *T. mentagrophytes* using MB as PS. Statically significant difference ($p < 0.05$) were obtained with (a) control group, (b) control and 1R groups, and (c) control, 1R and 2R groups.

Similarly for RB, a single repetition (1R) with 0.5 µM and light exposures of 5 J/cm^2 and 3 J/cm^2 did not show important inhibitory effects, except for 3R of 5 J/cm^2 that presented a growth inhibition around 40%, reducing 6 times the light energy density, halving the photons used and halving the PS concentration, compared with 1R of 1 µM–30 J/cm^2.

3.3. Repetitive MB-PDI in T. mentagrophytes

In order to prove that the effectiveness of repetitive exposures to light is not exclusive to *C. albicans,* we apply the same procedure to *T. mentagrophytes* starting with conditions determined in a previous work [19] that warranties > 99% inhibition (60 J/cm^2, 20 µM MB). Although all tested treatments showed some degree of growth inhibition (Fig. 3C) but we concentrate only on those achieving > 90%

- 5 µM–10 J/cm^2, where > 90% of inhibition was obtained with 3R, it means a half of the photons were used in comparison with 1R of 20 µM–60 J/cm^2 but a quarter of the PS concentration. This represents about the double of inhibition obtained with 1R at 5 µM–10 J/cm^2.
- 10 µM–10 J/cm^2, where > 95% of inhibition was obtained with 2R, it means one third of photons were used in comparison with 1R of 20 µM–60 J/cm^2, but halving the PS concentration. This represents about three times the inhibition obtained with 1R at 10 µM – 10 J/cm^2.
- 10 µM–20 J/cm^2, where > 98% of inhibition was obtained with 2R, it means two thirds of photons were used in comparison with 1R of 20 µM–60 J/cm^2, but halving the PS concentration. This represents about double the inhibition obtained with 1R at 10 µM – 20 J/cm^2.

3.4. Dark incubation time (DIT) between exposures

Since the choice of the DIT between irradiations was based on imitating the initial PS incubation time, we decided to try different DIT between irradiations (Fig. 1) looking to reduce the total treatment time as much as possible. It was corroborated that the inhibitory effect remains more or less constant, with lower efficiency as the DIT reduces: DIT of 30 min between irradiations showed the highest efficiency, followed by 15 min, 7.5 min and 4 min (Fig. 4).

4. Discussion

We have demonstrated that PDI with both MB and RB efficiently reduce *C. albicans* and *T. mentagrophytes* fungal strains growth. RB has proven to be more efficient than MB requiring less concentrations and light energy density in the same fungal strains, however, the molecular mechanism of such efficiency remains unclear. No significant dark toxicity was found for both PS at the evaluated concentrations. In the literature, there are plenty of reports that demonstrate the efficiency of PDI using both MB and RB in *Candida* strains (see Table 2) and also in many bacteria [20–22]. However, the correct dosage is still undetermined, for example, there are many reports were the PS concentrations and light energy density varied widely. The situation is not so different in the less studied *T. mentagrophytes* PDI, specifically with MB, although there are not enough reports to compare with; however, what is it clear is the efficiency of the *in vitro* and *in vivo* method against fungal infections caused by *T. mentagrophytes* and other dermatophyte fungi [19,28–30]. One possible explanation for this wide variety of PDI conditions is that the efficiency of PDI seems to depend on the product of light energy density and PS concentration under certain ranges that need to be determined. Too much concentration reduces the light penetration or may produce thermal damage or too little may be in inefficient. However, factors such as the origin of the strains, quality of the growth media and experimental procedure may also explain these discrepancies.

In this work, we observed that the use of repeated doses of light instead of a single dose, can improve the efficiency of the grow inhibition effect on the microorganisms evaluated. As an example, we can mention the case of PDI in *C. albicans* with MB as PS with 1R and 2R and a total of 20 J/cm^2 accumulated, that is, the same photons are applied to the cells. In the case of 2R, it implies two irradiations of 10 J/cm^2 were applied (Fig. 5). Using 5 µM about 17% of inhibition with 1R and about 20% with 2R as obtained, however, as the concentration of PS increases to 10 µM the inhibition increased to about 32% with 1R and about 60% (almost double) with 2R, *i.e.* doses fractionation increases its efficiency with the same photons. In Fig. 5 confirmed that as the density of light used increases, the inhibition effect obtained is also increased. A single dose using a low concentration of PS and low light energy density cause a slight inhibition on cell growth, but for subsequent exposure to light, the inhibition continues to increase. Branco et al. also found that by applying 3 repeated doses of light, (without PS)

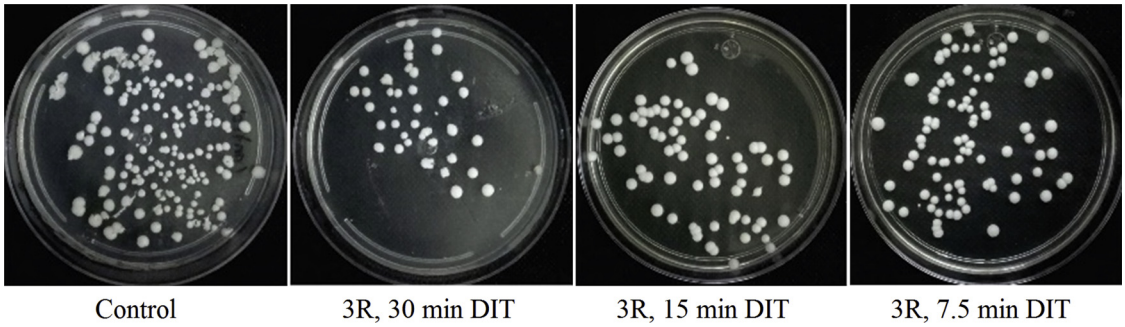

Fig. 4. *C. albicans* growing in SDA media 24 h after MB-PDI at 10 J/cm^2, 10 μM, by 3 exposures (3R) of irradiations and with different dark incubation time (DIT): 30, 15 and 7.5 min.

Staphylococcus aureus infection can be improved in an *ex vivo* experiment using pig skin [31] but the total amount of photons was three times higher contrary to our report. So, in principle, a sufficient number of repetitive low doses can achieve more than 99% of inhibition and even if PS concentration is further reduced.

It can be seen from Fig. 3, that for low PS concentrations, the inhibition from the first dose starts from a low value and it seems to growth in a superlinear fashion for three doses, however, it does not guarantee the efficacy of the treatment (Fig. 3). On the other hand, as the PS concentration increases, the inhibition for the first dose is higher, for example, the initial inhibition rate is 32–40% (for 10 μM in Fig. 3). Even if we assume a linear growth, the inhibition will increase to 64–80% with the second dose (2R) and over > 100% for the third dose (3R) which is not possible. For such reason, a sublinear response it is expected and two doses could be an option instead of three doses.

One wonder how many photons are being saved when repetitive light doses are applied but the concentration is reduced by a factor *k* and the total light dose remains the same? To answer this question, let's assume that J_0 is the total light energy density incident (delivered either in a single or several doses) onto sample with absorption coefficient α and thickness *l*, and then the transmitted energy density is $J = J_0 e^{-\alpha l}$. Now, if the absorption coefficient is changed to $k\alpha$, where *k* is a real constant, and the total incident light energy remains unchanged then the equation $ln(J_1/J_2) = (k\text{-}1)\varepsilon c1$, where ε is the molar absorptivity and c is the concentration of the PS, represents the fraction of saved photons. For example, if three light doses of 20 J/cm^2 and a concentration of MB at 10 μM (ε_{MB} (635 nm) = 40,084 1/(M cm)) and thickness 0.31 cm (depth of the well) requires ˜13% less photons than a single dose of 60 J/cm^2 and concentration of 20 μM to achieve 100% of inhibition. Similar calculations can be performed for other cases.

We believe that could be attributed to two reasons. First, there is the natural oxygen depletion as ROS and singlet oxygen are created. The average life time of the main ROS lies in the millisecond range (superoxide is 50 ms and H_2O_2 is ˜1 ms) [32] while the lifetime of singlet oxygen is approximately 3 μs in a cell [33]. So, the main limitation for

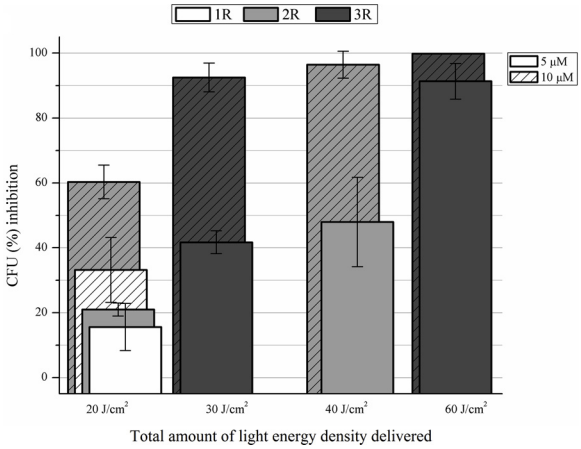

Fig. 5. Effect of MB-PDI in *C. albicans* CFU inhibition comparing the total amount of light energy density delivered to cells after the first (1R), second (2R) and third irradiation (3R) using MB at two concentrations (5 and 10 μM).

PDI is oxygen depletion in the illuminated region. Diffusion of oxygen from the surrounding media sets the DIT for the next irradiation. An upper limit for oxygen replenishment into a uniform illuminated area 1 mm^2 is ˜4 h assuming a molecular oxygen diffusion coefficient of D˜3 × 10^5 cm^2/s in water [34]. It was shown that DIT as short such as 4 min, the efficiency of inhibition is considerably reduced. The increased efficiency of PDI for DIT seems to be consistent with the oxygen replenishment assumption. The concentration of oxygen is one of the most important variables for the development of photodynamic therapy (PDT) [35]. It is known that low oxygen concentration reduces the photodynamic efficacy, preventing full therapeutic response [36] although it depends on the cell type or organisms in study, for example, Alves et al [21] showed that oxygen concentration did not significantly affect the photoinactivation of *Vibrio fischeri*. More studies are required

Table 2
Some PDI conditions used on *Trichophyton* and *Candida* species with MB and RB as PS.

REFERENCE	MICROORGANISM	PS	CONCENTRATION (μM)	LIGHT DOSE (J/cm^2)
Souza, 2006 [23]	*C. albicans, C. dubliniensis, C. krusei* and *C. tropicalis*	MB	313.47	28
Munin, 2007 [24]	*C. albicans*	MB	27 – 13,370	28
Carvalho, 2009 [25]	*C. albicans*	MB	31.26 – 312.64	28
Giroldo, 2009 [26]	*C. albicans*	MB	31.26 – 1560	28
Dai, 2011 [27]	*C. albicans*	MB	20	1.95 – 9.75
Costa, 2012 [7]	*C. albicans*	RB	10	95
Rossoni, 2010 [8]	*C. albicans*	RB	50	95
Soria-Lozano, 2015 [9]	*C. albicans*	RB	998 – 1997	95
Freire, 2014 [10]	*C. albicans*	RB	0.78 – 400	42.63
Morton, 2014 [11]	*C. albicans*	RB	140	24
Rodrigues, 2012 [14]	*C. albicans*	MB	1 – 12.5	5 – 30
Spezzia-Mazzocco, 2016 [19]	*T. mentagrophytes*	MB	10 – 100	20, 40 and 60

to understand the specific molecular mechanisms of oxygen for the photoactivation in different organisms. The possibility of reducing the DIT between the irradiations offers a more efficient and practical PDI since this would imply that patients would have to wait less time to take the complete treatment.

The second reason is that cells may suffer incremental damage with each irradiation. For the first irradiation, the cell may suffer little damage depending on the time of the following irradiation, so a second or third exposure to light could activate the remaining PS in the cells. In the treatment with several low light energy density dose could contribute to the efficiency of the process since there are reports that indicates that low light energy density rate is more effective than the higher one [37]. One reason why RB is more effective than MB may be explained by the higher quantum yield for singlet oxygen production of RB (0.7) than MB is 0.52 - i.e less fluorescent photons are produced [38]

The repetitive irradiation in PDI can be beneficial for the obvious advantages of being less invasive. Normally, neither the PSs nor light are harmful to the cells in the dark, as shown above. However, it is possible to find patients that may be extremely sensitive to certain photosensitizing compounds, exhibit delayed photosensitivity, reported burns sensation or pain during PDI treatment [18,39]. The suggested modifications for the PDI protocol allow reducing the concentration of PS and light energy density required. Likewise MB or RB alone, at high doses, already can induce slightly toxicity to normal cells, and both shown natural antifungal and antibacterial activity [40]. It is important to emphasize that increased toxicity occurs only when it is irradiated by light with the correct wavelength; hence this therapy will be of use mainly for local and topical applications. Lastly but not least, it is known that at higher concentrations MB may undergo self-aggregation in water media, thereby diminishing the singlet oxygen yield [41]. Furthermore, aggregated monomer and dimer forms of MB can show photodynamic activity *via* type I PDI, which is less effective than type II PDI [42–44]. Therefore, repetitive irradiation would increase the efficiency in PS dosing and activation.

In conclusion, PDI might be used as an effective procedure to treat *C. albicans* or *T. mentagrophytes in vitro* with MB or RB as PS. MB-PDI and RB-PDI with a light single dose. The results showed that RB was more effective than MB for *C. albicans* inactivation. It is possible to reduce significantly the concentration of PS and light energy density requirements amending the PDI application process *in vitro*, by using repetitive light energy density, regardless of the type of PS used or the type of microorganisms treated. We believe the efficiency of this method could be due to the alteration of the cell self-recovery process or the possibility to replenishment the oxygen; however, further studies are needed to test this hypothesis.

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