

Antimicrobial mechanisms behind photodynamic effect in the presence of hydrogen peroxide

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Received 1st April 2010, Accepted 8th November 2010

DOI: 10.1039/c0pp00082e

This study describes the use of methylene blue (MB) plus light (photodynamic inactivation, PDI) in the presence of hydrogen peroxide (H₂O₂) to kill *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. When H₂O₂ was added to MB plus light there was an increased antimicrobial effect, which could be due to a change in the type of ROS generated or increased microbial uptake of MB. To clarify the mechanism, the production of ROS was investigated in the presence and absence of H₂O₂. It was observed that ROS production was almost inhibited by the presence of H₂O₂ when cells were not present. In addition, experiments using different sequence combinations of MB and H₂O₂ were performed and MB optical properties inside the cell were analyzed. Spectroscopy experiments suggested that the amount of MB was higher inside the cells when H₂O₂ was used before or simultaneously with PDI, and ROS formation inside *C. albicans* cells confirmed that ROS production is higher in the presence of H₂O₂. Moreover enzymatic reduction of MB by *E. coli* during photosensitizer uptake to the photochemically inactive leucoMB could be reversed by the oxidative effects of hydrogen peroxide, increasing ROS formation inside the microorganism. Therefore, the combination of a photosensitizer such as MB and H₂O₂ is an interesting approach to improve PDI efficiency.

1 Introduction

The rapidly increasing emergence of antibiotic resistance amongst pathogenic bacteria may be bringing to an end of a period extending over the past 60 years, termed “the antibiotic era”.¹ In the 1940s, with the introduction of penicillin, antibiotic therapy began, but within just two years, pathogenic bacterial strains resistant to the drug were discovered.² Currently, the epidemiology of infectious diseases is in crisis due to the emergence of bacterial strains with multiple resistances to many conventional antibiotics^{3,4} and there is an urgent need for new treatments with novel mechanisms of action.^{5,6} Therefore, an alternative antimicrobial approach that could avoid the appearance of resistance and its side effects is clearly desirable.

Photodynamic therapy (PDT) involves the use of light sources to kill undesired cells or microorganisms and a photosensitizer

agent. The excited photosensitizer reacts with molecular oxygen, generating highly reactive oxygen species (ROS) that promote injury and death of microorganisms as well as tumor cells.^{7,8}

PDT has an efficient killing effect upon illumination of different microorganisms, such as Gram-positive bacteria, but less efficient killing of fungi and Gram-negative bacteria.^{9,10} The structure of the cellular wall seems to be fundamental to determine the difference in susceptibility to PDT between Gram+ and Gram– bacteria. The high susceptibility of Gram+ species is explained by their morphology, with a relatively porous layer of peptidoglycan and lipoproteic acid that allows the photosensitizer to cross their cytoplasmic membrane. On the other hand, the outer membrane of Gram– microorganisms forms a physical and functional barrier between the cell and its environment.

Hydrogen peroxide (H₂O₂) is used worldwide for cleaning wounds, removing dead tissue, or as an oral debriding agent, due to its strong oxidizing properties.¹¹ Previous studies from our group^{12,13} and from McCullagh and Robertson^{14,15} have indicated that the use of hydrogen peroxide associated with PDT gives increased killing of microorganisms. Therefore, the combination of a well-known antiseptic agent and a potential antimicrobial therapy could improve the efficiency of both treatments, achieving a better result especially for clinical applications. The enhancement of the killing effect may occur due to enhanced photochemistry of the dye, by a chemical reaction between H₂O₂ and the ROS produced during PDT, or by one treatment (H₂O₂ or dye + light) damaging the microbial cell and allowing the other treatment to penetrate better.

In this study, our aim was to investigate the enhancement of antimicrobial effect between photodynamic reactions in the presence of hydrogen peroxide. For this purpose, we used spectroscopic methods and fluorescence microscopy to examine ROS formation

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and photosensitizer uptake by cells. In addition, we also evaluated the optical characteristics of the dye in a biological environment to elucidate the possible mechanisms involved in this association.

2 Results

2.1 Investigation of the microbial reduction *in vitro*

Three different species of microorganisms with diverse physiology, morphology and different resistance were used in the photoinactivation studies, and all species are important human and animal pathogens. Methylene blue (MB) alone (60 μ M) and H₂O₂ solution (100 mM) were incubated with each of the microorganisms for 30 min to detect any inherent dark toxicity of the compounds. Both of them presented only slight toxicity for the three tested microorganisms.

Fig. 1 shows how the viability of the microorganisms changes with PDT in the presence of increasing H₂O₂ concentration. It can be observed that in the dark or using only H₂O₂, the bacterial inactivation was insignificant for any microorganism. When MB was diluted in PBS and the dye is photoactivated by red laser (PDT group), microbial reduction was less than 1 log. In fact, the decrease was approximately 70% for *Staphylococcus aureus* and *Candida albicans*. For *Escherichia coli*, the Gram-negative

species, the reduction was about 60%. However, for all examined microorganisms, PDT in the presence of increasing hydrogen peroxide concentrations gave increased microbial killing in an H₂O₂ dose-dependent manner.

2.2 Investigation of the mechanisms involved in the reaction

2.2.1 Singlet oxygen and total ROS detection in cell free system. As presented in Fig. 2A and 2B, there are important differences between the rates of ROS generation from illuminated MB in the presence and absence of H₂O₂, since the formation of singlet oxygen as measured by 1270 nm luminescence was almost inhibited (Fig. 2A) and total ROS production as measured by DCDHF oxidation was diminished (Fig. 2B). These results indicate that altered production of ROS by changes in photochemistry produced by H₂O₂ does not explain the increased microbial killing. However the photochemical reactions taking place inside the microorganisms may not be the same as those taking place in solution.

2.2.2 ROS production inside the microorganism. Surprisingly, as shown in the previous sections, the measurement of singlet oxygen and total ROS detection did not confirm our first assumption to explain the increased PDT effect in the presence of H₂O₂, *i.e.*, H₂O₂ would provide more molecules of oxygen for

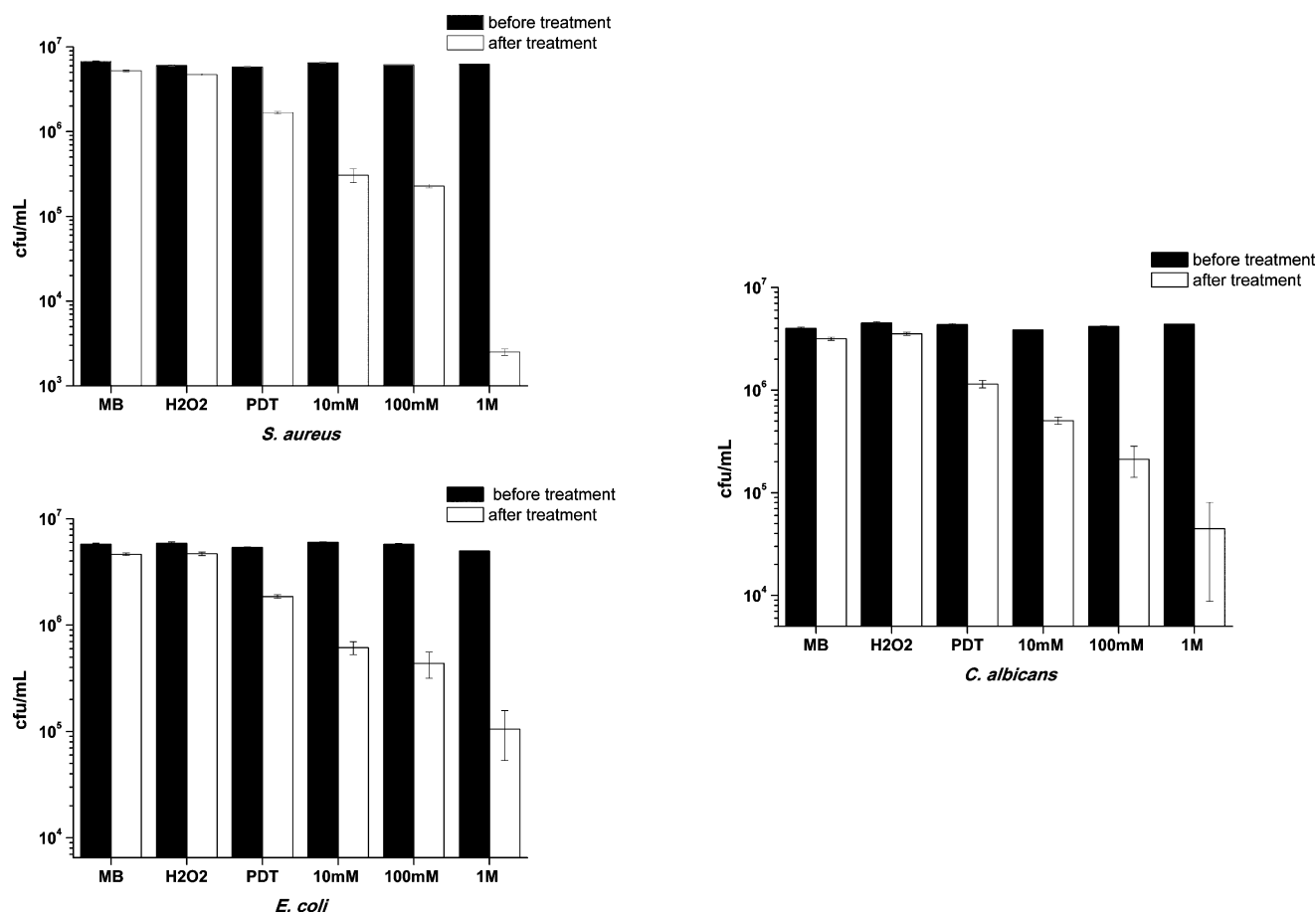


Fig. 1 Effect of MB (60 μ M during 10 min) in PBS or H₂O₂ (100 mM during 10 min) without irradiation and PDT using MB in PBS solution or with crescent concentrations of H₂O₂ solution under irradiation on the reduction of *S. aureus*, *E. coli* and *C. albicans*. Illumination parameters: $\lambda = 660$ nm, $P = 40$ mW, $\Delta t = 60$ s.

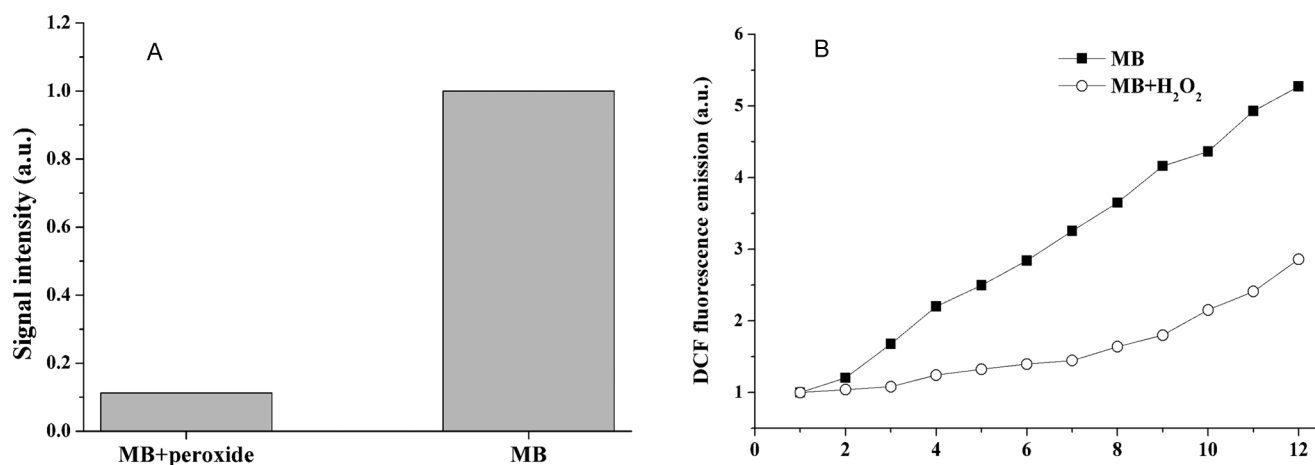


Fig. 2 ROS production by MB in the presence and absence of H₂O₂. (A) Direct measure of the phosphorescence of ¹O₂ at λ = 1270 nm (λ_{exc} = 532 nm); (B) Indirect measurement of total ROS formation by DCF fluorescence at λ_{emiss} 522 nm.

the photoreaction than in water solution and this should increase the amount of ROS produced. Thus, we decided to investigate what would happen with the photoreaction in the presence of a biological substance, since our microbiological experiments and also the literature^{12–15} agreed that H₂O₂ gave more microbial PDT killing.

Fig. 3 displays the fluorescence images of DCF (λ > 500 nm) as a function of the irradiation time inside the microorganism due to photo-oxidized MB taken up by *C. albicans*. Fig. 4 shows the formation of DCFH to DCF due to production of ROS under MB irradiation in presence or not of H₂O₂. To compare the rates of DCF formation, we assumed that the fluorescence can be written with the expression $F(t) = F_{\max}[1 - \beta e^{-t/k}]$,¹⁶ where $F(t)$ is the intensity of fluorescence at time, β is a fit parameter, and k is the time constant, *i.e.*, the inverse of the rate of DCF formation. Then, the rate of DCF formation can be calculated from expression: $V_F = dF(t)/dt$. Using this analysis, when $t \rightarrow 0$, the relative rate ($V_{\text{perox}}/V_{\text{PBS}}$) of DCF formation in the presence of hydrogen peroxide is approximately 4 times faster than in PBS solution.

2.3 Investigation of the mechanisms involved

2.3.1 MB uptake by bacteria. MB concentration from the supernatant removed from the *E. coli* suspension treated (experiment) or not (control) with H₂O₂ was calculated from absorbance spectra. The optical density at λ = 660 nm is higher when MB is diluted in PBS solution with a calculated concentration of approximately 22 μM and lower when the dye is in a peroxide solution (around 17 μM), which means that the amount of MB uptake by bacteria increases when H₂O₂ is combined with MB, *i.e.*, 72% and 63% of MB is taken up by cells in the presence of H₂O₂ and in PBS solution, respectively. These data (Fig. 5) indicate that there is more MB bound to the microorganism when they are treated with a peroxide solution, therefore maybe there is an increased probability of generating ROS inside the cells.

2.3.2 Reduction state of the dye. The contact of MB with biological environments containing reductase enzymes usually causes a small but reversible chemical reduction of the dye

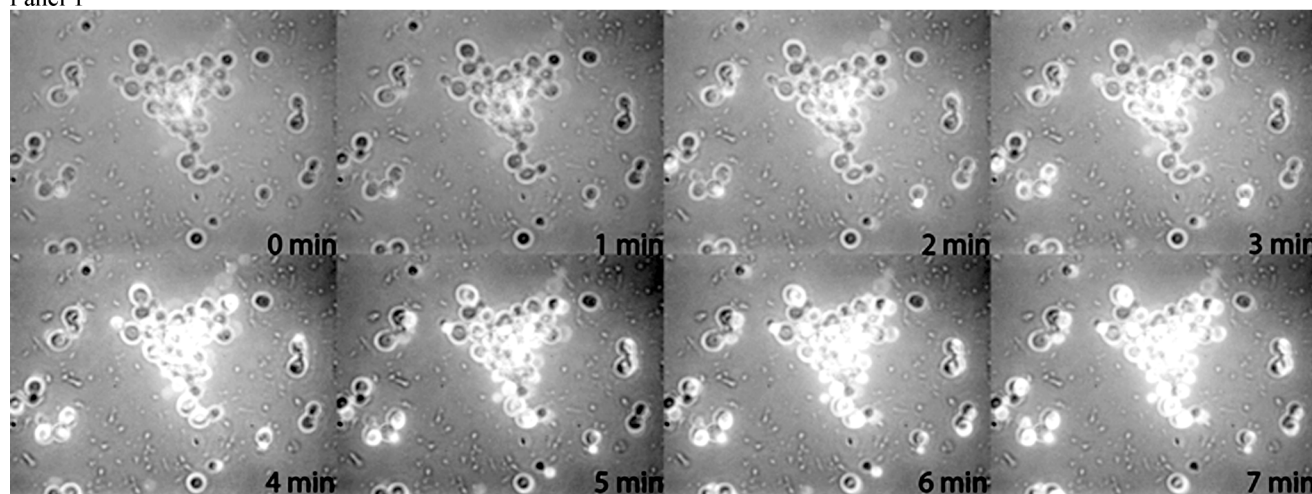
leading to an increase in the concentration of leucoMB.^{17,18} In our experimental conditions, this effect was visualized by putting MB in contact with *E. coli* and observing an increased absorption at 290 nm and a decrease in the absorption peak at 660 nm. Interestingly, when H₂O₂ was added to the cuvette there was a progressive inversion in the optical characteristics of the dye better visualized after 20 min of peroxide incubation (Fig. 6). The absorption peak at 290 nm diminished and the absorption peak at 660 nm increased showing oxidation of leucoMB and reappearance of “colored” form. In fact, the initial ratio 660/290 was 2.5 while following 20 min, the ratio increased to 2.7 ($p = 0.001686$). This finding could also be important to explain the enhancement in cell killing when MB and H₂O₂ are used during PDT.

2.3.3 Changing the sequence of addition of the components.

The presence of biological substrates affects the photoreaction of MB in a peroxide environment, as we can see at panel 2. We then asked if, as suggested by Funk and Krise,¹⁹ the H₂O₂ could alter the membrane permeability and improve the cellular accumulation of MB or, as suggested by Caetano and co-workers,²⁶ that the photoreaction would promote membrane disruption and facilitate the penetration of H₂O₂ into the cell. A possible explanation of the higher effect of PDT in the presence of H₂O₂ could be that MB has an easier transit across the cell membrane.²⁰ To test this hypothesis, we used a Gram-negative bacterium since these species have an outer membrane that provides a physical and functional barrier between the cell and its internal environment.

As shown in Fig. 7, the bacterial inactivation using MB in a PBS solution was only about 0.5 log and this value was significantly different from all the other groups. Concerning the other tested sequences of addition of H₂O₂ and PDT, a remarkable difference was observed among the groups. The group (MB+H₂O₂)+light showed a reduction of 1.8 log while the decrease for the group (MB+light)+H₂O₂ was about 1.4 log. Interestingly, the group H₂O₂+(MB+light) presented a diminution of 2.8 log suggesting that the H₂O₂ damages the bacterial outer membrane and improves the dye uptake.

Panel 1



Panel 2

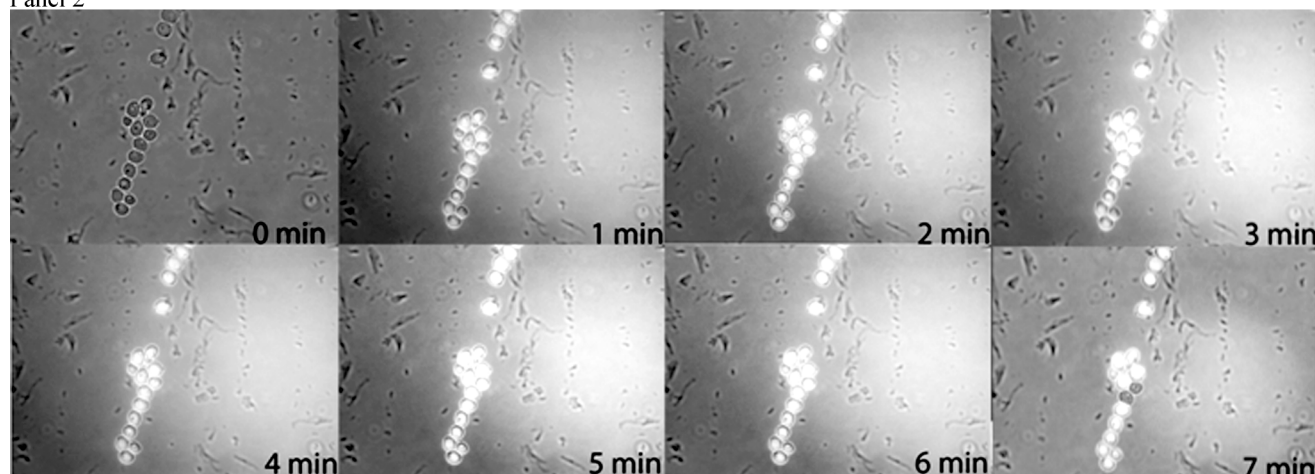


Fig. 3 Panel 1: Photomicrographs of suspension of *C. albicans* loaded with DCFH in the presence of MB in PBS solution. Compositions of fluorescence and optical microscopy of the cells following irradiation. Under irradiation, the DCFH fluorescence was increased in function of irradiation time (increased brightness due to DCFH oxidation by ROS formation). Panel 2: Photomicrographs of suspension of *C. albicans* loaded with DCFH in the presence of MB in H_2O_2 solution. Compositions of fluorescence and optical microscopy of the cells following irradiation. Under irradiation, the DCFH fluorescence was increased in function of irradiation time (increased brightness due to DCFH oxidation by ROS formation).

3 Discussion

McCullagh and Robertson first reported a possible improvement of microbial killing using H_2O_2 and PDT against cyanobacteria.¹⁴ Also in 2006, the same authors showed the photodestruction of *Chlorella vulgaris* by MB and nuclear fast red (NFR) combined with H_2O_2 under irradiation,¹⁵ presenting this interaction as an improvement in antimicrobial PDT. Hayek *et al.*¹² and Garcez *et al.*¹³ found similar results working with a urea peroxide paste associated with a photosensitizer against oral bacteria *in vitro* and *in vivo*. However, those studies did not compare PDT killing with PDT killing in the presence of H_2O_2 .

To test the combination of MB-PDT and H_2O_2 , we used more challenging conditions such as an increased number of bacteria, which diminishes the efficiency of the PDT, the microorganisms were used in stationary growth phase, when they have increased defenses against ROS, and sub-lethal PDT parameters were chosen in order to enhance the differences among the tested conditions.

Our results showed that the combination of MB with H_2O_2 solution can enhance the antimicrobial effectiveness of PDT for all species of microorganism tested, such as *S. aureus* (Gram+), *E. coli* (Gram-) and for the fungus *C. albicans*. The higher susceptibility of *S. aureus* was expected, since many reports have shown that Gram+ bacteria are more susceptible to PDT than other microbial cells.⁷⁻¹⁰ In addition, our findings indicate that the progressive increase in H_2O_2 concentration progressively increases microbial killing by PDT.

The first hypothesis to explain our results was that the increased amount of molecular oxygen, produced by H_2O_2 dissociation, could improve the likelihood of ROS formation. Because it is generally accepted that the type II reaction predominates in oxygenated system,²¹ singlet oxygen could be generated *via* an energy transfer process during a collision of the excited sensitizer with triplet oxygen and this process typically produces 10^3 to 10^5 molecules of singlet oxygen before being degraded.²² The amount of molecular oxygen in a H_2O_2 solution (at 1 M, normally used as

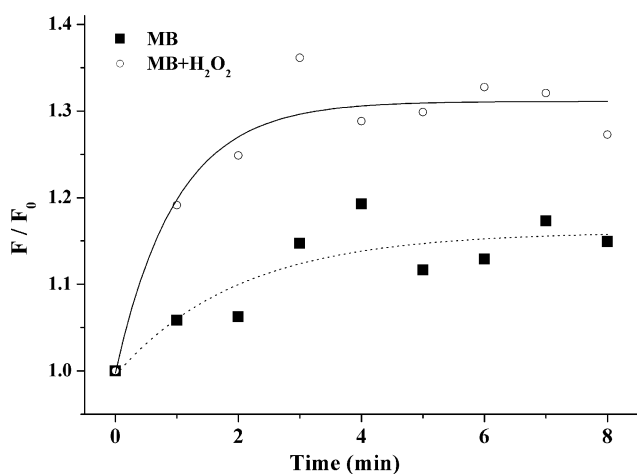


Fig. 4 Dynamic of relative ROS formation inside *C. albicans* cells by MB in PBS solution and MB in H_2O_2 solution evaluated by fluorescence intensity (F) of DCF formation under irradiation. F_0 is the fluorescence at initial irradiation time. Illumination parameters: $\lambda = 660$ nm, $P = 40$ mW, $\Delta t = 60$ s. Solid and dashed lines are the exponential data fits (see text).

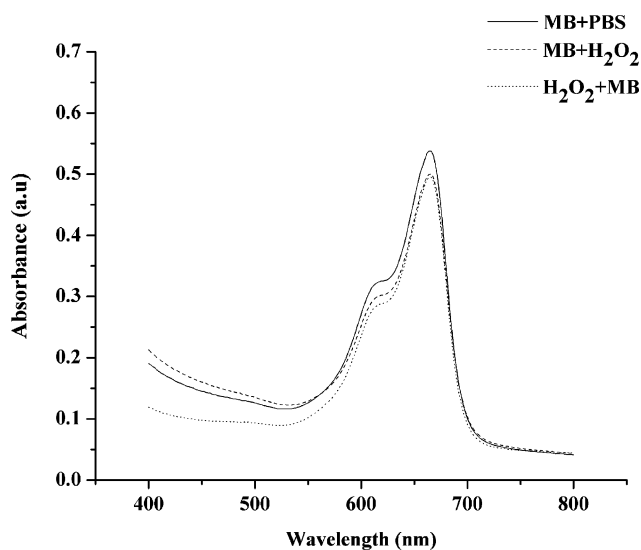


Fig. 5 Absorbance spectrum of *E. coli* supernatant after incubation with MB in PBS solution, incubation with MB in H_2O_2 solution, and incubation with H_2O_2 and then with MB.

an antimicrobial agent) is approximately 30% higher than in a PBS solution (data not shown). However, our findings undoubtedly demonstrate that ROS production in solution decreases rather than increases in the presence of H_2O_2 and the production of singlet oxygen is almost inhibited compared to the production of singlet oxygen in PBS solution. Therefore, the possibility of a type I mechanism was tested.

We tried to explain the enhanced microbial reduction by photochemical reaction. It could occur that the photo-excited MB reacts directly with H_2O_2 in a type I process *via* electron transfer generating either hydroxyl or more likely hydroperoxide radicals and that these ROS could attack the microorganisms. This hypothesis was supported by the report from Gak *et al.*²³ that investigated the quenching of triplet excited MB by H_2O_2 . The authors suggested two mechanisms for the quenching: electron transfer from the excited MB to H_2O_2 with the formation of a

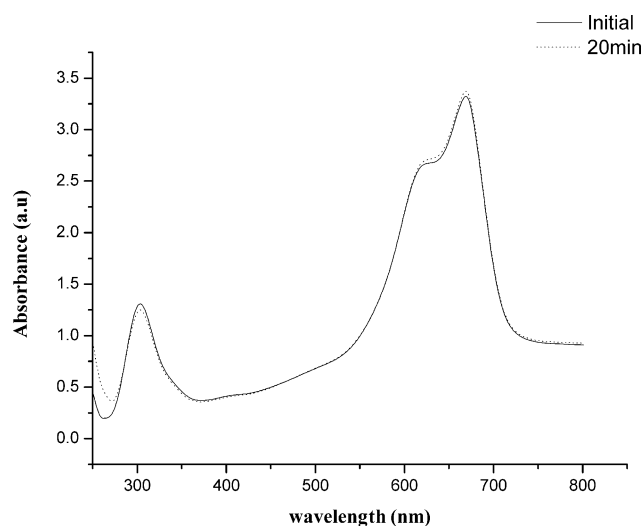


Fig. 6 Absorbance spectra of methylene blue inside *E. coli* (initial) and after 20 min of incubation in peroxide solution. Note the variation of the leucoMB and MB absorbance peaks ($p < 0.01$).

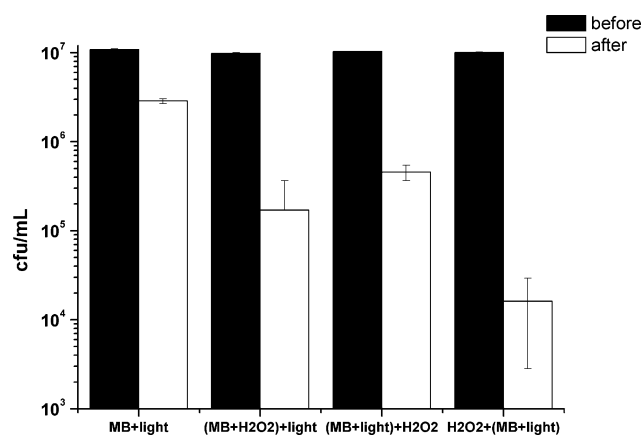


Fig. 7 Effects of different sequences of H_2O_2 , MB diluted in PBS or H_2O_2 and light on *in vitro* killing of *E. coli*. Illumination parameters: $\lambda = 660$ nm, $P = 40$ mW, $\Delta t = 60$ s.

MB^+ radical and OH^- or electron transfer from H_2O_2 to the MB molecule with the formation of the MB^- radical and HO_2^* radical. McCullagh and Robertson¹⁴ found an increase in the antimicrobial activity of MB/ H_2O_2 system when investigating the antimicrobial photodynamic effect of MB and H_2O_2 against cyanobacteria in an oxygen-free atmosphere. It was proposed that the process involved a type I mechanism with electron transfer from MB to H_2O_2 generating active radical species. However, the total ROS production measured in the present study indicated a decrease in ROS formation in a H_2O_2 environment.

Investigating the behavior and resistance of bacteria under extreme conditions and the physiological changes associated with oxidative stress, Baatout and collaborators²⁴ showed that bacterial strains exhibited varying sensitivities towards exogenous H_2O_2 . For all bacterial strains some physiological damage was noted, in particular with regard to their membrane permeability. Depending on the bacterial strains, moderate to high physiological damage was observed. Membrane potential, esterase activity, intracellular pH were considerably modified at high H_2O_2 concentrations. In

addition, a low micromolar, single dose of hydrogen peroxide was shown to cause dramatic increases in the apparent intracellular accumulation of fluorophores with different physicochemical properties in different cell types.¹⁹ The results were consistent with changes in lateral membrane diffusion caused by hydrogen peroxide.

In fact, Funk and Krise¹⁹ reported that exposure to H₂O₂ facilitated the accumulation of the test substrates (lucifer yellow, oregon green and daunorubicin) in different cell types (HL-60 and human foreskin fibroblasts). Our results agree with those of Funk and Krise, since the amount of MB uptake after incubation with H₂O₂ is higher (5 μM more compared to PBS solution). The enhancement on the amount of MB uptake by the microorganism results in a higher concentration of intracellular MB, therefore MB might be closer to the intracellular sites sensitive to PDT damage. It has been recognized for many years that MB binds to DNA, preferentially to G-C base pairs and it has been assumed that MB, being a tricyclic heterocyclic compound, would intercalate between the base pairs of DNA.²⁴ MB can also photo-oxidize several amino acids, specifically tryptophan, tyrosine, histidine, methionine and cysteine and this reaction can occur by type I or type II mechanisms. Some organelles as *E. coli* ribosomes are inactivated by MB photodynamic treatment and biological membrane contains proteins, phospholipids and cholesterol, which are susceptible to photodamage.²⁵

Increased MB uptake after H₂O₂ can explain the results found when changing the sequence of treatments. It is clear that using H₂O₂ before PDT or using MB in H₂O₂ solution improves the uptake of MB by the microorganism and leads to increased microbial killing. However, it is not totally clear how to explain improved killing when PDT was used first and subsequently H₂O₂ solution.

Caetano and coworkers²⁶ investigated the photodecomposition of phospholipid bilayers in aqueous solutions of methylene blue and observed giant unilamellar vesicles under an optical microscope, which revealed a consistent pattern of membrane disruption as a function of MB concentration and photon density for different substrates supporting the vesicles. Also, Giroldo *et al.*²⁷ studying the effect of antimicrobial PDT in *C. albicans* propose that the use of PDT in microorganisms results in an increase of membrane permeabilization, suggesting that antimicrobial PDT mechanism using MB may be related to damage in the plasma membrane of the cells.

Using this hypothesis, PDT alone could promote minor membrane disruption and this could increase the exposure of internal components of the bacteria to H₂O₂. Since H₂O₂ is currently used as antimicrobial agent,¹⁵ it was expected that facilitating the access of this agent to the interior of bacteria could increase the bacterial killing.

Investigating the production of ROS inside the microorganism and comparing the pictures in panel 1 and 2, the DCF fluorescence is higher inside the cells when the MB is in a H₂O₂ solution than in PBS solution. The dynamic of ROS production is different between the solutions. In a H₂O₂ environment, the intracellular concentration of MB may result in a faster and a higher amount of ROS production inside the microorganism and therefore a more efficient antimicrobial effect.

Apparently, these data are not consistent with the results of *in vitro* analysis of ROS and singlet oxygen photo-production

in the presence of H₂O₂. However, the results of the optical characteristics of the dye showed that the amount of leucoMB decreases when in contact with H₂O₂ and the oxidized “colored” form increases. May *et al.*²⁸ suggested that a reduction occurs on MB at the external face of the membrane through an interaction with reductase enzymes. The dye enters the cell by diffusion, and entry is facilitated because leucoMB is more lipophilic and it lacks the charge possessed by MB. It is proposed that once inside the cell, the leuco form is reoxidized.²⁹ Moreover, when in a H₂O₂ solution, the oxidation process is more intense and the amount of oxidized “colored” molecules inside the bacteria is enhanced. In aqueous solution, MB should be found in its leuco form, which is incapable of absorbing visible light ($\lambda = 660$ nm) and consequently initiate a photodynamic reaction.²⁷

Also, *C. albicans* is an aerobic microorganism and can deal with H₂O₂ by its expression of peroxidase and catalase enzymes, probably during the pre-irradiation time when the microorganism is in contact with MB and H₂O₂. The hydrogen peroxide improves the uptake of the photosensitizer and part of the H₂O₂ may be inactivated by the yeast enzymes. Moreover, there are a lot of different sites to be oxidized by H₂O₂ or PDT inside the cells and the quenching proposed by Gak *et al.*²³ (electron transfer from the excited MB to H₂O₂ or electron transfer from H₂O₂ to the MB molecule) is probably lower than *in vitro* or even there was no quenching. Therefore, the photoreaction inside the microorganism was not decreased by the presence of H₂O₂. Indeed, the superior amount of MB inside the cells produces an enhanced photoreaction.

These data indicate that the photochemistry of the MB is most unfavorable in the presence of H₂O₂ in cell free systems. However, the exposure of microorganisms to H₂O₂ leads to a higher uptake of MB inside the cells resulting in an improved ROS production close to vital structures and, consequently, to a higher microbial death.

4 Experimental procedure

4.1 *In vitro* antimicrobial experiments

Suspensions of three microorganisms, Gram-positive (*Staphylococcus aureus*, ATCC 27659), Gram-negative (*Escherichia coli*, HB 101) and yeast (*Candida albicans*, 51393) were grown in brain heart infusion (BHI) broth at 37 °C with shaking (150 rpm) for 48 h, until they formed a stationary growth phase suspension.

The suspensions were diluted in PBS to a cell density of 10⁷ per mL, and 1 mL aliquots were added to wells of a 24-well plate. The three microorganisms were incubated with 60 μM of methylene blue (MB) (Sigma Aldrich, Milwaukee, USA) in a PBS solution and in 100 mM solution of H₂O₂ for 30 min in the absence of light to verify intrinsic (dark) toxicity.

When the results showed a similar microbial killing for the dye and for the H₂O₂, around 20% after 30 min (data not shown), we tested the effect of photoreaction when 60 μM of MB were incubated in a solution of PBS or in a solution of H₂O₂ (10 mM, 100 mM and 1 M) for 10 min (pre-irradiation time) followed by illumination with a diode laser at $\lambda = 660$ nm, output power of 40 mW (MMOptics, São Carlos, Brazil) and exposure time of 60 s corresponding to the delivery of 60 J cm⁻².

Since 30 min of contact with a solution of 100 mM H₂O₂ did not affect the viability of the microorganisms more than the dark toxicity of the MB, we tested the microbial reduction in the presence of 10 times less and 10 times more H₂O₂ than the dark exposure to verify if more or less oxygen available in the solution could kill more microorganisms.

A control group was incubated by the same time without any irradiation or H₂O₂ challenge. Survival fractions were determined from the colony forming units (CFU) in the initial inoculums and compared with the experimental CFU.

4.2 *In vitro* singlet oxygen and total ROS detection

The first hypothesis that we tested concerned about oxygen concentration. In H₂O₂ solution the molecules of oxygen available for a photoreaction could be greater than in water solution, as a consequence, this would increase the amount of ROS, especially singlet oxygen.

To validate this hypothesis, a direct singlet oxygen detection was performed, based on its phosphorescence at $\lambda = 1270$ nm. A cuvette containing 30 μ M of MB in a deuterated (D₂O) solution or D₂O plus H₂O₂ solution was irradiated using a Nd:YAG laser (Surelite III, Continuum, Santa Clara, USA) at $\lambda = 532$ nm (pulse-energy of 30 mJ and pulse-length of 10 ns). The transient emission of singlet oxygen phosphorescence was accumulated and integrated over 20 pulses by a fluorimeter linked to an infrared photomultiplier tube.³⁰ A D₂O solution was used to enhance the lifetime of singlet oxygen and therefore increase its detection by the equipment.

In addition, an indirect total ROS detection was performed, based on the oxidation of diacetate of dichlorofluorescein (DCFH-DA) to its fluorescent form dichlorofluorescein (DCF form). The fluorescence was measured ($\lambda = 522$ nm) with a standard fluorimeter. Initially, 10 μ L of sulfuric acid (Aldrich, Milwaukee, USA) was added to a cuvette containing 20 μ L of DCFH-DA for 20 min in dark conditions. Then, ten- μ L of sodium hydroxide was added to neutralize the acid. MB at 60 μ M in PBS or H₂O₂ solution completed the cuvette and a baseline was registered. Illumination was performed using a $\lambda = 660$ nm diode laser with $P = 40$ mW for periods of 1 min performing 12 min of irradiation and DCF fluorescence was measured (at $\lambda = 522$ nm) following excitation with a $\lambda = 490$ nm lamp.¹⁶

4.3 Total ROS detection inside *C. albicans*

To evaluate if biological material could affect the photoreaction of MB in a peroxide environment, a suspension of *C. albicans* was incubated with 60 μ M of MB in PBS solution for 10 min. First, the microorganism incubated with MB was washed and centrifuged for 10 min at 4000 rpm; the supernatant was removed and the cells re-suspended in a PBS solution. Then, a solution of 10 μ M of DCFH-DA was added for 15 min and again washed to remove all DCFH-DA not bound to the cells. The cells were irradiated using the same laser as above mentioned with a fluence of 60 J cm⁻² ($\Delta t = 1$ min) and the rate of DCF formation was evaluated at $\lambda = 522$ nm by fluorescence microscopy images (inverted Zeiss microscope). The images were analyzed using the software Image J (National Institute of Health, USA) and Adobe Photoshop CS3 for Macintosh (Adobe, USA). Each picture has 640 \times 480 pixels and 8 bits black and white image. The total

area of each image was selected and analyzed by fluorescence intensity through the histogram and the values were analyzed as a function of time. The relative brightness (maximum and extension) of fluorescence images was evaluated as a function of irradiation time, and plotted as intensity *versus* irradiation time. The same experimental procedure was repeated using MB in a 100 mM H₂O₂ solution. The use of yeast instead of bacteria is justified because the morphology of *C. albicans* is more complex and it is bigger than *S. aureus* or *E. coli* to be observed by optical microscopy.

4.4 Mechanisms involved in the combination

4.4.1 MB uptake by bacteria. Suspensions of *E. coli* were diluted in 60 μ M MB in PBS solution to a cell density of 10⁷ per mL for 10 min. The suspension was centrifuged for 10 min at a rate of 4000 rpm sedimenting bacteria with bound dye. In the other group, suspension of *E. coli* was diluted in 100 mM of H₂O₂ associated to 60 μ M MB solution to a cell density of 10⁷ per mL for 10 min, and then the suspension was also centrifuged for 10 min at 4000 rpm and the supernatant was removed. In the third group, bacterial suspension was firstly incubated with 100 mM of H₂O₂ for 10 min. The peroxide solution was washed and replaced by MB at 60 μ M in PBS solution. The suspension was again centrifuged for another 10 min at 4000 rpm. The supernatant of all groups was removed and analyzed by optical spectroscopy to evaluate the MB uptake by bacteria.

4.4.2 Reduction state of the dye. To evaluate if H₂O₂ would alter the balance between MB and leucoMB,¹⁸ the optical characteristics of the MB inside an *E. coli* suspension in aqueous or H₂O₂ solution were analyzed *via* absorbance spectroscopy in a computer-interfaced double-beam spectrophotometer (Cary – 17D Spectrophotometer Conversion, On-Line Instrument System Inc, USA) in 1.0 cm optical path length quartz cuvettes. The dye was dissolved in 3 mL of de-ionized water or H₂O₂ at the concentration of 60 μ M in the presence or absence of 10⁴ cfu mL⁻¹. The optical density at $\lambda = 290$ nm to $\lambda = 660$ nm was obtained from each wavelength. A second assay was performed replacing 1 mL of aqueous solution by peroxide solution to keep the same MB molarity.

Optical densities were measured every 1 min and analyzed regarding MB/leucoMB ratio; the ratio was calculated by absorbance at 660 nm/absorbance at 290 nm, since the absorbance of MB monomers is around $\lambda = 660$ nm and for leucoMB is around $\lambda = 290$ nm. The different values found at each sample time were statistically compared using *t*-test with Microsoft Excel. Results were considered significant when $p < 0.01$.

4.4.3 Changing incubation sequence. Different combination sequences of MB and H₂O₂ were performed to verify the hypothesis that different mechanism of killing could occurs depending on the sequence used for the antimicrobial effect. Suspensions of *E. coli* in the stationary phase were diluted in PBS to a cell density of 10⁷ per mL, and 100 μ L aliquots were added to wells of a 96-well plate. Regarding to the experimental groups, one group [(MB+H₂O₂)+light] received the 60 μ M MB in a 100 mM solution of H₂O₂ for 10 min and was irradiated by the same diode laser used above (light fluence: 60 J cm⁻²). The second group [H₂O₂+(MB+light)] received 100 mM of H₂O₂ for 10 min, and then the suspension was centrifuged for 10 min at 4000 rpm. The

supernatant was removed and replaced by a 60 μM of MB solution in PBS for further 10 min and then irradiated (light fluence: 60 J cm^{-2}). The third group [(MB+light)+ H_2O_2] was incubated with 60 μM of MB for 10 min in a PBS solution followed by irradiation. Then, this group received a 100 mM solution of H_2O_2 in the absence of light for further 10 min. Survival fractions were determined from the CFU in the initial inoculums and compared with the experimental CFU.

Conclusion

The combination of methylene blue and H_2O_2 has been shown to be an interesting approach to improve PDT efficiency against Gram+ bacteria, Gram- bacteria and fungi. The mechanism involved seems to be related with the increase in cell membrane permeability, MB reduction by the cell during photosensitizer uptake followed by oxidation of the leucoMB form as a result of the hydrogen peroxide, therefore increasing ROS formation inside the microorganism.

Acknowledgements

The authors kindly thank Prof. Karin do Amaral Riske for the use of the optical microscope, Helena Junqueira for her collaboration, and FAPESP (grant 05/51598-7) for financial support. R. Itri, M. S. Baptista and M. S. Ribeiro also acknowledge CNPq for research fellowship. N. A. Daghanli was supported by a post-doctorate fellowship from FAPESP (05/00253-0). Research in Dr Hamblin's laboratory is supported by US NIH grant R01AI050875, US Air Force MFEL Program (FA9550-04-1-0079).

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