Antimicrobial mechanisms behind photodynamic effect in the presence of hydrogen peroxide

Aguinaldo Silva Garcez[a], **Silvia Cristina Núñez**[b], **Mauricio S. Baptista**[c], **Nasser Ali Daghastanli**[d], **Rosangela Itri**[e], **Michael R. Hamblin**[f,g,h], and **Martha Simões Ribeiro**[i]

[a] Centro de Pesquisa e Pós-Graduação São Leopoldo Mandic, Campinas-SP, Brazil

[b] Centro de Estudos, Treinamento e Aperfeiçoamento em Odontologia, São Paulo-SP, Brazil

[c] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo-SP, Brazil

[d] Universidade Federal do ABC, Santo André-SP, Brazil

[e] Departamento de Física Aplicada, Instituto de Física, Universidade de São Paulo, São Paulo-SP, Brazil

[f] Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA, USA

[g] Department of Dermatology, Harvard Medical School, Boston, MA, USA

[h] Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA, USA

[i] Centro de Laser e Aplicações, Instituto de Pesquisas Energéticas e Nucleares, IPEN-CNEN/SP, Av. Lineu Prestes, 2242, Cidade Universitária, 05508-000, São Paulo-SP, Brazil. marthasr@usp.br; Fax: +55-11-31339374; Tel: +55-11-31339197

Abstract

This study describes the use of methylene blue (MB) plus light (photodynamic inactivation, PDI) in the presence of hydrogen peroxide (H_2O_2) to kill *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. When H_2O_2 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 H_2O_2. It was observed that ROS production was almost inhibited by the presence of H_2O_2 when cells were not present. In addition, experiments using different sequence combinations of MB and H_2O_2 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 H_2O_2 was used before or simultaneously with PDI, and ROS formation inside *C. albicans* cells confirmed that ROS production is higher in the presence of H_2O_2. 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 H_2O_2 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 cytoplasmatic 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 (H_2O_2) 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 H_2O_2 and the ROS produced during PDT, or by one treatment (H_2O_2 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 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 μM) and H_2O_2 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 H_2O_2 concentration. It can be observed that in the dark or using only H_2O_2, 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 H_2O_2 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 H_2O_2, 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 H_2O_2 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 PDI effect in the presence of H_2O_2, *i.e.*, H_2O_2 would provide more molecules of oxygen for 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 H_2O_2 gave more microbial PDT killing.

Fig. 3 displays the fluorescence images of DCF ($\lambda > 500$ 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 H_2O_2. 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 $t \to 0$, the relative rate (V_{perox}/V_{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 H_2O_2 was calculated from absorbance spectra. The optical density at $\lambda = 660$ 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 H_2O_2 is combined with MB, *i.e.*, 72% and 63% of MB is taken up by cells in the presence of H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 into the cell. A possible explanation of the higher effect of PDT in the presence of H_2O_2 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 H_2O_2 and PDT, a remarkable difference was observed among the groups. The group (MB+H_2O_2)+light showed a reduction of 1.8 log while the decrease for the group (MB+light)+H_2O_2 was about 1.4 log. Interestingly, the group H_2O_2+ (MB+light) presented a diminution of 2.8 log suggesting that the H_2O_2 damages the bacterial outer membrane and improves the dye uptake.

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 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 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 Robertson[14] 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 collaborators[24] 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 Krise[19] reported that exposure to H_2O_2 facilitated the accumulation of the test substrates (lucifer yellow, oregon green and daunorubincin) 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 H_2O_2 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 H_2O_2 can explain the results found when changing the sequence of treatments. It is clear that using H_2O_2 before PDT or using MB in H_2O_2 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 H_2O_2 solution.

Caetano and coworkers[26] 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 H_2O_2. Since H_2O_2 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 H_2O_2 solution than in PBS solution. The dynamic of ROS production is different between the solutions. In a H_2O_2 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 H_2O_2. However, the results of the optical characteristics of the dye showed that the amount of leucoMB decreases when in contact with H_2O_2 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 H_2O_2 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 H_2O_2 by its expression of peroxidase and catalase enzymes, probably during the pre-irradiation time when the microorganism is in contact with MB and H_2O_2. The hydrogen peroxide improves the uptake of the photosensitizer and part of the H_2O_2 may be inactivated by the yeast enzymes. Moreover, there are a lot of different sites to be oxidized by H_2O_2 or PDT inside the cells and the quenching proposed by Gak *et al.*[23] (electron transfer from the excited MB to H_2O_2 or electron transfer from H_2O_2 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 H_2O_2. 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 H_2O_2 in cell free systems. However, the exposure of microorganisms to H_2O_2 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 10^7 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 H_2O_2 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 H_2O_2, 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 H_2O_2 (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 H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 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 μM of MB in a deutered (D_2O) solution or D_2O plus H_2O_2 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 D_2O 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 μL of sulfuric acid (Aldrich, Milwaukee, USA) was added to a cuvette containing 20 μL of DCFH-DA for 20 min in dark conditions. Then, ten-μL of sodium hydroxide was added to neutralize the acid. MB at 60 μM in PBS or H_2O_2 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 μM 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 μM 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 640×480 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 H_2O_2 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 μM MB in PBS solution to a cell density of 10^7 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 H_2O_2 associated to 60 μM MB solution to a cell density of 10^7 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 H_2O_2 for 10 min. The peroxide solution was washed and replaced by MB at 60 μM 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 H_2O_2 would alter the balance between MB and leucoMB,[18] the optical characteristics of the MB inside an *E. coli* suspension in aqueous or H_2O_2 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 H_2O_2 at the concentration of 60 μM in the presence or absence of 10^4 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 H_2O_2 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 10^7 per mL, and 100 μL aliquots were added to wells of a 96-well plate. Regarding to the experimental groups, one group [(MB+H_2O_2)+light] received the 60 μM MB in a 100 mM solution of H_2O_2 for 10 min and was irradiated by the same diode laser used above (light fluence: 60 J cm^{-2}). The second group [H_2O_2+(MB+light)] received 100 mM of H_2O_2 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.

Acknowledgments

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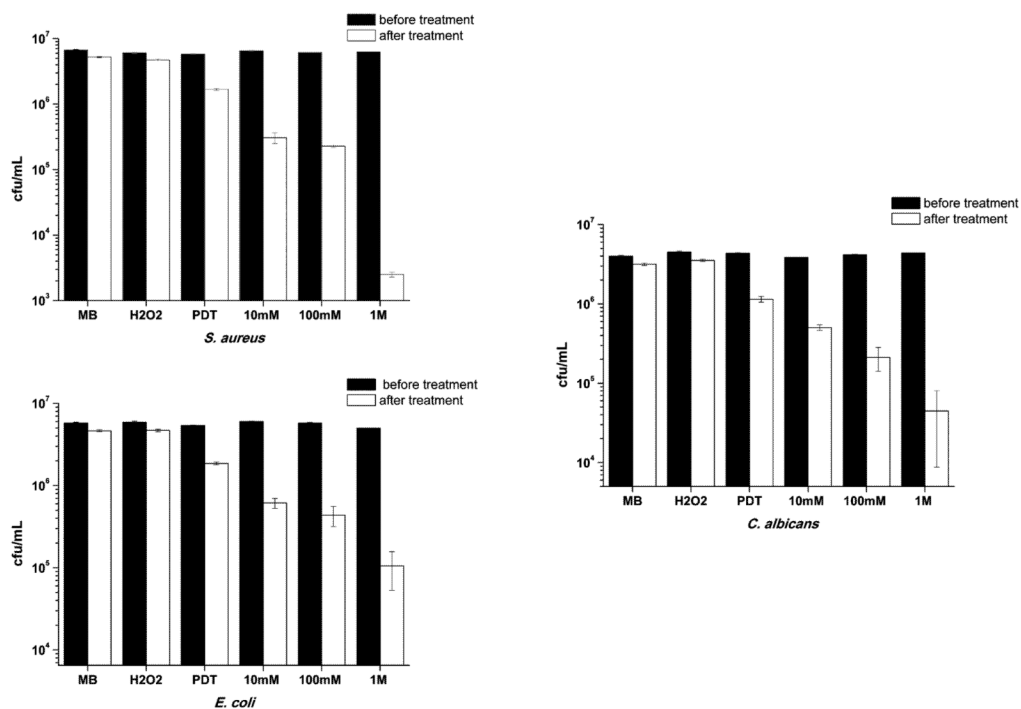
Fig. 1.
Effect of MB (60 μM during 10 min) in PBS or H_2O_2 (100 mM during 10 min) without irradiation and PDT using MB in PBS solution or with crescent concentrations of H_2O_2 solution under irradiation on the reduction of *S. aureus*, *E. coli* and *C. albicans*. Illumination parameters: $\lambda = 660$ nm, $P = 40$ mW, $\Delta t = 60$ s.
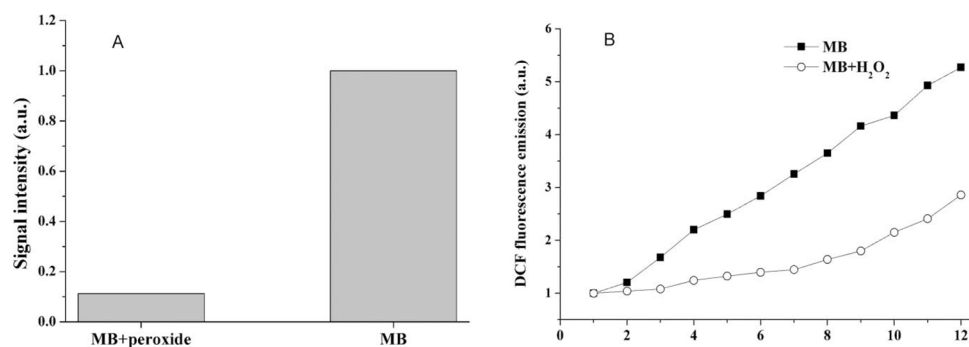
Fig. 2.
ROS production by MB in the presence and absence of H_2O_2. (A) Direct measure of the phosphorescence of $^1O_2$ at λ = 1270 nm (λ_{exc} = 532 nm); (B) Indirect measurement of total ROS formation by DCF fluorescence at λ_{emiss} 522 nm.
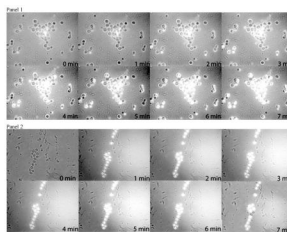
Fig. 3.
Panel 1: Photomicrographs of suspension of *C. albicans* loaded with DCFH in the presence of MB in PBS solution. Compositions of fluorescence and optical microscopy of the cells following irradiation. Under irradiation, the DFC fluorescence was increased in function of irradiation time (increased 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 DFC fluorescence was increased in function of irradiation time (increased brightness due to DCFH oxidation by ROS formation).
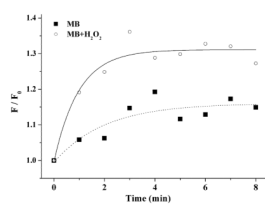
Fig. 4.
Dynamic of relative ROS formation inside *C. albicans* cells by MB in PBS solution and MB in H_2O_2 solution evaluated by fluorescence intensity (*F*) of DCF formation under irradiation. F_0 is the fluorescence at initial irradiation time. Illumination parameters: $\lambda = 660$ nm, $P = 40$ mW, $\Delta t = 60$ s. Solid and dashed lines are the exponential data fits (see text).
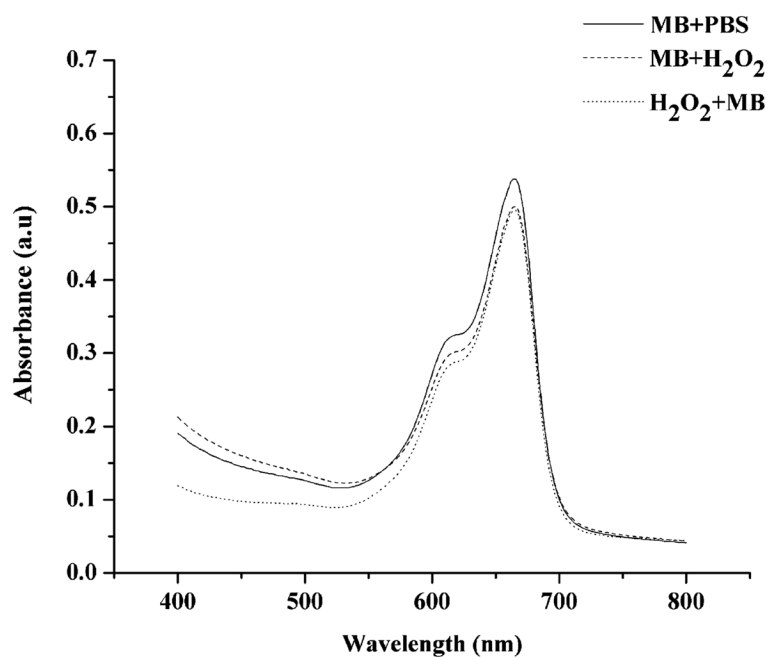
Fig. 5.
Absorbance spectrum of *E. coli* supernatant after incubation with MB in PBS solution, incubation with MB in H_2O_2 solution, and incubation with H_2O_2 and then with MB.
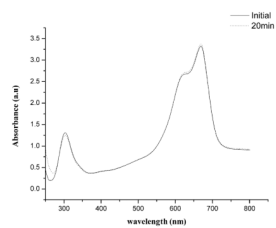
Fig. 6.
Absorbance spectra of methylene blue inside *E. coli* (initial) and after 20 min of incubation in peroxide solution. Note the variation of the leucoMB and MB absorbance peaks ($p <$ 0.01).
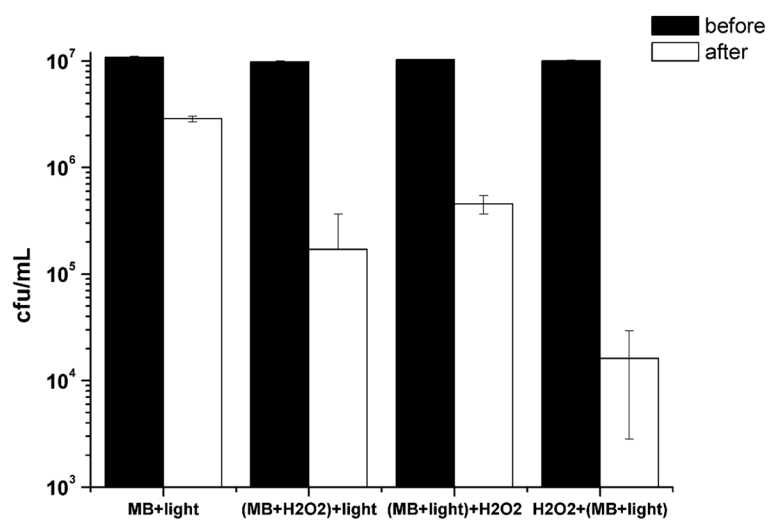
Fig. 7.
Effects of different sequences of H$_2$O$_2$, MB diluted in PBS or H$_2$O$_2$ and light on *in vitro* killing of *E. coli*. Illumination parameters: $\lambda = 660$ nm, $P = 40$ mW, $\Delta t = 60$ s.