Photodynamic effect of light-emitting diode light on cell growth inhibition induced by methylene blue

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The aim of this study was to propose the use of red light-emitting diode (LED) as an alternative light source for methylene blue (MB) photosensitizing effect in photodynamic therapy (PDT). Its effectiveness was tested against Staphylococcus aureus (ATCC 26923), Escherichia coli (ATCC 26922), Candida albicans (ATCC 90028) and Artemia salina. The maximum absorption of the LED lamps was at a wavelength of 663 nm, at intensities of 2, 4, 6 and 12 J.cm⁻² for 10, 20, 30 and 60 min of exposure, respectively. Assays with and without LED exposure were carried out in plates containing MB at concentrations of 7 to 140.8 µM for microorganisms and 13.35 to 668.5 µM for microorganisms or microcrustaceans. The LED exposure induced more than 93.05%, 93.7% and 93.33% of growth inhibition for concentrations of 42.2 µM for S. aureus (D-value=12.05 min) and 35.2 µM for E. coli (D-value=11.51 min) and C. albicans (D-value=12.18 min), respectively after 20 min of exposure. LED exposure for 1 h increased the cytotoxic effect of MB against A. salina from 27% to 75%. Red LED is a promising light device for PDT that can effectively inhibit bacteria, yeast and microcrustacean growth.


1. Introduction

Photodynamic therapy (PDT) has been successfully used for the treatment of several diseases involving abnormal cell growth such as cancer, rheumatoid arthritis, vitiligo, pathological myopia, age-related macular degeneration and arteriosclerosis (Levy 1995; Sternberg and Dolphin 1996; Sternberg et al 1998). The greatest advances have been in cancer treatment, but PDT has also shown effectiveness as an antibacterial and antifungal agent (Wainwright 1998; Phoenix et al 2003).

PDT is based on the interaction of visible light and a photosensitizer agent, which under photo-activation generates short-lived cytotoxic species in situ. Under excitation, the photosensitizer is converted from singlet to triplet state by an intersystem crossing process which, in turn, reacts with surrounding molecules to yield radical species and hydrogen peroxide – known as the type 1 mechanism, or transfers its energy to molecular oxygen (¹O₂) to produce singlet oxygen (¹O₂) – known as the type 2 mechanism. Both types of PDT result in localized cell death; however, the principal photodynamic mechanism has been shown to be the type 2 mechanism, which generates the highly toxic singlet oxygen (Weishaupt et al 1976).

Clinically, once the photosensitizer drug reaches its maximum level in the target tissue, a carefully regulated light dose is shone to activate the compound and start the photodynamic attack. For superficial skin diseases the light is applied directly to the affected tissue, while for internal diseases the light source is connected to an optical fibre catheter, which allows it to reach the target organ.

High-potency laser sources are preferable, but their high costs make PDT inaccessible for many institutions and therefore make the dissemination of this technology
difficult in most countries. The low cost of lasers based on
diode technology is very attractive and has been employed
for PDT, although they are still expensive for poor nations
(Machado 2000; Mang 2004). Recently, however, various
alternative light sources have been researched to replace
the laser in PDT. One of the most interesting of these is
the light-emitting diode (LED) because of its low price.
It is possible to find different colours of LED light in the
market, with radiations covering almost all of the visible
electromagnetic spectrum, including red light (650 nm
region). This alternative, despite having a lower potency
than laser, has several economic advantages and can be used
to optimize the investigation of new compounds for PDT
(Mang 2004).

The high cost of PDT in clinical application does not
result only from the price of the equipment, but also from
the high cost of the drugs used for PDT. Therefore, it is desirable
to develop new medicines, whose properties should include
high efficiency, low side-effects and reduced costs. Following
these requirements, some organic dyes have been proposed
as PDT candidates. Methylene blue (MB, structure shown
in figure 1), a well-known dye with high light absorption
at 665 nm, is effective in PDT, showing ability to generate
'O2 and photodynamic activity for clinical applications
against several diseases (Tardivo et al. 2004, 2005). Even
without light, MB has natural antifungal and antibacterial
activity (Wainwright and Crossley 2002; Calzavara-Pinton
et al. 2005), whose toxic potential can be increased by light
activation.

Bacteria and fungi are commonly used in preclinical
studies for modelling cell growth. Another bioassay that
may be useful for the investigation of new PDT compounds
is the in vitro assay of lethality using brine shrimp (Artemia
salina). This experiment is quick and inexpensive, and can be
adapted for the evaluation of photodynamic activity. Artemia
salina is a tiny crustacean used in many physiological
and bioassay studies, including the general toxicity of
polluted water, investigation of bioactive plant extracts,
mycotoxins, dinoflagellate toxins, anaesthetics, toxicity of
oil dispersants, di-n-alkyl phthalates and co-carcinogenic
phorbol esters (Kinghorn et al. 1977). The eggs of brine
shrimp are readily available commercially at low cost and
remain viable in their dry state for years. This bioassay has a
good correlation with cytotoxic activity in some human solid
tumours and with pesticide activity (McLaughlin et al. 1998;
Hostellmann 2001).

Therefore, the aim of the present paper is to contribute
to PDT development by researching alternative light
sources using LED light and its effect on the photodynamic
activity of MB in biological experiments. The investigation
of the effectiveness of PDT with MB and a LED device
was carried out on cultures of Staphylococcus aureus,
Escherichia coli (prokaryotic cells), Candida albicans and
A. salina (eukaryotic cells).

2. Materials and methods

2.1 Photosensitizer and light

MB (Vetec, C16H18ClN3S3H2O, MW 373.90 g mol−1) and all
other reagents and solvents used were of analytical grade and
used without purification. The MB stock solution of \(2.67 \times
10^{-2} \text{ mol l}^{-1}\) was prepared in deionized water and then diluted
by the appropriate volume to obtain the test solutions. The
LED (EverLight Co., Taiwan, ROC) light system was
constructed using 4 units (in series) that emitted red light, and
their individual potency was determined using a handheld
laser power meter (Edmund Optics Inc., Barrington, NJ,
USA). The fluorescence emission spectrum was obtained
in a SPEX Fluorolog2, model 1680. The absorption spectra
were obtained in a Cary 50 spectrophotometer (Varian Inc.,
Palo Alto, CA, USA).

2.2 Bacterial strains and culture conditions

Staphylococcus aureus (ATCC 26923), E. coli (ATCC
26922) and C. albicans (ATCC 90028) were used in the
experiments. The bacteria were kept in tryptic casein
agar and the yeast in Sabouraud dextrose agar in stock
culture plates in a fridge at −4°C. Before the experiments,
the microorganisms were grown in Mullen–Hinton broth
(MHB) at 37°C for 15 h, then harvested by centrifugation
and suspended in 0.85% saline at a stock concentration of
\((1–2) \times 10^6\) colony-forming units (CFU)/ml.

2.3 Assay with bacteria and yeast

All experiments were carried out in two groups: one group
exposed to the light and other kept in the dark. Two control
groups were prepared: one by mixing \(400 \mu\text{l}\) of inoculum +
100 \(\mu\text{l}\) of sterile saline (positive control), and the other by
mixing \(100 \mu\text{l}\) of MB at different concentrations + \(400 \mu\text{l}\) of
MHB without microorganisms (negative control). The test
groups were prepared by adding \(100 \mu\text{l}\) of MB solution in
different concentrations to \(400 \mu\text{l}\) of the calibrated inocula

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**Figure 1.** Structure of methylene blue.

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\text{H}_3\text{C} - \text{N} - \text{S} - \text{N} - \text{CH}_2 - \text{CH}_3
\]

\[
\text{H}_3\text{C} - \text{N} - \text{S} - \text{N} - \text{CH}_2 - \text{CH}_3
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\text{J. Biosci. 33(2), June 2008}
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Photodynamic effect of LED light on cell growth inhibition induced by methylene blue

2.3 Materials

in 4-well (15 mm diameter) sterile polystyrene plates, giving final concentrations varying from 7 to 140.8 $\mu$M. The light sources were placed vertically above the wells containing the samples (figure 2) and the plates were illuminated (one LED light for each well), under shaking at 25°C for 30 min. After illumination, all the samples were transferred to tubes, diluted with MHB to a final volume of 3.5 ml and incubated at 37°C. The growth rate was quantified by a spectrophotometer and the percentage of transmittance (%T) at 580 nm measured at 0, 2, 4 and 6 h until confluent growth of the positive control group was obtained. The results were expressed in %T as a function of turbidity resulting from microbial cell growth. At time 0, all test tubes were 100%T, and increased turbidity was therefore inversely proportional to the %T.

Inhibition of 50% (IC50) of the microbial cell growth for each microorganism was calculated from the exact correlation between MB concentration and the percentage of growth inhibition, obtained from the %T of each treatment in comparison with the %T of the positive control group. The results were expressed in %T as a function of turbidity resulting from microbial cell growth. At time 0, all test tubes were 100%T, and increased turbidity was therefore inversely proportional to the %T.

Comparison of the data was carried out using an ANOVA one-way test with the software Statistica® followed by a Tukey test, using the final %T value and number of CFUs.

2.4 Assays with A. salina

The A. salina eggs were hatched in saline water (NaCl 3.8 g l$^{-1}$). After 48 h at room temperature, 20 mature crustaceans were transferred to test-tubes containing 1.5 ml of aqueous MB solution and 1.5 ml of saline, with the final MB concentrations in the tubes being: 13.35, 67, 133.5, 343 and 668.5 $\mu$M. The samples were separated into two identical groups, with one group being illuminated (1 h) and the other group, including the control tubes without MB, being kept in the dark for 24 h. The numbers of live and dead crustaceans were then counted. All experiments were carried out in triplicate.

2.5 Statistical analysis

The slopes of the killing curves were measured by regression analysis and D-values were obtained from the slope of the killing curve line.

3. Results

The constructed LED system (4 x LED units – see figure 2) proved to be very effective and easy to use. The emission spectrum of red LED light and the aqueous MB absorption spectrum are shown in figure 3. The maximum absorption band of the dye, centred at 665 nm, is almost coincident with the maximum light emission of the red LED (figure 3). The measured potency of each LED was around 5 mwatts at 663 nm. As the illuminated area used in this work was around 1.5 cm$^2$, the energy dose calculated for 30 min...
illuminatation at this wavelength was 6 J cm\(^{-2}\); for 20 min it was 4 J cm\(^{-2}\) and for 10 min it was 2 J cm\(^{-2}\).

In the microorganism assay (figure 4), confluent growth (approximately 20–40\%T) was reached after 4 h of incubation for *S. aureus* and *E. coli*, and after 6 h for *C. albicans* in MHB (positive control group).

In a second experiment, MB was added to the cells under the same experimental conditions. The percentage growth rate of the microorganisms decreased proportionally as the MB concentration increased, as could be seen by the decrease in turbidity of the medium and the increase in %T values (figures 4A, B and C). The inhibition of 50\% (IC50) of the microbial cell growth for each microorganism was 43.35 ± 6.75 \(\mu\)M for *S. aureus*, 56.40 ± 11.48 \(\mu\)M for *E. coli* and 63.20 ± 11.30 \(\mu\)M for *C. albicans*.

Then, after the IC50 for each microorganism with MB was determined, in a third experiment using the same experimental conditions and the same concentrations of MB as described above, the cultures were submitted to LED light for 30 min. When the LED light irradiated the cultures without MB, no effect was seen on the growth of any of the microorganisms. However, for the cultures treated with MB, after 30 min of light activation, significant growth inhibition could be seen through the unchanged %T values for all the microorganisms from the beginning to the end (4 or 6 h of incubation) of the assay (figures 4D, E and F).

The effect of exposure time to LED irradiation was also investigated for the cultures with MB. A concentration lower than the IC50 was chosen because of the remarkable photosensitizing effect seen at lower concentrations for all the microorganisms tested. The cultures were illuminated for 10, 20 and 30 min (figure 5).

It can be seen that inhibition is dependent on exposure time to the LED. The response to MB and LED light calculated by the killing-rate curve and D-value was similar for all microorganisms.

**Figure 4.** Effect of LED light and different concentrations of MB on microbial cell growth. The *S. aureus*, *E. coli* and *C. albicans* cells were cultured with MB at various concentrations, and the growth rates of non-irradiated samples (A, B and C) and samples irradiated with LED light for 30 min (D, E and F) were measured by transmittance at 580 nm. The values are shown in %T but to calculate the IC50 the %T were transformed to % of growth inhibition using the control group as reference for 0% of inhibition. The concentrations of MB used are shown in the figure. Each point represents the mean ± SD of triplicate measurements.
Additionally, to demonstrate that the photoactivation effect of LED light on MB solution is not exclusive to microorganisms, the photodynamic effect on another live organism, the crustacean *A. salina*, was evaluated. The experiments with *A. salina* were modified for PDT assays and the results are shown in figure 6.

MB alone showed a toxic effect on the crustaceans similar to that observed for microorganisms in the non-exposed samples, and the combination of LED light on MB solution increased the death percentage of *Artemia*, confirming that the LED light was effective in PDT for both the microorganisms and the crustaceans.

4. Discussion

In this study, it was found that red LED light increased the inhibitory PDT effect of MB in prokaryotic and eukaryotic cells. The photosensitizing mechanism of the LED light on MB follows the same pattern as the majority of other light sources that generate singlet oxygen and act against a wide range of target cell molecules, including membrane lipids, cytoplasmic enzymes and nucleic acids (Wainwright 1998).

MB has no difficulty in crossing bacterial cell walls. Because of its cationic charge, it binds easily to the negative charge of the lipopolysaccharides of Gram-negative bacteria (Livermore 1990). Gram-positive bacteria have only capsular material and peptidoglycan outside of the cytoplasmic membrane, enabling MB to cross it easily as well (Zelina *et al* 2001). Its penetration into *C. albicans* cells seems to be more difficult, as studies carried out with cationic porphyrin have
shown that it can enter the cytoplasm only after membrane damage has been induced by light (Lambrechts et al. 2005), or under the action of antifungal agents (Sud and Feingold 1981). In the present study, no difference was observed in the ability of the combination of MB and red LED light to inhibit and kill the three types of microorganism cells tested.

No photodynamic study has been carried out with the microcrustacean *A. salina* until now, but this model has been extensively used for investigating plant compound cytotoxicity, and a good relationship has been found between brine shrimp lethality and detection of antitumoral compounds in terrestrial plant extracts (McLaughlin et al. 1998; Hostettmann 1991; Carballo et al. 2002).

The IC50 of MB for the non-irradiated samples in this study increased in the following order: *S. aureus* < *E. coli* < *C. albicans* < *A. salina* (figures 4–6), and it was observed that increasing concentrations of MB resulted in proportionately increased effects in all the experiments.

Studies carried out by Zeina et al. (2001) suggest that the source of the light is not important for PDT in cases where the wavelength covers the absorption maxima. For instance, Zeina et al. did not observe a difference in photodynamic effect between sunlight and a projector light. Our results, using LED light, were comparable with those of studies using lasers, and partially support Zeina’s conclusion.

The results showed that LED light was as effective against *S. aureus*, *E. coli* and *C. albicans* as those of experiments using laser (Wilson and Mia 1993; Usacheva et al. 2001) or an Exal light box (Wainwright et al. 1997). Concentrations of 42 μM MB exposed to diode laser (665 nm) with a potency of 15 J cm−2 (Usacheva et al. 2001) and 0.90 μM MB exposed to an Exal light box with a potency of 6.3 J cm−2 led to the total death of *S. aureus* (Wainwright et al. 1997). In this experiment, MB concentrations of 7 and 14 μM exposed to red LED light (potency of 6 J cm−2) for 30 min led to 79% and 84% inhibition, respectively, against *S. aureus*. For *E. coli*, a MB concentration of 180 μM exposed to diode laser (665 nm) with a potency of 40 J cm−2 (Usacheva et al. 2001) and 90 μM MB exposed to an Exal light box (6.3 J cm−2) (Wainwright et al. 1997) led to total cell death. In the present study, a concentration of 35.2 μM MB exposed to LED light at intensities of 2 and 4 J cm−2 led to 88.8% and 93.7% inhibition, respectively, and at a concentration of 70.4 μM MB with 6 J cm−2, induced 96% inhibition. Experiments on *C. albicans* using MB at 130 μM exposed to: (i) a laser light (685 nm) intensity of 28 J cm−2 induced 88.6% inhibition (Souza et al. 2006) and (ii) a laser light (660 nm) of 2.04 J cm−2 induced 42% inhibition (Wilson and Mia 1993). In the present study, the LED light (6 J cm−2) with MB at 70.4 μM induced 95.14% inhibition.

The experiments with *A. salina* using red LED light plus MB produced the same effects as those seen for the microorganisms (figure 6). For samples using a concentration of 13.35 μM of MB, the light increased deaths by 126%, while at a higher concentration (668.5 μM) the effect was only 15% greater than that on the non-illuminated cells. This result may be explained by the fact that MB alone, at high doses, already induces a high death percentage (Wainwright and Crossley 2002; Calzavara-Pinton et al. 2005). Another possible explanation is that, at higher concentrations, MB may undergo a self-aggregation process in water media, thereby diminishing the singlet oxygen yield (Sternberg et al. 1998). Furthermore, aggregated monomer and dimer forms of MB (whose peaks appear at 610 nm and 664 nm, respectively) can show photodynamic activity via type I PDT, which is less effective than type II PDT (Usacheva et al. 2003; Panda and Chakraborty 1997; Borissevitch and Gandini 1998).

Since MB is known to be slightly toxic to normal cells at high concentrations, it is important to emphasize that increased toxicity occurs only when it is irradiated by the LED, hence this therapy will be of use mainly for local and topical applications. For usage by the intravenous route, it is important to know the exact site where the drug will accumulate in the body, and then expose that site to LED irradiation.

LED light appears to be a promising light source for PDT and is not just a monochromatic form of radiation, a special characteristic compared with lasers. This is advantageous for PDT because it increases the overlay of the spectrum of LED irradiation and the light absorption by MB (figure 3), which means that MB absorbs high doses of light from this source.

In the present study, the dose of the light energy from the LED was calculated by considering only the maximum peak of light absorption, without adding the smaller doses of light absorbed in wavelengths lower than 650 nm and higher than 670 nm (figure 3).

The investigation of the light exposure time indicates that the balance between MB concentration and illumination time can control the efficiency of PDT. For non-perfused tissues where the accumulation of MB is poor, the irradiation time should be prolonged to allow a similar therapeutic effect.

Exposure time is fundamental to PDT activity, as can be observed by the effects on cell death rate at different lengths of exposure.

Compared with other light sources, the LED appears to be a very good option for PDT because of its high efficacy and very low cost.

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Photodynamic effect of LED light on cell growth inhibition induced by methylene blue

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