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Singlet oxygen release due to different concentrations of photosensitizer

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ABSTRACT. Photodynamic therapy is a technique that consists of activating a Photosensitizing Agent (PS) to form reactive oxygen species, which are important for antimicrobial action. This research aimed to carry out laboratory tests to measure singlet oxygenand superoxide radicals release as a function of different formulations and concentrations of methylene blue (MB), eosin Y (EY) and fluorescein (FL) dyes, and to compare their photodynamic efficiency. Solutions containing these compounds in a MIX solvent (glycerol, ethanol and water) irradiated with low power laser (λ= 660 nm) were analyzed. The production of singlet oxygen ($^1O_2$) was photometrically evaluated through the consumption rate of 1-3 diphenylisobenzofuran (DPBF), a $^1O_2$ sequestering substance. Statistical analyses applied were the ANOVA and Duncan's complementary test using the Statistical Package for Social Sciences (SPSS) software. MB had greater photodynamic activity, as it presented higher values for the decrease in DPBF for the three concentrations evaluated. The mean concentration was 89.9% at 150 μM, 87.6% and 2.9 at 15 μM, and 77.3% and 2.5 at 1.5 μM. EY and FL presented similar results, with no significant difference between the concentrations. The MB at 150 μM expressed greater production of $^1O_2$, which suggests a greater antimicrobial effect and highlights its relevancecompared to the other dyes.

Keywords: photodynamic therapy; photosensitizers; singlet oxygen.

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Introduction

Photodynamic therapy (PDT) is a non-invasive therapeutic alternative with satisfactory results in the treatment of localized infections, with low recurrence of bacterial development (Hamblin & Hasan, 2004). It is based on the local application of a photosensitizing agent (PS), locally activated by a light source at a suitable wavelength. This generates a state of molecular stimulation (Pagonis et al., 2010). Its bactericidal effect is directly related to the formation of free radicals and superoxides. The main product is singlet oxygen ($^1O_2$), a highly cytotoxic species responsible for the antimicrobial action of photodynamic treatment (De Rosa & Bentley, 2000; Konopka &Goslinski, 2007).

PS are compounds with specific characteristics, such as kinetic and thermodynamic stability, do not aggregate in biological media, rapid synthesis and low levels of toxicity (Castano, Demidova, & Hamblin, 2004). Its antimicrobial effect is related to its action potential with the target site, described in the literature, in which Gram-positive bacteria have different susceptibility to PDT compared to Gram negative bacteria (George & Kishen, 2007). Different compounds, such as methylene blue (phenothiazine derivative), eosin Y and fluorescein (xanthene derivatives) are promising for PDT due to their chemical and physical characteristics. Thus, PS, in the presence of light and oxygen, originates reactive species, which are harmful to cells. These types of species are capable of damaging structures like membranes, proteins, and nucleic acids, promoting irreversible destruction. Thus, the technique is highly selective, as only cells exposed to PS and light will be affected bythis cytotoxic effect (Sharman, Allen, & Van Lier, 1999).

In Dentistry, one of the main goals in endodontic and periodontal treatments is the elimination of microorganisms, including those present in biofilms (Gomes et al., 2004; Stuart, Schwartz, Beeson, & Owatz, 2006). PDT has been shown to be an effective alternative in combating these infections (Wainwright & Crossley, 2004; Meisel, & Kocher, 2005). Some studies have shown 99% reduction in root canal bacteria when treatment with PDT was implemented (Fimple et al., 2008; Tennert et al., 2015).

However, although laboratory studies indicate a satisfactory bactericidal effect of PDT, the results found in clinical studies are not recognized. This is because it is a subject little disseminated in the field of activity, and because it does not present established protocols for use in Dentistry. Studies on different irradiation protocols and with different types and concentrations of PS have been carried out (Foschi et al., 2007; Pagonis et al., 2010; Street, Pedigo, & Loebel, 2010).However, there is little known about the development of new formulations that can effectively act in the uptake of PS by bacterial cells, as well as their bactericidal action when exposed to irradiation, which has beneficial effects to PDT (Castano, Demidova, & Hamblin, 2004). Thus, the objective of this study was to measure the release of singlet oxygen and superoxide radicals as a function of different concentrations of methylene blue (MB), eosin Y (EY) and fluorescein (FL).

Material and Methods

The production rate of singlet oxygen ($^1O_2$) from the different dye formulations was photometrically analyzed through the reaction with 1,3-diphenylsobenzofuran (DPBF), a substance recognized for being a $^1O_2$ sequestrant. Dyes EY, FL and MB (Sigma Aldrich, San Luis, Missouri, USA) were tested as PS. The PS were dissolved in a mixture of glycerol, ethanol and water (30:20:50), called MIX solvent, in three different concentrations (1.5, 15 and 150 μM).

For the assay, 100 μL dye solutions (MB, EY and FL) were added to the wells and added with 100 μL 200 μM 1,3-diphenylisobenzofuran (DPBF) solution in cereal alcohol. Solutions were placed in a 96-well plate, using six per row, always leaving the well between the solutions empty to minimize the dissipative effect of irradiation. Each row was prepared before its respective reading to avoid external influence on the result due to instability of the solutions. Then, each well was irradiated using a LaserSmile (LaserSmile®, Biolase Technology, Inc., Irvine, USA) as irradiation source for 180 seconds, at 100 mW intensity.

Subsequently, the absorbance in all wells was read using a Spectramax Paradigm multi-reader spectrophotometer (Molecular Devices Sunnyvale, CA, USA), at 420 nm. Each dye was also monitored at its maximum absorption wavelength: MB (664 nm), FL (495 nm) and EY (535 nm). The $^1O_2$ production rate was assessed in relation to the percentage reduction in absorbance of DPBF at 420 nm relative to 100%, which is the DBPF solution irradiated without contact with the dyes. As controls, dye solutions (with and without irradiation), DPBF solution (with and without irradiation) and MIX solvent without irradiation were used. The formulations were kept protected from light throughout the experiment.

Each experiment was performed in triplicate and the results were analyzed using Excel (Microsoft Office). The normal distribution of data was tested by the Shapiro Wilk test. As data were normal, ANOVA ($p < 0.05$) was adopted for the data, using the Statistical Package for Social Sciences (SPSS) software (version 22.0, Chicago IL. USA).

Results and discussion

MB in all concentrations presented higher photodynamic potential as PS in relation to the consumption of DBPF when compared to other dyes. Still, the concentrations of 150 μM (89.9%) and 15 μM (87.6%) were the most efficient in the photodynamic potential.

The xanthene PS, on the other hand, presented very similar results to each other, in which EY obtained greater results in the more diluted concentrations, at 1.5 μM (85.3%), and FL consumed a higher rate of DPBF in the formulations at 1.5 μM (85.2%) and 15 μM (85.3%), but with no statistical difference ($p \geq 0.05$) (Table 1).

Table 1. Values (%) of PS at the concentrations investigated after irradiation (MB- Methylene Blue; EY Eosin Y; FL Fluorescein).

Concentration (μM)	Dye (%)		
	MB	EY	FL
150	89.9	81.5	81.1
15	87.6	84.4	85.3
1.5	77.3	85.3	85.2

PDT is becoming a promising treatment alternative due to its ability to produce reactive oxygen species (ROS). The formulation of MB in MIX solution at 150 μM presented a higher photodynamic potential considering the photophysical and photochemical characteristics, which was also dose-dependent, that is, the action decreased with decreasing concentration (150 > 15 > 1.5 μM). Regarding the photodynamic activity of xanthene dyes, EY showed better photodynamic potential compared to FL. Differently from MB, solutions with lower concentrations of xanthenes consumed larger amounts of DPBF, so that more dilute solutions of these PS were more effective in producing $^1O_2$ and reacting with DBPF.

A recent study evaluated the use of EY in PDT to combat *Staphylococcus aureus* and *Salmonella typhimurium* microorganisms. At all concentrations (0.1, 0.25, 0.5, 1.0 and 5.0 μM) and irradiation times evaluated, there was a significant reduction in the number of microorganisms (CFU/mL) compared to the control. The greatest reductions were observed at concentrations of 0.5 and 1.0 μM when combined with 15-minute irradiation (Bonin et al., 2018).In another investigation, which compared EY with rose bengal in *Candida albicans* culture, photodynamic effect was observed from the concentration of 12.5 μM (Juzeniene, Peng, & Moan, 2007).

Although xanthenes are good $^1O_2$ generators, they are less cytotoxic than phenothiazines. As PS are hydrophilic and have a negative charge (-2) they tend to be very polar and do not have the ability to penetrate the cell, limiting their action to the plasma membrane (Castano et al., 2004). This can lead to a greater probability of cellular response, increasing the chances of survival. For a PS to be effective when used in PDT, it must present a spectrum of light absorption within what is known as a "therapeutic window" (600-800 nm). Wavelengths shorter than the therapeutic window are spread and absorbed by endogenous chromophores, such as hemoglobin (Allison et al., 2004; Kübler, 2005). On the other hand, wavelengths greater than 800 nm are absorbed by water and do not have enough energy to produce $^1O_2$ (Ravanat, Di Mascio, Martinez, Medeiros, & Cadet, 2000; Meisel & Kocher, 2005). Thus, the studied xanthene dyes require structural changes that make their wavelength be within 600-800nm for greater effectiveness in humans (Wang, Lu, Zhu, Li, & Cai, 2006).

On the other hand, MB phenothiazine dye has maximum absorption at 660 nm and, as it is a lipophilic compound and has a positive charge, it can diffuse through the plasma membrane with its oxidative effect also present in the intracellular environment (Harris, Sayed, Hussain, & Phoenix, 2004). Studies show that MB has its primary action on lysosomes and then penetrates the cell nucleus (Harris et al., 2004; Walker et al., 2004).

The results of the present investigation showed greater potential for the production of singlet oxygen from MB at a concentration of 150 μM. This production was higher compared to all concentrations of xanthene PS. Future studies should investigate the MB/MIX formulation at different times of irradiation, as well as their antimicrobial potential, aiming at advances in PDT.

Conclusion

Based on our findings, it can be concluded that the MB/MIX solution at 150 μM presents greater efficacy among the substances and concentrations tested. Regarding the xanthene PS, although good photodynamic activities are observed, they are smaller than the MB activity, which is more favorable for use in the context of PDT in Dentistry.

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