Photodynamic inactivation of planktonic cultures of *Streptococcus mutans* using erythrosine irradiated by LED

Inativação fotodinâmica de culturas planctônicas de *Streptococcus mutans* usando eritrosina irradiada por LED

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ABSTRACT

Objective: The aim of this *in vitro* study was to evaluate the efficacy of photodynamic inactivation (PDI) with erythrosine (E), using a light-emitting diode (LED) on planktonic cultures of *Streptococcus mutans*. **Material and Methods**: A *Streptococcus mutans* strain (UA 159) was used to prepare the suspensions containing 10^7 cells/mL, which was tested under different experimental conditions: a) LED irradiation in the presence of erythrosine as a photosensitizer (E+L+); b) LED irradiation only (P-L+); c) treatment with erythrosine only (E+L-); and d) no LED irradiation or photosensitizer (P) treatment, which served as a control group (P-L-). After treatment, strains were seeded onto MSBS agar for determination of the number of colony-forming units (CFU/mL). **Results:** The results were submitted to analysis of variance and the Tukey test (p < 0.05). No reduction in the number of CFU/mL was observed in the treatment group with erythrosine (E+L+) when compared to the control (P-L-). **Conclusion:** PDI using erythrosine did not reduce the number of CFUs per millimeter within the parameters in this study.

KEYWORDS

Erythrosine; Decay; Photodynamic inactivation; Light-emitting diode.

RESUMO

Objetivo: o objetivo deste estudo in vitro foi avaliar a eficácia da inativação fotodinâmica (PDI) com a eritrosina (E), usando diodo de emissão de luz azul (LED) em culturas planctônicas de *Streptococcus mutans*. **Material e métodos:** a cepa de *Streptococcus mutans* (UA 159) foi usada para o preparo das suspensões padrões contendo 10^7 células/mL, as quais foram testadas em diferentes condições experimentais a) irradiação com LED em presença da eritrosina como fotossensibilizador (E+L+); b) irradiação com LED apenas (F-L+); c) tratamento com eritrosina apenas (E+L-); e d) tratamento sem irradiação com LED ou fotossensibilizador (F), que serviu como grupo controle (F-L-). Após o tratamento, as cepas foram semeadas em ágar MSBS para determinação do número de unidades formadoras de colônias (UFC/mL). **Resultados:** os resultados foram submetidos à análise de variância e teste de Tukey (p < 0.05). Não foi observada redução no número de UFC/mL no grupo de tratamento com eritrosina (E+L+) quando comparado ao grupo controle (F-L-). **Conclusão:** a PDI usando etritrosina e LED não reduziu o número de UFCs por milímetro com os parâmetros utilizados neste estudo.

PALAVRAS-CHAVE

Eritrosina; Cárie; Inativação fotodinâmica; LED; *Streptococcus mutans*.

INTRODUCTION

Dental caries is a global oral health concern caused by an imbalance in the physiological equilibrium between tooth minerals and oral microbial biofilm [1]. Dental plaque is highly complex, formed of 30 bacterial genera and over 300 taxa, with each individual dentition site consisting of a unique microbial composition [2].

The high consumption of carbohydrates can lead to the appearance of aciduric saccharolytic flora like *Streptococcus mutans*, *Lactobacillus* species, and certain non-*mutans streptococci*, leading to the development of dental caries [3,4]. *Streptococcus mutans* have been known as the first bacteria that colonize oral surfaces and might make up 70% of bacteria found in dentogingival biofilm [5-7]. *S. mutans* is considered a cariogenic bacteria because its high prevalence in biofilm before the appearance of dental caries, its ability to produce a great amount of acid after the metabolism of carbohydrates, and for its survival in the environments with low pH [1,2, 8]. The production of acid changes the pH below the critical value, causing disequilibrium and demineralization of the tooth surface if no methods of prevention take place [9-11]. The usual methods to remove the oral microbial biofilm are tooth brushing, the use of dental flossing and antiseptics. However, biofilm removal depends on patient compliance and antiseptics could produce drug-resistant microbes and lead to other side effects [12].

The use of photodynamic inactivation (PDI) can be an alternative for microbial control in order to prevent dental caries and periodontal diseases [13]. PDI uses a light source (halogen lamps, laser or LED) to activate a specific photosensitizer in the presence of oxygen, producing reactive radicals that induce cell death [14]. Blue LED is commonly used in the dental office for the curing of composite resin based materials and tooth whitening, as it does not damage the oral tissues. Moreover, it is small, lightweight, low cost, has a broad spectrum output, and is easy to operate [14,15]. Dentists could use their usual LEDs to perform PDI in their offices, reducing their practice costs of purchasing lasers or different types of LEDs.

Erythrosine dye has been widely used in Dentistry for biofilm detection and also as a photosensitizer because it is not toxic and is approved for clinical use [16,17].

Therefore, the purpose of this study was to evaluate the effect of PDI using erythrosine photosensitizer irradiated by a blue LED on the viability of *Streptococcus mutans* in planktonic cultures. The null hypothesis tested is that erythrosine do not decrease the numbers of CFU/mL.

MATERIAL AND METHODS

Photosensitizers and light source

Erythrosine (Sigma-Aldrich, Steinheim, Germany), at a final concentration of $10\mu M$ was used for the sensitization of *S. mutans* strain. The photosensitizer solution was prepared by dissolving each dye in saline solution (0.85% NaCl). After filtration through a sterile 0.20 μm Millipore membrane (GVS, Sanford, USA), the photosensitizer solution was stored in the absence of light. Figure 1 shows the absorption spectra for erythrosine and its chemical structure.
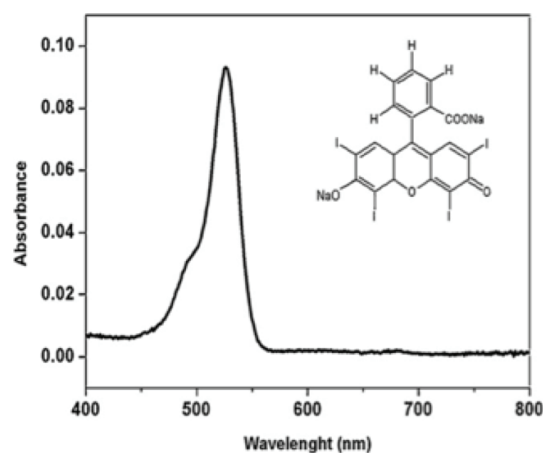

Figure 1 - Chemical structure and absorption spectrum of erythrosine [18].

The light source used was a wireless blue LED (Emitter C, Schuster, Santa Maria, RS, Brazil) at a wavelength of 420-480 nm, a power output of 625mW and an illuminated area of 0.5 cm². A fluence of 75 J.cm^{-2} (energy of 37.5 J and irradiation time of 60s as recommended by Costa et al. [19] and a fluence rate of 1250 mW.cm^{-2} were used. Figure 2 shows the maximum emission peak of the LED used to irradiate the samples.
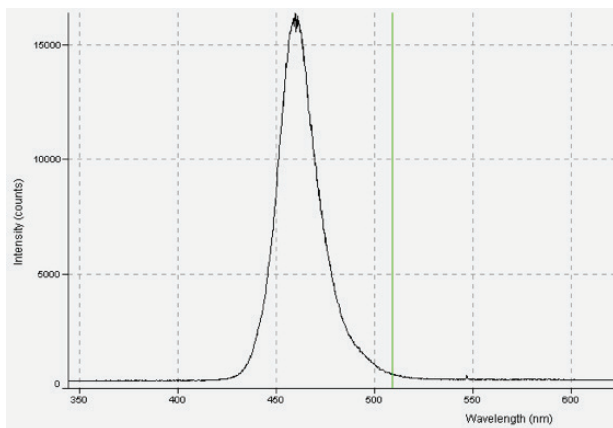

Figure 2 - Emission spectrum of the blue LED (Emitter C, Schuster, Santa Maria, RS, Brazil).

Bacteria Strain

The planktonic culture methodology was proposed by Costa et al. [19], as follows. The reference strain (UA 159) of *S. mutans* maintained in our laboratory stock collection was included in the study. A standard suspension containing 10^7 cells/mL was prepared by seeding *S. mutans* onto a brain heart infusion (BHI) and incubation for 24 h at 37° C (± 1° C) under microaerophilic conditions. After incubation, the bacteria were cultured in 7.5mL BHI broth (Himedia) for 24 h at 37 °C (± 1° C) under microaerophilic conditions. All incubations were carried out at 37°C (± 1° C) under 5% CO_2. The bacterial cultures were then centrifuged at 4700xg for 5 min, and the supernatant was discarded and cleaned using 5 mL of sterile saline solution (0.85% sodium chloride). This procedure was repeated, and the sediment was resuspended in 5mL of sterile physiological solution.

The number of cells in each suspension was measured using a spectrophotometer (B582, Micronal, São Paulo, Brazil) at a wavelength of 398nm until an optical density of 0.560 was reached.

The assays were divided into four experimental conditions (n=10) as described in Table I.

Table I - Experimental groups

Evaluated groups	Description
E+L+	LED irradiation using erythrosine as photosensitizer
P-L+	LED irradiation only
E+L-	Treatment with erythrosine only
P-L-	No LED irradiation or photosensitizer treatment

In vitro photosensitization

According to the experimental conditions described, 0.1mL of the bacterial suspension was added to each well of sterile 96-well flat-bottom microtiter plates (Costar Corning, New York, USA). After that, 0.1 mL of the photosensitizer was added for groups E+L+ and E+L-, whereas 0.1mL sterile physiological solution was added for the groups P-L+ and P-L-. The plates were shaken for 5 min (during the pre-irradiation time) using an orbital shaker (Solab, Piracicaba, Brazil). The well contents of groups E+L+ and P-L+ were irradiated according to the protocol described above. The distance between the light source and the bacterial cells was approximately 6mm. Irradiation was performed under aseptic conditions in a laminar flow hood in the dark. The plates were covered with a black screen with an orifice whose diameter corresponded to the size of the well opening in order to prevent the spreading of light to neighboring wells. After irradiation, serial dilutions were prepared, and 0.1mL aliquots of each dilution were seeded in duplicate onto MSBS agar plates and incubated for 48 h at 37° C (± 1° C) under microaerophilic conditions. After incubation, the number of

colony-forming units per milliliter (CFU/mL) was determined. The results were log transformed and statistical analyses were carried out with GraphPad Prism 6.0 program (GraphPad Software, Inc., San Diego, CA, USA). Analysis of Variance (one-way ANOVA) and the Tukey's test were used. A *p* value < 0.05 was considered to indicate a statistically significant difference.

RESULTS

Figure 3 presents the mean values, standard deviations and the Tukey's test of the number of CFU/mL ($\log_{10}$) obtained for the different groups. Figure 4 and Table II present the median values (crossbar), interquartile range (IQR box), minimum and maximum values of CFU/mL ($\log_{10}$) obtained for the different groups. PDI with erythrosine did not induce a reduction in the numbers of CFU/mL when compared to the control group (P-L-) (p = 0.3644).
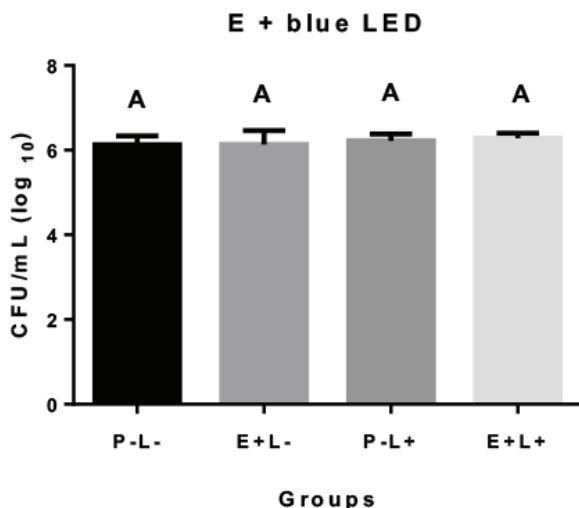

Figure 3 - Mean and standard deviation of the number of CFU/mL ($\log_{10}$) obtained from PDI of planktonic cultures of *S. mutans* mediated by erythrosine 10 µM and blue LED, p = 0.3644. Different letters indicate statistical differences among groups.
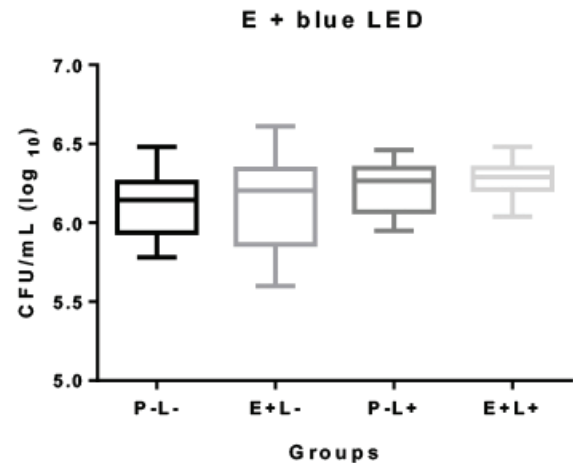

Figure 4 - Median values (crossbar), interquartile range (IQR box), minimum and maximum values of CFU/mL ($\log_{10}$) obtained from PDI of planktonic cultures of *S. mutans* mediated by erythrosine 10 µM and blue LED.

Table II - Descriptive statistical analysis of data from the CFU/mL of *S. mutans* treated with physiologic solution alone (P-L-), erythrosine (E+L-), physiologic solution and LED (P-L+), erythrosine and LED (E+L+)

Values	P-L-	E+L-	P-L+	E+L+
Minimun	5.78	5.60	5.95	6.04
First Q	5.98	6.01	6.10	6.24
Median	6.15	6.21	6,27	6.29
Third Q	6.23	6.32	6.34	6.32
Maximum	6.48	6.61	6.46	6.48
Mean	6.13	6.14	6.22	6.28

DISCUSSION

Photodynamic inactivation (PDI) is an alternative technique that uses a specific photosensitizer and light source to control microorganisms, including oral pathogens [12,20]. It is an interesting method to prevent the bacterial resistance of antibiotic drugs and chemical agents that is minimally invasive and nontoxic. There are some advantages of PDI when comparing to conventional antimicrobial agents, such as killing of the target microorganisms is quick (related to the parameters used), the lack of resistance by the specific organisms, and the antimicrobial effects can be localized to a specific site, preventing disruption of normal microflora in other regions [14,21-23].

Currently, PDI has been widely investigated as an adjunct therapy to control the growth of *S. mutans*, and consequently, the development of dental caries. Several previous studies demonstrated that PDI is capable of reducing biofilms formed by *S. mutans*, becoming a very useful strategy for dental biofilm control [13,18,24]. In addition, PDI can be used as a supplementary approach for killing remaining isolated cells of *S. mutans* in the contaminated dentine prior to a definitive restoration [25]. In this context, this study investigated the antibacterial photodynamic effect of erythrosine that is a common dye used as a photosensitizer for PDI.

The *in vitro* investigation using a planktonic model is a major step for guiding protocols/parameters for more future detailed studies, when considering illumination aspects (related to the light sources) and concentration (related to the dyes) [26]. The *in vitro* planktonic essays are also essential to determine the efficacy of potential antimicrobial agents. Most methods for evaluating antimicrobial activity of new antimicrobial candidates are based on determining cell viability in microbial suspensions [27]. According to the American Society of Microbiology, a CFU-reduction of 3 $\log_{10}$ is necessary to use the term "antimicrobial" [25].

In this study, the light source used was a blue LED that presents many advantages: it is usually found in the dental office for light-curing composite resin materials and tooth whitening and it does not damage the oral tissues. Moreover, it is inexpensive, has a broad spectrum output, and is easy to operate [14,15]. Dentists could use their usual LEDs to perform PDI in their offices, thereby reducing the need to acquire a laser or different types of LEDs. Some studies have showed that the use of LEDs alone exerts little or no antimicrobial effect [28,29]. Erythrosine has been used in dental practice for the detection of dental biofilms at concentrations of 9-25 μM [30]. The current study showed no effect with

erythrosine in planktonic culture of *S. mutans*. Then, the null hypothesis was rejected for erythrosine.

At the moment, there are many photosensitizers based on porphyrin, phenothiazinium, phthalocyanine, and chlorin derivatives. However, the future of PDI is highly dependent on the development of more efficient photosensitizers [31]. In addition to the photochemical yield, there are several factors that are important for PDI efficiency, such as tissue/cell localization and membrane binding [29]. Therefore, having a large photochemical yield is certainly a necessary characteristic required for developing new photosensitizers [32].

Esper et al. [33] showed photodynamic inactivation of planktonic cultures and *Streptococcus mutans* biofilms for prevention of white spot lesions during orthodontic treatment using hematoporphyrin IX and modified hematoporphyrin IX. The study showed an interesting result with hematoporphyrin IX and modified hematoporphyrin IX, killing 54% and 100% (p < 0.0001) of *S. mutans* in planktonic culture, respectively. In addition, no reduction of *S. mutans* biofilms was observed with the use of hematoporphyrin IX on both types of brackets. However, modified hematoporphyrin IX showed a significant inactivation of *S. mutans* biofilms, with 44% and 53% survival rate (P = 0.0020 and P = 0.0004), respectively, seen on metallic and ceramic brackets. This is the first study using hematoporphyrin IX and modified hematoporphyrin IX as photosensitizers on planktonic culture and biofilms of *S. mutans*. In the present study was used the same wavelength (Figure 2), the same energy and strain of *S. mutans* (UA 159).

Costa et al. [19] tested the effect of PDI using rose bengal and erythrosine with a LED over planktonic cultures of *S. mutans* (ATCC 35688) and Pereira et al. [13] over a biofilm of S. mutans (ATCC 35688) and *S. sanguinis* (ATCC 10556). The first authors concluded that PDI with both photosensitizers exerted an antimicrobial effect,

with a reduction of 6.86 (rose bengal) and 5.16 $\log_{10}$ (erythrosine) on the strain studied. The last study showed a reduction of 0.62 (rose bengal) and 0.52 $\log_{10}$ (erythrosine) for *S. mutans* biofilms and 0.95 (rose bengal) and 0.88 $\log_{10}$ (erythrosine) for *S. sanguinis* biofilms. Both studies used a wavelength range of 440-460 nm, similar to this study. The concentrations of the photosensitizer were 2 and 5μM, respectively, and the concentration used in this experiment was 10μM. However, in the present study, no effect was observed with erythrosine, despite of the use of a similar wavelength (Figure 2). The same energy was used in both studies but a different strain of *S. mutans* was used (UA 159).

Metcalf et al. [34] used a light dose fractionation (500-550 nm) and erythrosine (22μM) over *S. mutans* biofilms (NCTC 10449). Those authors observed a reduction of 2 $\log_{10}$ after 5 min of continuous light irradiation and a reduction of 3.7 $\log_{10}$ when using fractionation of the light dose. They highlighted the clinical potential of this photosensitizer because, with the light dose fractionation, irradiation time could be reduced to clinically acceptable levels. In the current study, a reduction of 6.78 $\log_{10}$ was observed for the HP+L+ group when compared to the control (P-L-) and the irradiation time of 60 seconds was clinically acceptable. However, PDI with erythrosine did not induce a reduction in the numbers of CFU/mL when compared to the control group (P-L-).

Another in vitro study [12], evaluated the effect of PDI on *S. mutans* biofilm (ATCC 25175), using a halogen light-curing unit (400-520 nm) and erythrosine (20 μM). The results showed a significant decrease in *S. mutans* biofilm formation and a potential for preventing dental caries. They used a higher concentration of erythrosine and a light source with a higher emission spectrum when compared with this work.

Habiboallah et al. [35] investigated the periodontal bactericidal effect on *P. gingivalis* and *F. nucleatum* using erythrosine and an LED (440-480 nm) compared with a diode laser (800 nm). Those authors concluded that erythrosine at 22 μM in the presence of a blue light and infrared diode laser could be considered as a potential approach of PDI against Gram-negative periodontal pathogenic species. Nagata et al. [36], in a review of PDI for dental caries, reported that erythrosine is the most hydrophilic photosensitizer and the Gram-positive bacteria allow better penetration of this dye, but Habiboallah et al. [35] and Silva et al. [37] showed good results with Gram-negative bacteria.

Ishiyama et al. [38] used an Nd-YAG laser and three different photosensitizers: rose bengal, erythrosine, and phloxine at 10 μM to compare their ability to produce singlet oxygen in relation to their bactericidal activity on *Streptococcus mutans* (JCM 5705). Rose bengal showed the highest bactericidal activity against *S. mutans*, followed by erythrosine and phloxine. One of the main reasons for the discrepancy between the singlet oxygen generating ability and bactericidal activity was the incorporation efficiency of the photosensitizers into the bacterial cells. They used the same concentration of the dye as in our study, but also used an Nd-YAG laser (532 nm).

Fracalossi et al. [39] evaluated singlet oxygen quantum yield of erythrosine solutions illuminated with a halogen light source (375–525 nm) in comparison to a LED array (480–560 nm), and the photodynamic effect of erythrosine dye in association with the halogen light source on *S. mutans* (UA 159). They concluded that photodynamic response of erythrosine induced by the halogen light was capable of killing *S. mutans*. It is within the range used in the present study with the same strain.

There are two basic mechanisms related to the lethal damage caused to bacteria by PDI. The first is related to the DNA damage and the second to the damage caused to the cytoplasmic membrane, allowing leakage of cellular contents or inactivation of membrane transport systems and enzymes. Breaks in both single and double stranded DNA and

the disappearance of the plasmid supercoiled fraction have been detected in both gram-positive and gram-negative bacteria. Another potential causes of cell death include the alteration of cytoplasmic membrane proteins, the disturbance of cell wall synthesis, and the appearance of a multi lamellar structure near the septum of dividing cells; loss of potassium ions from the cells may also be a possible method of bacterial death [40].

In this study, no significant difference in the number of CFU/mL was observed between the control group (P-L-) and the groups treated in the dark with the photosensitizers only (HP+L- and E+L-) or the group irradiated in the absence of the dye (P-L+). These findings agree with the basic principles of PDI, in which application of the dye or the light source alone has no antibacterial effect [41].

No reduction in *S. mutans* was observed in the groups submitted to PDI (E+L+) when compared to the others. Erythrosine absorb light in the 400-550 nm ranges, and the light source used in the present study emits light in the 420-480 range. The association between erythrosine and light do not resulted in cellular death.

Since it was observed that erythrosine had no antimicrobial activity against *S. mutans* cells, the next steps will be to test other concentrations of erythrosine photosensitizer and/or to using a modified erythrosine in planktonic cultures and on biofilms of *S. mutans* formed *in vitro*, on dental surfaces, and on oral biofilms developed *in vivo*, in animal models.

The synthesis of new and more efficient photosensitizers is an interesting approach to improve results in PDI and for clinical research in the future.

CONCLUSION

The results showed that *Streptococcus mutans* cells were not sensitive to PDI using erythrosine and a blue LED, in the parameters used.

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