

**Ação antimicrobiana da terapia fotodinâmica  
mediada por eritrosina e LED de alta potência  
sobre suspensão planctônica de *Streptococcus  
mutans***

Salma Ivanna Araújo Cavalcante

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Ação antimicrobiana da terapia fotodinâmica mediada por eritrosina  
e LED de alta potência sobre suspensão planctônica de  
*Streptococcus mutans*

Dissertação apresentada ao Programa de  
Pós-Graduação em Odontologia da  
Universidade CEUMA para obtenção do título  
de Mestre em Odontologia.

Área de concentração: Odontologia Integrada

Orientador: Prof.º Dr. Marco Aurélio Benini  
Paschoal

São Luís  
2016

Nome: Salma Ivanna Araújo Cavalcante

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Aprovado em: \_\_\_\_/\_\_\_\_/\_\_\_\_

Banca Examinadora

Prof. Dr. Marco Aurélio Benini Paschoal  
Universidade CEUMA

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Prof.<sup>a</sup> Dra. Letícia Machado Gonçalves  
Universidade CEUMA

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Prof.<sup>a</sup> Dra. Cecilia Claudia Costa Ribeiro  
Universidade Federal do Maranhão

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## **DEDICATÓRIA**

Ao Senhor Jesus que me sustentou até aqui e que me fez vitoriosa;

Aos meus pais pelo incentivo, amor e força;

A todos aqueles que de forma direta ou indireta contribuíram com a minha formação.

## AGRADECIMENTOS

“Todos acham que esta é a pior parte para escrever, talvez porque a vida não se coloca em análise de regressão e não é pelo valor p que descobrimos a significância das pessoas na nossa trajetória.”

Agradeço a Deus por ter me dado condições para prosseguir com mais esta etapa. Onde não tive força para concluir o que me era devido foi Jesus quem me carregou e me deu toda a condição necessária.

À minha espetacular família, papai, mamãe e Hector, pelo infinito apoio e eterno amor. Eu não teria conseguido se não tivesse a base sólida que com tanto esforço construíram. A presença de vocês significou segurança e certeza de que não estou e nunca estarei sozinha nessa caminhada. Mãe, eu estou indo voar sozinha, mas sempre voltarei para o teu ninho.

Ao prof. Dr. Marco Aurélio Benini Paschoal, cujas palavras NUNCA serão suficientes para mostrar o quão agradecida eu sou por todas as oportunidades que me foram dadas. Obrigada pela paciência, pela compreensão, pela ajuda e, sobretudo, pela confiança. Além de tudo, por ter se tornado referência de pesquisador e professor. Não posso esquecer de agradecer por acreditar em mim, mesmo quando nem eu mesma acreditava. Desculpe pelas entregas em cima dos prazos e a falta de jeito em algumas coisas, mas quero que você saiba que até suas broncas (principalmente elas) me fizeram ser uma pessoa melhor. Eu desejo de todo o coração, poder ser pelo menos um pouquinho, para os meus futuros alunos, o que você foi para mim.

*(Nesse momento, as lágrimas me tomam...)*

Ao meu amor, companheiro e parceiro de todas horas, Erick Machado. Não só pela convivência diária, mas pelo estímulo,

palavras de apoio e paciência nos dias de estresse, tpm e agonia nessa etapa. Principalmente pelo companheirismo que só me surpreende cada dia mais... Como um cordão de três dobras que dificilmente se rompe, assim sempre seremos.

Aos meus colegas de turma, Adriana, Débora, Mônica, Ricardo e Sâmara; que apesar do pouco tempo, se tornaram muito especiais. Sinto que nós percorremos este caminho juntos, nos complementando e nos fortalecendo. Obrigada pela rica troca e cumplicidade. Em especial, à Giovana, que se tornou uma amiga para vida toda, além de companheira de laboratório.

Ao Prof. Dr. Matheus Bandéca, por coordenar com maestria o Programa, por estar disposto a nos ouvir e ajudar em todos os momentos. O mestrado me proporcionou mais que uma simples busca de conhecimento técnico e científico, mas uma lição de vida. E uma mudança também.

A Erymônica, por toda a paciência nesses anos e que sempre prontamente e educadamente nos atendeu.

A Margareth, que foi essencial no começo dessa caminhada, que ensinou os primeiros passos no laboratório e a desvendar cada dispositivo, cada solução... Por ter sido companheira neste e em outros projetos desenvolvidos e nunca ter medido esforços para nos ajudar.

A FAPEMA, pelo apoio financeiro concedido para realização deste projeto e de tantos outros em que estive envolvida.

E eu não poderia esquecer (deixei para o final de propósito) de agradecer à Hadda e Iasmym, que foram peças fundamentais na realização desse trabalho como um todo. Nada, e repito, NADA teria acontecido se não fosse com a ajuda de vocês. No sentido laboratorial, como no psicológico. Eu espero que a nossa amizade verdadeiramente só cresça. Obrigada por me aturarem, me

incentivarem e me ouvirem. Obrigada por aguentar meu mau humor matinal, minhas reclamações (a respeito da dissertação) e desabafos.

Das coisas boas que o mestrado me deu, as amizades e o conhecimento foram os principais pilares dessa conquista. Possuir amigos e tutores que pensam de formas tão distintas, enriqueceu significativamente a minha formação. Agradeço a enorme diversidade que me rodeia que, apesar de me desorientar às vezes, me ajuda a captar diferentes olhares sobre a mesma realidade.

Estou certa que esta realização não é somente minha, mas de todos aqueles que participaram comigo desta caminhada, minha família, amigos, professores e colegas.

Nesse momento eu estou simplesmente envolvida por um enorme sentimento: gratidão.

## EPÍGRAFE

“Faço o melhor que sou capaz  
Só pra viver em paz...”  
(O vencedor – Los Hermanos)



Cavalcante SIA. Ação antimicrobiana da terapia fotodinâmica mediada por eritrosina e LED de alta potência sobre suspensão planctônica de *Streptococcus mutans*. [dissertação]. São Luís. Universidade CEUMA; 2015

## RESUMO

**Introdução:** Para o controle da população de microrganismos cariogênicos, devido à dificuldade de um eficaz método de controle mecânico e químico do biofilme oral, o desenvolvimento e a inserção de novas estratégias terapêuticas podem contribuir para o domínio dessa população. A partir disso, a terapia fotodinâmica antimicrobiana (TFD) ganha grande destaque. Porém, um dos grandes desafios clínicos da TFD é o longo tempo de irradiação das fontes de luz necessários para uma eficácia antimicrobiana.

**Objetivos:** Determinação de um parâmetro ótimo da aplicação da TFD e comparação de dois protocolos de iluminação (pulsado X contínuo) por meio da combinação do corante eritrosina (E) e fotossensibilizada com uma fonte de luz LED de alta intensidade de potência (L) no comprimento de onda azul (420-480 nm; P = 1.200 mW/cm<sup>2</sup>) sobre suspensão planctônica de *S. mutans*. **Materiais e**

**Métodos:** Suspensões de *S. mutans* foram tratadas em quatro situações experimentais: 1) Situação E-L- (grupo controle); 2) Situação E+L- (E em 2 concentrações diferentes - 2µM e 4µM); 3) Situação E-L+ (L em 3 dosimetrias diferentes - 48 J/cm<sup>2</sup>, 96 J/cm<sup>2</sup> e 144 J/cm<sup>2</sup> (correspondente à 40, 80 e 120 segundos, respectivamente) e 4) Situação E+L+ (TFD – cruzamento das 2

concentrações de E com as 3 doses de L, totalizando 6 grupos). As amostras de cada condição experimental foram cultivadas em placas de ágar sangue a 37° C e 5% CO<sub>2</sub> durante 48 horas. Os resultados dessas situações experimentais denominaram a primeira fase do estudo, onde o protocolo em que se obteve um efeito bactericida eficaz a um tempo de irradiação mais curto foi submetido a comparação de dois protocolos de iluminação (iluminação contínua – TFD+C+ e iluminação pulsada - TFD+P+) e essa comparação denominou a segunda fase do estudo. Para ambas as fases foi realizada a contagem de unidades formadoras de colônia por mililitro (UFC/mL) e os dados transformados em log<sub>10</sub> e analisados por ANOVA e teste de Tukey a 5%. **Resultados:** O grupo submetido à TFD apresentou erradicação bacteriana total em comparação ao grupo controle, exceto o grupo a 2µM para todas as doses testadas (p > 0,05). O resultado letal deu-se pela combinação de 4µM de E e 48 J/cm<sup>2</sup> de L (40 s). Eritrosina ou fonte de luz utilizadas isoladamente não demonstraram efeito antimicrobiano significativo no número de bactérias viáveis. Adicionalmente, ambos os protocolos de iluminação não demonstraram diferença estatística após aplicação do protocolo determinado (4µM de E / 48 J/cm<sup>2</sup> de L). **Conclusão:** A terapia fotodinâmica aplicada *in vitro* na presença de baixa concentração de eritrosina a um tempo de exposição curto atingiu fotoativação letal de *S. mutans*.

Palavras-chave: Cariologia; *In vitro*; Fotoquimioterapia.

Cavalcante SIA. Antimicrobial action of photodynamic therapy mediated by erythrosine and high potency LED on planktonic suspension of *Streptococcus mutans*. [dissertation]. São Luís. Universidade CEUMA; 2015

## **ABSTRACT**

**Background:** To control the population of cariogenic microorganisms, because of the difficulty of effectiveness mechanical and chemical control method of oral biofilm, the development and introduction of new therapeutic strategies may contribute to the domination of this population. From there, the antimicrobial photodynamic therapy (PDT) wins highlight. However, a major clinical challenge of PDT is the long time irradiation of light sources required for antimicrobial effectiveness. **Objective:** Determining an optimal application parameter of PDT and comparison of two lighting protocols (pulsed X continuous) through a combination of erythrosine dye (E) and photosensitized with a LED light source of high intensity power in the blue wavelength (420-480 nm; P = 1.200 mW/cm<sup>2</sup>) on plankton suspension of *S. mutans*. **Materials and Methods:** *S. mutans* suspensions were treated in four experimental situations: 1) Situation E-L- (control group); 2) Situation E+L- (E in 2 different concentrations - 2µM and 4µM); 3) Situation E-L+ (L in 3 different dosimetries - 48 J/cm<sup>2</sup>, 96 J/cm<sup>2</sup> and 144 J/cm<sup>2</sup> (corresponding to 40, 80 and 120 seconds, respectively) and 4) Situation E+L+ (PDT - combination of 2 concentrations of E with 3 doses of L, a total of 6 groups). Samples from each experimental condition were cultured on

blood agar plates to 37°C and 5% CO<sub>2</sub> for 48 hours. The results of the experimental situations dominated the first phase of the study, where the protocol was obtained an lethal bactericidal effect in a shortest irradiation time was subjected to the comparison of two illumination protocols (continuous illumination – PDT+C+ and pulsed lighting – PDT+P+), and this comparison denominated the second phase of the study. For both phases was done counting colony forming units per milliliter (CFU/ml) and the data transformed in log<sub>10</sub> and analyzed by ANOVA and 5% Tukey test. **Results:** The group submitted to PDT presented a total eradication of bacterial rate in comparison with control group, except the group at 2µM to all tested dosages (p > 0.05). The lethal result was due to the combination of 4µM of E and 48 J/cm<sup>2</sup> of L (40 s). Erythrosine or light source used alone did not demonstrate a lethal antimicrobial effect on the number of viable bacterial counts. Additionally, both illumination protocols did not show statistical difference after application of the determination protocol (4µM of E / 48 J/cm<sup>2</sup> of L). **Conclusion:** Photodynamic therapy applied *in vitro* in the presence of low concentrations of erythrosine a short exposure time lethal hit to photoinactivation *S. mutans*.

Key words: Cariology; *In vitro*; Photochemotherapy.

## LISTA DE TABELAS

Tabela 1. Média dos valores obtidos após contagem das unidades formadoras de colônia por mililitro (UFC/mL) na base logarítmica nas diferentes situações experimentais avaliadas	24
Tabela 2. Média dos valores após contagem das unidades formadoras de colônia por mililitro (UFC/mL) na base logarítmica para comparação dos protocolos pulsado e contínuo.	25

## SUMÁRIO

1. RESUMO .....	16
2. INTRODUÇÃO .....	18
3. RESULTADOS .....	23
4. DISCUSSÃO .....	25
5. CONCLUSÃO .....	28
6. REFERÊNCIAS .....	29
7. METODOLOGIA DETALHADA .....	37
9. NORMAS DA REVISTA .....	44

# ***CAPÍTULO 1***

## **Antimicrobial action of photodynamic therapy mediated by erythrosine and high potency LED on planktonic suspension of *Streptococcus mutans***

### **Abstract**

**Background:** To control the population of cariogenic microorganisms, because of the difficulty of effectiveness mechanical and chemical control method of oral biofilm, the development and introduction of new therapeutic strategies may contribute to the domination of this population. From there, the antimicrobial photodynamic therapy (PDT) wins highlight. However, a major clinical challenge of PDT is the long time irradiation of light sources required for antimicrobial effectiveness. **Objective:** Determining an optimal application parameter of PDT and comparison of two lighting protocols (pulsed X continuous) through a combination of erythrosine dye (E) and photosensitized with a LED light source of high intensity power in the blue wavelength (420-480 nm; P = 1.200 mW/cm<sup>2</sup>) on plankton suspension of *S. mutans*. **Materials and Methods:** *S. mutans* suspensions were treated in four experimental situations: 1) Situation E-L- (control group); 2) Situation E+L- (E in 2 different concentrations - 2µM and 4µM); 3) Situation E-L+ (L in 3 different dosimetries - 48 J/cm<sup>2</sup>, 96 J/cm<sup>2</sup> and 144 J/cm<sup>2</sup> (corresponding to 40, 80 and 120 seconds, respectively) and 4) Situation E+L+ (PDT - combination of 2 concentrations of E with 3 doses of L, a total of



6 groups). Samples from each experimental condition were cultured on blood agar plates to 37°C and 5% CO<sub>2</sub> for 48 hours. The results of the experimental situations dominated the first phase of the study, where the protocol was obtained an lethal bactericidal effect in a shortest irradiation time was subjected to the comparison of two illumination protocols (continuous illumination – PDT+C+ and pulsed lighting – PDT+P+), and this comparison denominated the second phase of the study. For both phases was done counting colony forming units per milliliter (CFU/ml) and the data transformed in log<sub>10</sub> and analyzed by ANOVA and 5% Tukey test. **Results:** The group submitted to PDT presented a total eradication of bacterial rate in comparison with control group, except the group at 2µM to all tested dosages (p > 0.05). The lethal result was due to the combination of 4µM of E and 48 J/cm<sup>2</sup> of L (40 s). Erythrosine or light source used alone did not demonstrate a lethal antimicrobial effect on the number of viable bacterial counts. Additionally, both illumination protocols did not show statistical difference after application of the determination protocol (4µM of E / 48 J/cm<sup>2</sup> of L). **Conclusion:** Photodynamic therapy applied *in vitro* in the presence of low concentrations of erythrosine a short exposure time lethal hit to photoinactivation *S. mutans*.

Key words: Cariology; *In vitro*; Photochemotherapy.

## INTRODUCTION

A variety of microorganisms are largely colonized in the oral cavity and organized in ecosystems relatively complex, including aerobic and anaerobic bacterial species, both Gram positive and Gram negative as well as fungi and virus (1). Biofilms are highly structured and spatially organized, and are often composed by microbial consortia (2, 3).

For oral biofilm control and diseases associated with its presence, various methods are proposed: the mechanical features (is the most efficient and cost effective method consists of brushing and flossing) and chemical, associated with diet control (3). However, these methods are not always carried out properly and its efficiency is directly related to skill, maturity of coordination and patient compliance. Thus, many substances have been used for chemical plaque control as adjuncts to mechanical procedures (chlorhexidine and triclosan) (4-6) but, many studies show alteration in taste, staining of both teeth and restorations, and burning sensation as the main side effects related to the use of this substances (7,8). From the need to study and implementation of new alternatives for this control, photodynamic therapy gained great prominence.

Alternative tools such as PDT have entered the dentistry field as a therapeutic option to killing bacteria in oral biofilms or dental caries (9-13). The PDT is based on a photochemical reaction, non-thermal, local, involving concurrently photosensitizer, light and

oxygen (14). Alone, neither the drug or light has the ability to produce antimicrobial effect (15, 16).

Xanthene dyes such as erythrosine and Rose Bengal show strong absorption of light in the spectral range of 500–550 nm, a range corresponding to that emitted by light-emitting diodes (LEDs; blue and green light). LEDs represent an alternative light source for PDT because of their low cost, more portable, low thermal component and monochromatic light with a bandwidth in the order of 600 nm (11) and are widely used in dentistry for light-curing of composite resins (17).

Erythrosine has oral antimicrobial activity against Gram-positive and Gram-negative and is used for plaque staining (plaque disclosing agent). Below other dyes, erythrosine has three major advantages: a) non-toxic to the host; b) approved by the FDA (Food and Drug Association) for use in food products; c) approved for use in dentistry, which makes its easier clinical application (18). Additionally, other advantage of using this dye is that presents an absorption peak very close to emission of the light sources used in dental offices for composite resins activation (LED light sources or halogen with wavelengths between 400-600 nm), allowing the inclusion of this practice in the dental context (19). Furthermore, recent studies using this dye have shown photodynamic efficiency by reducing the number of cariogenic microorganisms in suspension and in the biofilm counterparts (18, 20).

The current photodynamic studies achieve a photodynamic efficacy at the expense of long-time illumination by the use of low power lights (18, 20-22). Thus, there is a need to find new protocols for the establishment of a more favorable clinical time.

Given the promising results from the application of PDT, it becomes a viable proposition to investigate the use of erythrosine associated a source of high intensity light on cariogenic microorganism. Thus, the present study aimed to determine an *in vitro* protocol using a combination of low concentrations of erythrosine and short exposure illumination times and to test whether the mode of illumination may influence the bacterial viability of planktonic suspension of *S. mutans*.

## **MATERIALS AND METHODS**

### ***Bacterial strain***

The microorganism used in this study was *S. mutans* UA159 (ATCC 700610). An aliquot was inoculated in BHI (Brain Heart Infusion, Merck - Darmstadt, Germany), containing 1% (wt/vol) glucose and grown overnight at 37° C, 5% CO<sub>2</sub>. Then, the bacterial suspension was centrifuged at 3000rpm for 5 min with the supernatant discarded. The cell pellet was resuspended in sterile phosphate-buffered saline (PBS). The cell numbers were adjusted at an optical density (OD) at 540 nm, equivalent to  $\sim 2 \times 10^9$  colony-forming units (CFU/mL) (23).

### ***Photosensitizer (PS)***

Erythrosine (Sigma Aldrich, St Louis, MI, USA) was dissolved in 100mL of PBS. All stock solutions were kept in the dark, and were diluted immediately before the experiments.

### ***Light source***

The light used was a LED of high potency at blue wavelength. This dispositive (3M ESPE, SP, Brazil) provides an emission at 420-480nm wavelength, power density of 1200mW/cm<sup>2</sup>. Each sample was irradiated with a dosimetry of 48 J/cm<sup>2</sup>, 96 J/cm<sup>2</sup> and 144J/cm<sup>2</sup>, achieving a energy dose of 24J, 48J and 72J (corresponding to 40, 80 and 120 seconds, respectively), and the working distance used was 5 mm (distance between the light source tip and the microorganism) aiming to avoiding thermal effect. (23) This distance was adjusted and maintained with the aid of a digital caliper (Digimess, São Paulo, Brazil).

### ***Photodynamic therapy application study design***

All solutions were diluted to obtain final concentrations at 2µM and 4µM (20). In a microcentrifuge cap (diameter = 8 mm; area = 50 mm<sup>2</sup>) was added 100 µL of PS and 100 µL of preadjusted microorganism and kept in the dark for 60 seconds (pre-irradiation time). After this period, the suspensions were irradiated by the above irradiation times. The PDT situation were treated with PS and light (E+L+, where E = erythrosine, L = light). To determine whether PS alone induce any toxic effect on the bacteria viability, bacterial suspensions were exposed to the PS under identical conditions to

those described, but without exposure to light (situation E+L-). Likewise, the microorganism was exposed to the irradiation, determining the isolated effect of the light source, without previous exposure to the PS (E-L+). Moreover, suspensions of *S. mutans* were not exposed to the PS or light (E-L-) representing the control group.

After the treatment application, aliquots of treated suspensions were used to perform 10-fold serial dilutions, and the diluted samples were plated on blood agar and then incubated at 37°C, 5% CO<sub>2</sub> for 48h to investigate the number of viable microorganisms. After incubation, the total number of CFU was determined and number of CFUs per milliliters of suspension (CFU/mL) was obtained and transformed into logarithmic scale ( $\log_{10}$ ).

To investigate whether there are differences in the efficacy of two modes of light emission, the results from the combination of shortest dye concentration at the shortest illumination time (e.g. energy dose) that achieved a photobactericidal mortality was subjected to two types of light emission protocols: pulsed and continuous. For this, the application of PDT was divided into two groups: PDT+P+ (light pulsed regimen) and PDT+C+ (continuous irradiation) and both groups were compared to control group (not exposed to any protocol).

### ***Statistical analysis***

In order to verify the differences among the studied groups, the variable reduction in viable bacterial colony counts promoted by

each group was analyzed by ANOVA and Tukey's test with a p value of 5% for statistical significance. The program BioEstat 5.0 software for Windows (Sociedade Civil Mamirauá, Manaus, AM, Brazil) was used for data analysis.

## RESULTS

The results of reduction in viable bacterial colony counts (CFU/ml) in  $\log_{10}$  are summarized in the Table 1. The association of dosimetries of LED and 4 $\mu$ M of erythrosine (E+L+) were able to reduce the number of viable bacteria compared to the control groups (E+L-; E-L+ and E-L-) ( $p < 0.05$ ), confirming the efficacy of the photodynamic treatment. On the other hand, the effect of the light source alone (E-L+), and erythrosine (E+L-) produced no reduction in bacterial counting compared to the control group (E-L-) ( $p > 0.05$ ) (Table 1). Thus, the concentration of 4  $\mu$ M of erythrosine irradiated for 40s (shortest irradiation time) - 48J/cm<sup>2</sup> was considered the protocol to be applied on the second phase of this study (different illumination modes).

When compared the pulsed (PDT+P+) and continuous protocol (PDT+C +), there was no statistical difference between the tested groups achieving an efficient bacterial mortality, attesting the efficiency of both mode emissions in comparison to control group ( $p < 0.05$ ).

**Table 1.** Mean values of log<sub>10</sub> (CFU/ml) for all experimental conditions. E+L+: erythrosine and LED; E+L-: erythrosine alone; E-L+: LED alone; E-L-: control group.

<b>Groups</b>	<b>Experimental situations</b>	<b>CFU/ml (log<sub>10</sub>)</b>	<b>p value</b>
<b>E-L-</b>	Control group	6.67 <sup>a*</sup>	-
<b>E+L+ /E = 2uM</b>	48 J/cm <sup>2</sup> (40s)	5.25 <sup>a*</sup>	p > 0,05
	96 J/cm <sup>2</sup> (80s)	4.77 <sup>a*</sup>	p > 0,05
	144 J/cm <sup>2</sup> (120s)	4.30 <sup>a*</sup>	p > 0,05
<b>E+L+ /E = 4uM</b>	48 J/cm <sup>2</sup> (40s)	0.00 <sup>b*</sup>	p < 0,05
	96 J/cm <sup>2</sup> (80s)	0.00 <sup>b*</sup>	p < 0,05
	144 J/cm <sup>2</sup> (120s)	0.00 <sup>b*</sup>	p < 0,05
<b>E+L-</b>	2μM	6.77 <sup>a*</sup>	p > 0,05
	4μM	6.77 <sup>a*</sup>	p > 0,05
	8μM	6.67 <sup>a*</sup>	p > 0,05
<b>E-L+</b>	48 J/cm <sup>2</sup> (40s)	6.69 <sup>a*</sup>	p > 0,05
	96 J/cm <sup>2</sup> (80s)	6.77 <sup>a*</sup>	p > 0,05
	144 J/cm <sup>2</sup> (120s)	6.72 <sup>a*</sup>	p > 0,05

Data analyzed using ANOVA followed by Tukey test, considering a significance level of 5%; \*different lowercase denote statistical significance.



**Table 2.** Mean values of  $\log_{10}$  (CFU/ml) for all experimental conditions. E-L-: control group; PDT+C+: continuous mode irradiation; PDT+P+: pulsed mode irradiation.

Groups	Experimental situations	CFU/ml ( $\log_{10}$ )	p value
E-L-	Control group	6.67 <sup>a*</sup>	-
PDT+C+	48 J/cm <sup>2</sup> (40s)	0.00 <sup>b*</sup>	p < 0,05
E = 4 $\mu$ M			
PDT+P+	48 J/cm <sup>2</sup> (40s)	0.00 <sup>b*</sup>	p < 0,05
E = 4 $\mu$ M			

Data analyzed using ANOVA followed by Tukey test, considering a significance level of 5%; \*different lowercase denote statistical significance.

## DISCUSSION

A large number of studies have shown that bacteria are susceptible to the effects of PDT when held in planktonic suspension (24-27). *In vitro* studies have demonstrated the susceptibility of bacteria (10, 28-30) and fungi (32, 33) to PDT. In the analyzed articles, *S. mutans* was the most studied bacteria, since they are the main specimen related to dental caries.

As in the literature there are inconclusive outcomes regarding efficiency of different modes of irradiation and a lack of *in vitro*

protocol using erythrosine, it is necessary define parameters aiming to apply on its biofilm counterparts and on *in vivo* applications as well.

Studies have shown a successful rate of photodynamic approach using low and high concentrations of the studied dye (18, 21, 37-40). Most evaluated the efficacy of PDT on *in vitro* biofilm. As the concentration of the used dye to be higher due to greater organization of bacteria involved, it was decided to work with lower concentrations, since the PDT was performed in planktonic suspension. Thus, the PS concentrations were used at a very low concentrations (2, 4 and 8 $\mu$ M). It can be speculated that this aspect represent an advantage to the photodynamic field due to be not harmful to oral tissues and not stain restorative structures (e.g. resin composite) and teeth.

The LED has been cited as an alternative source of light relative to the laser, because it is inexpensive compared to complex system, in addition of being a simple technology (10, 41). Studies have shown that the isolated use of LED has little or none microbicidal activity (10, 43, 44). Furthermore, they are smaller and lighter equipment, have low cost, have greater flexibility in time of irradiation and easy handling (11, 43).

Therefore, thinking about the use of photodynamic procedures for the inactivation of *S. mutans*, the use of LED may be suggested, considering its capacity of not changing the temperature allied to its high dose energy supply. In association with LED, the most

appropriate photosensitizer would vary according to the spectrum of wavelength of the selected light source wavelength. (44)

Currently, most studies achieve efficacy in PDT using a long exposure light (long time) by the use of low power lights (18, 20-22). Thus, in our study we seek to reduce the lighting time when using a source of high-power light.

This study aimed to demonstrate the antimicrobial effect of PDT when using the erythrosine and LED light. The results showed that treatment of PDT with an LED light from the irradiation at  $48\text{J}/\text{cm}^2$  in the presence of  $4\mu\text{M}$  erythrosine achieved a lethal photoinactivation rate when compared with the control group. The group treated only with the PS (E+ L-) or only irradiated (E-L+) showed no reduction of microorganisms. These results are consistent with basic principles of PDT where in the isolated use of the PS or the light source has no antibacterial effect (15, 16, 45).

Metcalf *et al.* (21) demonstrated success in PDT using erythrosine in the concentration of  $0,019\text{mg}/\text{mL}$  (corresponding to  $0,022\mu\text{M}$ ) combined with a white light (550-550nm and  $6.75\text{ J}/\text{cm}^2$ ) after 10 minutes of exposure. Wood *et al.* (18) also uses erythrosine in the same concentration, but using a tungsten filament (500-550 and  $20.43\text{ J}/\text{cm}^2$ ) after exposure of 15 minutes concluding that PDT at these parameters is also effective but with no clinical application due to long irradiation time.

In relation to both different illumination protocols, Metcalf *et al.* (21) demonstrated that the fractionation of white light irradiation

biofilm of *S. mutans* treated with erythrosine solution of 22  $\mu\text{M}$  resulted in a 1.7  $\log_{10}$  more than the continuous light irradiation in killing these organisms. According to the authors, this may be due to oxygen replenishment, during periods of dark, for excitation of the PS. In this same way, it was tested two different irradiation protocols achieving no statistical difference between pulsed and continuous irradiation protocols to PDT group (Table 2). This result demonstrated that the set erythrosine and LED showed great potential of PDT in planktonic culture *S. mutans* apart of light illumination mode.

## **CONCLUSION**

Given the parameters used, according to the combination of dye concentrations and irradiation doses and light emission modes with a specific light wavelength, it can be concluded that the photodynamic therapy (PDT) represented by photosensitization with low concentration of erythrosine and irradiate at short exposure of time, showed lethal antimicrobial effect on planktonic suspension of *S. mutans*.

## **ACKNOWLEDGEMENTS**

The authors would like to thank FAPEMA for the financial aid granted (proc # 01595/14, scholarship to Salma Cavalcante).

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*Anexos*