
**UNIVERSIDADE FEDERAL DO CEARÁ**

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**CURSO DE ODONTOLOGIA**

**PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA**

**RAMILLE ARAÚJO LIMA**

# ESTUDOS DA AÇÃO DA TERAPIA FOTODINÂMICA ANTIMICROBIANA EM BIOFILMES DE *Streptococcus mutans* - EFEITO NA VIABILIDADE BACTERIANA E NA MATRIZ DE POLISSACARÍDEOS.

**FORTALEZA**
**2014**

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# ESTUDOS DA AÇÃO DA TERAPIA FOTODINÂMICA ANTIMICROBIANA EM BIOFILMES DE *Streptococcus mutans* – EFEITO NA CÉLULA E NA MATRIZ DE POLISSACARÍDEOS

Tese apresentada ao Programa de Pós-graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, como requisito parcial para obtenção do título de doutor em Odontologia.

Área de concentração: Clínica Odontológica

Orientadora: Profa. Dra. Iriana Carla Junqueira Zanin dos Santos

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## RESUMO

A terapia fotodinâmica antimicrobiana (TFDA) surge como um tratamento alternativo ao uso de antimicrobianos tradicionais diante da emergência de cepas resistentes a estes agentes. Este estudo está dividido em três capítulos, cujos objetivos foram: Capítulo 1) Comparar o efeito antimicrobiano da TFDA realizada com o fotossensibilizador azul de orto-toluidina (TBO) e duas diferentes fontes de luz vermelha {Laserbeam® (LB) ou LumaCare® (LC)} em biofilmes maduros de *Streptpcoccus mutans*; Capítulo 2) Quantificar as espécies reativas de oxigênio (ERO) encontradas a nível intracelular em biofilmes de *S. mutans* como resultado da TFDA realizada com TBO e a fonte de luz vermelha LC; e Capítulo 3) Avaliar os efeitos da TFDA realizada com TBO e a fonte de luz vermelha LC na viabilidade microbiana, na matriz de polissacarídeos extracelulares (PEC) e na topografia de biofilmes de *S. mutans* maduros e em formação. Biofilmes foram crescidos em discos de hidroxiapatita imersos em caldo de peptona caseína soja e extrato de levedura com 1% de sacarose por cinco dias. No capítulo 1, biofilmes maduros foram submetidos à TFDA utilizando o fotossensibilizador TBO (100$\mu$g/mL) e duas fontes de luz diferentes: LB (56,6 J/cm$^2$) e LC (56,6 J/cm$^2$; 158,5 J/cm$^2$; 317,0 J/cm$^2$; 475,6 J/cm$^2$). No capítulo 2, os biofilmes foram expostos a terapia usando o LC nas densidades de energia 211.37 J/cm$^2$ e 422.74 J/cm, e a produção de ERO como consequência da terapia foi determinada utilizando o marcador dihidrorodamina 123. No capítulo 3, as mesmas densidades de energia do capítulo 2 (211.37 J/cm$^2$ e 422.74 J/cm$^2$) foram testadas em biofilmes maduros e em formação. Verificamos que não existe diferença na redução da viabilidade bacteriana quando LB e LC são utilizados na mesma dose de energia, porém LC é superior nas exposições 317,0 J/cm$^2$ e 475,6 J/cm$^2$ (capítulo 1). A TFDA gerou 3x mais ERO que o grupo controle negativo. A TFDA nas densidades de energia 211.37 J/cm$^2$ e 422.74 J/cm$^2$ obteve reduções da viabilidade bacteriana de 2 a 5 logs, respectivamente, em biofilmes maduros, e de 6 a 6,5 logs em biofilmes em formação. A TFDA aplicada sobre biofilmes em formação, além de promover redução da viabilidade bacteriana, também reduziu a formação de PEC e o número de conglomerados bacterianos, além de promover a perda de estruturas de conexão dos PEC. (capítulo 3). Conclui-se que TFDA, nos parâmetros utilizados, apresenta-se como uma promissora abordagem terapêutica na inibição da formação de biofilmes de *S. mutans* e na inativação de biofilmes já estabelecidos.



Palavras-chaves: Cárie Dentária. Placa Dentária. Fotoquimioterapia.

## ABSTRACT

In front of the emergence of strains resistant to traditional antimicrobial agents, photodynamic antimicrobial chemotherapy (PACT) arises as an alternative treatment to these agents. This study was divided into three chapters, whose objectives were: Chapter 1) compare the antimicrobial effect of PACT performed with the photosensitizer TBO and two different sources of red lights {Laserbeam® (LB) or LumaCare® (LC)} in *S. mutans* mature biofilms, Chapter 2) verify the amount of intracellular reactive oxygen species (ROS) found in *S. mutans* biofilms as a result of PACT performed with TBO and light source LC and Chapter 3) evaluate the effects of PACT performed with TBO and red LED LC on microbial viability, polysaccharide matrix and topography of *S. mutans* mature biofilm and *S. mutans* biofilm formation. Biofilms were formed on saliva-coated hydroxyapatite discs using batch culture method at 37ºC, 5% $CO_2$. Tryptone-yeast extract broth containing 1% sucrose was changed daily. In chapter 1, the mature biofilms were subjected to PACT using TBO (100µg/mL) and two different light sources: LB (56.6 J/cm2) and LC (56.6 J/cm$^2$; 158.5 J/cm$^2$; 317.0 J/cm$^2$; 475.6 J/cm$^2$). The results were expressed as colony forming units (CFU) per mg of biofilm. In chapter 2, the biofilms were exposed to PACT using TBO and LC energy densities of 211.37 J/cm2 and 422.74 J/cm$^2$ and the reactive oxygen species (ROS) production, as a result of the therapy, was determined using dihidrorhodamine 123 dye. On chapter 3, these same energy densities (211.37 J/cm$^2$ and 422.74 J/cm$^2$) were tested in mature biofilms and forming-biofilms. We verified that there is no difference in the reduction of bacterial viability when LB and LC were used in the same energy dose, however LC was superior when the exposures 317.0 J/cm$^2$ and 475.6 J/cm$^2$ were tested (Chapter 1). PACT generated 3-times more ROS than negative control group. PACT using energy densities 211.37 J/cm$^2$ and 422.74 J/cm$^2$ achieved reductions in bacterial viability up to 2-5 logs in mature biofilms, respectively, and 6 to 6.5 logs in forming-biofilms. Therapy in forming-biofilms also promoted a reduction in the PEC formation, while reducing the number of bacterial clusters and promote the lost of PEC connection structures (Chapter 3). We conclude that photodynamic therapy, in the parameters used, comes as a promising therapeutic approach for inhibiting the cariogenic biofilms formation and inactivation of biofilms already established.


Key- words: *Streptococcus mutans*. Biofilm. photodynamic antimicrobial chemotherapy.

**SUMÁRIO**

## 1 INTRODUÇÃO GERAL

A cárie dentária continua sendo um grande problema na Odontologia e deve receber muita atenção na prática diária. É uma das doenças crônicas mais prevalentes no mundo, atingindo cerca de 60% a 90% das crianças em idade escolar e quase 100% da população adulta (PETERSEN et al., 2005). Apesar do declínio da prevalência de cárie entre crianças, os índices de cárie aumentam com a idade e permanece um problema na população adulta (BERNABÉ e SHEIHAM, 2014).

A produção de ácidos pelas bactérias acidogênicas e acidúricas no biofilme dental é um pré-requisito essencial para o desenvolvimento das lesões de cárie. Os biofilmes bacterianos são formados quando micro-organismos unicelulares se tornam irreversivelmente aderidos a uma superfície sólida e envolvida por uma matriz de polissacarídeos extracelulares, podendo haver a formação de biofilmes a partir de uma ou múltiplas espécies bacterianas (SPRATT e PRATTEN, 2003). A diversidade anatômica existente na cavidade oral e a interdependência entre as suas estruturas tornam o desenvolvimento dos biofilmes particularmente interessante. Contrariamente ao que ocorre nas superfícies mucosas, onde há uma constante descamação do epitélio e consequente renovação dos micro-organismos aderidos, os dentes constituem superfícies duras favoráveis à formação e maturação do biofilme bacteriano tanto na região supragengival como na região subgengival (MARSH, 1994). A placa dental é um biofilme bacteriano encontrado naturalmente na superfície dos dentes, apresentando composição bacteriana e bioquímica que pode variar em decorrência de fatores intrínsecos e extrínsecos ao hospedeiro (KOLENBRANDER, 2000).

Nos últimos anos, tem sido observado que bactérias inseridas nos biofilmes passam a exibir características fisiológicas distintas que resultam no aumento de resistência dos biofilmes aos agentes antimicrobianos quando comparadas às mesmas bactérias crescidas de forma planctônica (SVENSATER et al., 2001). Aparentemente, o crescimento dos biofilmes de forma organizada sobre a superfície dos dentes pode funcionar como uma proteção contra as forças mecânicas da mastigação, contra os mecanismos de defesa inata e adquirida do hospedeiro, bem como contra agentes agressores externos. Além disso, os mecanismos de defesa dos micro-organismos podem incluir a formação de uma barreira física constituída especialmente por polissacarídeos, dificuldade de difusão dos agentes antimicrobianos no interior dos biofilmes, transição para uma taxa de crescimento mais lento decorrente das limitações nutricionais no

interior dos biofilmes, a ativação de mecanismos gerais de resposta ao estresse, bem como a expressão de fenótipos específicos para microrganismos organizados na forma de biofilmes (MAH e O'TOOLE, 2001).

Apesar da complexidade da comunidade bacteriana que coloniza o biofilme dental, existem evidências consideráveis de que a presença de *Streptococcus mutans* esteja diretamente relacionada aos estágios iniciais da formação das lesões de cárie em humanos. Isso se dá devido a sua presença em altos níveis imediatamente antes do surgimento das lesões; a sua habilidade em rapidamente degradar carboidratos fermentáveis promovendo a formação abundante de ácido; além da sua capacidade de tolerar ambientes com baixo pH (SVENSATER *et al*., 2001). Em adição, *S. mutans* tem alta afinidade à superfície dentária mediada pela presença de adesinas e polissacarídeos extracelulares que contribuem para a sua patogenicidade (SENADHEERA *et al*., 2005).

Encontra-se estabelecido na comunidade científica o conceito de que o surgimento das duas doenças mais prevalentes na cavidade bucal de humanos, a cárie dental (LOESCHE, 1986) e a doença periodontal (SOCRANSKY & HAFFAJEE, 1991), estejam intimamente relacionadas à presença dos biofilmes organizados sobre a superfície dos dentes. O tratamento das doenças relacionadas à presença dos biofilmes bacterianos envolve basicamente a remoção mecânica desses biofilmes e o uso de antibióticos e/ou agentes anti-sépticos. Entretanto, diante da emergência de cepas resistentes aos agentes antimicrobianos tradicionais, têm aumentado o interesse da comunidade científica em desenvolver terapias antimicrobianas alternativas que tornem o surgimento de cepas geneticamente resistentes improvável (HAMBLIN; HASAN, 2004).

Neste sentido, a terapia fotodinâmica antimicrobiana surge como um tratamento alternativo ao uso de agentes antimicrobianos tradicionais. Durante esse processo, componentes celulares fotossensíveis passam para um estado excitado quando expostos a uma luz de comprimento de onda complementar, que é caracterizado pela passagem dos elétrons para níveis de energia superiores. Neste estado excitado, o fotossensibilizador pode interagir com o oxigênio molecular, iniciando a formação de oxigênio singleto altamente reativo (fotoprocesso Tipo II) ou interagir com outras moléculas, como aceptores de elétrons, resultando na produção de hidroxilas e outros radicais orgânicos (fotoprocesso do Tipo I) (DOUGHERTY et al., 1998). Os produtos

dessas reações fotoquímicas podem então danificar componentes essenciais das células ou alterar as atividades metabólicas de maneira irreversível resultando na morte bacteriana.

Como a maioria das espécies bacterianas não apresenta componentes fotossensíveis, a utilização de um agente cromóforo que atraia para si a luz e inicie a formação de radicais livres é importante para a efetividade da terapia fotodinâmica. No que diz respeito à interação entre fotossensibilizador e bactéria, a efetividade da terapia está relacionada a três principais aspectos: (1) capacidade do fotossensibilizador em interagir com a membrana bacteriana; (2) capacidade do fotossensibilizador em penetrar e agir dentro da célula; e (3) formação de oxigênio singleto ao redor da célula bacteriana (NAGATA et al., 2012). Assim, células desprovidas de componentes fotossensíveis endógenos podem se tornar sensíveis à luz se forem coradas com fotossensibilizadores exógenos como derivados das porfirinas, azul de orto-toluidina (TBO) ou compostos de cloro conjugado (ROLIM et al., 2012). Dentre esses, o uso do azul de orto-toluidina torna-se interessante devida sua natureza catiônica e, portanto, capaz de inativar tanto bactérias Gram-positivo quanto Gram- negativo (NAGATA et al., 2012).

Com relação à fonte de luz utilizada, a muitos dos estudos sobre o efeito antimicrobiano da terapia fotodinâmica utiliza lasers de baixa potência com diversos meios ativos e comprimentos de onda. O uso de dispositivos semicondutores que quando polarizados adequadamente emitem luz na faixa visível ou invisível, os chamados diodos emissores de luz (LED) surgiram como fontes alternativas de luz para essa terapia (ZANIN et al., 2005; ZANIN et al., 2006). Embora tanto os lasers quanto os LED produzam luzes monocromáticas, os LED apresentam colimação e coerência menos eficientes, resultando em bandas de emissão mais largas que acabam por favorecer a obtenção de efeito antimicrobiano, uma vez que a luz é emitida em todo o espectro de absorção dos fotossensibilizadores durante a realização da terapia fotodinâmica (NAGATA et al., 2012).

Entre as vantagens da terapia fotodinâmica antimicrobiana em relação ao uso dos agentes antimicrobianos tradicionais, temos primeiramente que a morte da célula bacteriana pode ser rápida, não sendo necessária a manutenção do agente químico em altas concentrações sobre as lesões por longos períodos de tempo como ocorre com os agentes anti-sépticos e antibióticos. Além disso, a morte bacteriana mediada pela liberação de radicais livres torna o desenvolvimento de resistência pelos microrganismos improvável (WILSON, 2004). Resultados promissores mostram que a terapia fotodinâmica antimicrobiana foi efetiva contra micro-organismos

antibiótico-resistentes, e repetidas aplicações não resultam em seleção de cepas resistentes (GURSOY et al., 2013). Finalmente, o uso do fotossensibilizador ou da luz sozinhos não apresentam efeito significativo sobre a viabilidade das bactérias, de modo que a terapia pode ser confinada à área da lesão pela aplicação tópica cuidadosa do corante e restrição da irradiação por meio do uso de fibra ótica (WILSON, 2004).

Estudos prévios têm demonstrado o efeito antimicrobiano da terapia fotodinâmica em bactérias orais em culturas planctônicas (WILLIAMS *et al*., 2003; ROLIM et al. 2012), biofilmes desorganizados (O'NEILL et al., 2002) e organizados (ZANIN et al., 2005; ZANIN et al., 2006; TEIXEIRA et al., 2012) e em lesões de cárie dentinária formadas *in vitro* (MELO et al., 2010) e *in situ* (LIMA et al., 2009) quando a combinação apropriada de fotossensibilizador e luz é utilizada. No entanto, a inativação total das bactérias presentes nos biofilmes e lesões de cárie não tem sido possível e a presença da matriz de polissacarídeos extracelulares produzida por *S. mutans* tem sido apontada como o principal responsável pela efetividade parcial da terapia (ZANIN et al., 2005). Dessa forma, este estudo teve por objetivo avaliar o efeito antibacteriano da terapia fotodinâmica realizada com a combinação do fotossensibilizador TBO e LED vermelho, e analisar os efeitos dessa terapia tanto na viabilidade bacteriana quanto na matriz de polissacarídeos de biofilmes de *S. mutans*.

## 2 PROPOSIÇÃO

Esta tese de doutorado é apresentada em três capítulos, tendo como objetivos:

**Capítulo 1**: Comparar o efeito antimicrobiano da terapia fotodinâmica realizada com o fotossensibilizador TBO e duas diferentes fontes de luz vermelha (Laserbeam® ou LumaCare®) em biofilmes maduros de *S. mutans*.

**Capítulo 2**: Verificar a quantidade de espécies reativas de oxigênio encontradas a nível intracelular em biofilmes de *S. mutans* como resultado da terapia fotodinâmica antimicrobiana realizada com TBO e a fonte de luz vermelha LumaCare®, e assim verificar o stress oxidativo promovido por essa terapia.

**Capítulo 3**: Avaliar o efeitos da terapia fotodinâmica realizada com TBO e a fonte de luz vermelha LumaCare® na viabilidade microbiana, na matriz de polissacarídeos e na topografia de biofilmes de *S. mutans* maduros (tratamento em dose única) e em formação (tratamento diário).

## 3 CAPÍTULOS

Esta tese está baseada no artigo 46 do regimento Interno do Programa de Pós-graduação em Odontologia da Universidade Federal do Ceará, que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado, e permite a inserção de artigos científicos de autoria ou co-autoria do candidato. Dessa forma, esta tese é composta por três capítulos, contendo artigos a serem submetidos para publicação em revistas científicas, conforme descrito abaixo:

Capítulo 1

"Photodynamic antimicrobial chemotherapy on *S. mutans* mature biofilms: comparative effect of two red lights sources". Iriana Carla Junqueira Zanin, Ramille Araújo Lima, Denise Lins de Sousa, Simone Duarte. Este artigo será submetido à publicação no periódico "Caries Research".

Capítulo 2

"Intracellular Reactive Oxygen Species in *S. mutans* biofilm after Photodynamic Antimicrobial Chemotherapy – PACT". Ramille Araújo Lima, Juliana Aparecida Delben, Iriana Carla Junqueira Zanin, Simone Duarte. Este artigo será submetido à publicação no periódico "Lasers in Medical Science".

Capítulo 3

"Photodynamic Antimicrobial Chemotherapy on *S. mutans* Mature and Forming Biofilms" Ramille Araújo Lima, Denise Lins de Sousa, Simone Duarte, Iriana Carla Junqueira Zanin. Este artigo será submetido à publicação no periódico "Journal of Dental Research".

### 3.1 Capítulo 1

**Title:** Photodynamic antimicrobial chemotherapy on *S. mutans* mature biofilms: comparative effect of two red light sources.

**Short Title:** Effect of PACT using different lights on *S. mutans* biofilms

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**Key-words:** Photodynamic therapies; *Streptococcus mutans*; biofilm; extracellular polysaccharides

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There are no potential conflicts of interest relating to this study.

**Abstract**

Photodynamic antimicrobial therapy (PACT) promotes bacterial death as a result of the photosensitization of microbial components. This study evaluated the comparative antimicrobial effect of PACT performed with different red light sources on *in vitro S. mutans* mature biofilms. For that, *S. mutans* UA159 biofilms were formed on saliva-coated hydroxyapatite discs using batch culture method at 37ºC, 5%$CO_2$. Tryptone-yeast extract broth containing 1% sucrose was changed daily. The antimicrobial effect of toluidine blue O (TBO) (100ug/ml), associated with two different red light sources, LB (Laserbeam®, λ ≅638.8nm) or LC (LumaCare®, λ ≅630nm) was evaluated. On the 5th day, biofilms were treated with sensitizer-S (TBO) and/or light-L in the test ($S^+L^+$) and control ($S^-L^-$, $S^+L^-$, $S^-L^+$) groups. LB was tested using 900 sec irradiation time (56.6 Jcm-$^{-2}$) while LC was tested using 22 sec (56.6 Jcm$^{-2}$); 60 sec (158.5 Jcm$^{-2}$), 120 sec (317.0 Jcm$^{-2}$); and 180 sec (475.6 Jcm$^{-2}$) irradiation time. After treatments, biofilms were colleted, weighted and disrupted using ultrasonic pulses. Ten-fold serial dilutions were carried out and aliquots were plated onto 5% Blood agar which were then incubated at 37°C, 5% $CO_2$ for 48 hours before enumerating the viable microorganisms. The results were expressed as colony forming units (CFU) mg$^{-1}$ of biofilm and the transformed to $\log_{10}$ of CFU mg$^{-1}$ of biofilm. The results demonstrated that groups receiving the PACT ($S^+L^+$) were statistically different from all tested controls ($S^-L^-$, $S^+L^-$, $S^-L^+$) for both red lights used ($p < 0.05$). Also, there were no statistical difference among all tested control groups ($p > 0.05$). Comparing microbial counts after PACT for the pair LB 900 sec (5.84±0.41) and LC 22 sec (6.09±0.20), both using energy density of 56.6 Jcm-$^{-2}$, there was no difference between them ($p > 0.05$). Also LC 120 sec (4.88±0.18) and LC180 sec (4.98±0.35) presented similar results ($p > 0.05$). Furthermore, considering the clinical irradiation time and also the antimicrobial effect observed, PACT with λ ≅630nm red light, irradiation time of 120 sec, seems to be the most suitable for reducing *S.mutans in vitro* biofilms when the photosensitizer TBO is used.

## Introduction

*Streptococcus mutans* is the major etiological agent in dental caries [Bowen, 1999] and it has a great influence on the formation and composition of pathogenic biofilms mainly due its ability to quickly degrade fermentable carbohydrates and synthesize extracellular polysaccharides (EPS) [Paes Leme et al., 2006]. Bacteria in biofilms show distinct physiological characteristics that result in increased resistance to antimicrobial agents when compared to the planktonic forms [Svensater et al., 2001]. Defense mechanisms of biofilm microorganisms may include the formation of a physical barrier (polysaccharides), difficulty dissemination of antimicrobial agents within the biofilm, as well as the expression of specific phenotypes [Mah and O'Toole, 2001].

Front the emergence of strains resistant to traditional antimicrobial agents, the photodynamic antimicrobial chemotherapy (PACT) appears as an alternative therapy to killing oral bacteria. It is based on the use of extrinsic photosensitizers (PS), light-absorbing molecules that initiate a photochemical reaction when exposed to light of a specific wavelength. This photochemistry process leads to reactive oxygen species (ROS) formation, which may cause irreversible damage to essential bacterial cell compounds and may change cell metabolism resulting in bacterial death [Dougherty et al., 1998]. The use of ortho-toluidine blue (TBO) as PS seems to be interesting due its cationic nature, and therefore able to inactivate both gram-positive as gram-negative bacteria [Nagata et al. 2012; Rolim et al. 2012].

Red light sources (630–700 nm) have been used extensively in PACT due to their relatively wide irradiation wavelengths, which can effectively penetrate biological tissues [Wilson, 1986]. The light emitting diode (LED) is a semiconductor device that emits non-coherent spectrum light. It has become an effective alternative to laser systems for reasons including lower cost, availability in a variety of wavelengths ranging from ultraviolet to near-infrared region of the spectrum, and a light fluence rate that can achieve hundreds of $mW/cm^2$. In addition, the arrays can be constructed in various sizes to accommodate large areas, and they do not emit considerable heat, which may cause additional tissue damage [Zanin et al., 2006; Nagata et al., 2012]. Previous studies have demonstrated the antimicrobial effect of PACT performed with TBO and red light on *S. mutans* biofilms [Zanin et al., 2005; Zanin et al., 2006; Teixeira et al. 2012]. However, the treatment protocols used in these studies required long irradiation times,

ranging from 10 to 20 minutes, which precluded the possibility of clinical use of this therapy. Thus, there is the need of alternatives protocols more effective and less time-demanding. Therefore, the aim of this study was to compare the antimicrobial effect of PACT performed with TBO and two different red light sources (Laserbeam® 900 sec; or LumaCare® 22sec, 60sec, 120 sec or 180 sec) on *S. mutans* mature biofilms.

## Materials and methods

**Experimental Design** For this *in vitro* experiment, 108 hydroxyapatite (HA) sterile discs (0.74 $cm^2$) were randomly allocated into 12 groups, with 3 experimental units per set of group. Biofilms of *Streptococcus mutans* UA159 were grown on HA discs immersed in batch culture and the mature biofilm was submitted to PACT after 5 days [Duarte et al. 2008]. To minimize the inherent bias related to microbiological procedures, three independent experiments were performed at different time points. The treatment conditions in which biofilms were exposed are as follows: biofilms were exposed to both TBO and light under different conditions ($S^+L^+$), biofilms were not exposed to sensitizer or light ($S^-L^-$), biofilms were exposed only to sensitizer ($S^+L^-$) or biofilms were exposed only to light ($S^-L^+$) using different light sources and energy densities (figure 1).

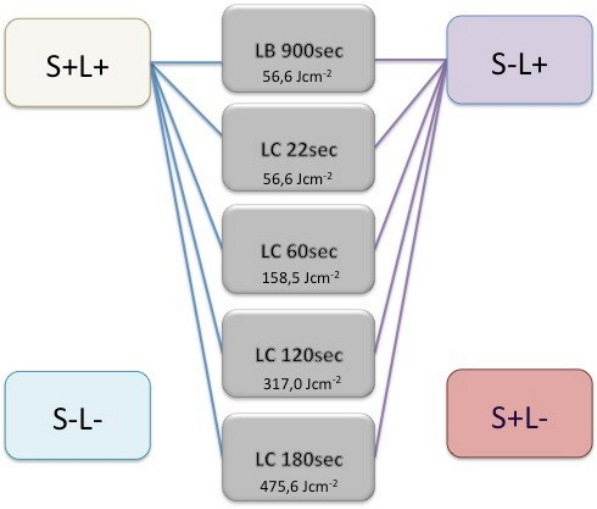


Figure 1. Experimental design. LB represents the Led device Laserbeam® and LC represents the non-coherent light LumaCare®

**Light Sources and Parameters:** A red light-emitting diode (LED, Laserbeam®, Rio de Janeiro, RJ, Brazil) with spectrum of emission ranging from 620 to 660 nm and predominant wavelength of 638.8 nm, fixed output power of 40 mW and optic fiber spot size of 0.69 $cm^2$ was used as one of the light sources. Biofilms were exposed to a 56.6 $Jcm^{-2}$ energy density after 900 seconds of irradiation. For comparing purpose, a non-coherent red light LumaCare® (Model LC-122, Medical Group, Newport Beach, CA) with spectrum of emission ranging from 570 to 690 nm and predominant wavelength of 630 nm, fixed output power of 1.68W, and optic fiber spot size of 1.13 $cm^2$ was also tested. Biofilms were exposed to four different energy densities and respectively energies doses as follows: 56.6 $Jcm^2$, 35.9 J (22sec); 158.5 $Jcm^{-2}$, 100.64 J (60sec); 317.05 $Jcm^{-2}$, 201.32 J (120 sec); 475.58 $Jcm^{-2}$, 3012.99 J (180 sec). Irradiation was performed in a noncontact mode with a focused beam at 10 mm of working distance.

**Photosensitizer:** The photosensitizer (S) used was toluidine blue ortho (TBO, CI 52040, Sigma Chemicals, Poole, UK), dissolved in deionized water (100 $\mu gmL^{-1}$) and stored at room temperature in the dark. The pre-irradiation time was 5 minutes [Zanin et al., 2005].

**Inoculum and biofilm model:** *Streptococcus mutans* UA159 (ATCC 700610) was obtained from single colonies isolated on 5% blood agar plates, inoculated in tryptone yeast-extract broth containing 1 % glucose (w/v) and incubated for 18–24 h at 37 °C under microaerophilic conditions in partial atmosphere of 5 % of $CO_2$. Biofilms of *S. mutans* UA159 were formed on saliva-coated hydroxyapatite discs (HA) (0.635$cm^2$) placed in batch cultures at 37 °C in 5 % $CO_2$ for 5 days. The biofilms were grown in tryptone yeast-extract broth containing 1 % sucrose (w/v) and were kept undisturbed for 24 h to allow initial biofilm formation. After that, the culture medium was replaced once daily [Duarte et al., 2006].

**Photodynamic antimicrobial chemotherapy of *in vitro* biofilms***:* After 5 days of biofilm formation, the biofilms were dip-washed three times in a plate containing of NaCl 0.89% solution in order to remove the loosely bound biofilm. After that, HA discs containing the biofilms were transferred to other 24-well polystyrene plates containing TBO (groups $S^+L^+$ and $S^+L^-$) or sterile miliQ water (groups $S^-L^-$ and $S^-L^+$) during the pre-irradiation time of 5 minutes in the dark. Following this time, the biofilms were exposed to red light irradiation (groups $S^+L^+$ and $S^-L^+$) or

maintained at room temperature during the same period (groups $S^+L^-$ and $S^-L^-$). The biofilms were then scrapped with a sterile spatula and transferred to a pre-weighed microtube containing 1 mL of NaCl 0.89% solution. To disperse the biofilm, 2 pulses of 10 seconds with 1 min of interval at an output of 7W were performed (Branson Sonifier 150; Branson Ultrassonics, Danbury, CT). Ten-fold serial dilutions were carried out and aliquots were plated onto Blood agar which were then incubated at 37°C, 5% $CO_2$ for 48 hours before enumerating the viable microorganisms. The results were expressed as colony forming units (CFU) $mg^{-1}$ of biofilm.

*Statistical analysis:* The normality distribution of data was checked using the Kolmogorov–Smirnov statistical test. The mean and the standard deviation of numbers of surviving microorganisms for each treatment were calculated. Colony forming units (CFU) were transformed in $\log_{10}$ CFU in order to reduce variance heterogeneity. One-way analysis of variance test (ANOVA) was used to detect differences, followed by a Tukey–Kramer test for pairwise comparisons. Significance level was set at 5% ($p< 0.05$) and the confidence interval was set at 95%. Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA, USA) was used to perform the analyses.

**Results**

The results indicated that neither incubation with TBO alone ($S^+L^-$), nor treatment with light alone ($S^-L^+$), had a significant effect on the viability of *S. mutans* ($p > 0.05$). Significant decreases in the viability of monospecies biofilms were only observed when biofilms were exposed to both TBO and irradiation at the same time. At same energy density (56.6 $Jcm^{-2}$), Laserbeam® (900 sec) and LumaCare® (22 sec) showed the same performance, with no difference between the groups, with median viable counts of $9.54 \times 10^5 \pm 6.21 \times 10^5$ (SD) in the group submitted to LB ($S^+L^+$) and $1.37 \times 10^6 \pm 6.35 \times 10^5$ (SD) in the group submitted to LC ($S^+L^+$); contrasting with the control groups: $2.43 \times 10^7 \pm 5.53 \times 10^6$ ($S^-L^-$), $2.36 \times 10^7 \pm 6.45 \times 10^6$ ($S^+L^-$), $4.62 \times 10^7 \pm 4.26 \times 10^7$ ($S^-L^+$ LB) and $3.35 \times 10^7 \pm 2.13 \times 10^7$ ($S^-L^+$ LC) (Figure 2).

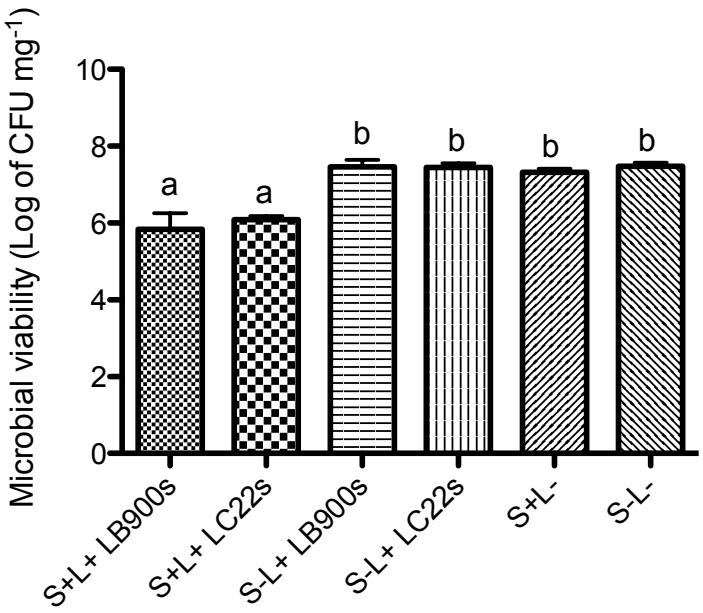


Figure 2. Microbial viability (Log of CFU $mg^{-1}$) after photodynamic therapy performed with LB and LC (energy density 56.6 $Jcm^{-2}$ ) + TBO (S+L+) compared with negative controls (S-L+ LB900s; S-L+ LC22s, S+L-, S-L-). Vertical bars denote standard deviation and different letters represent statistically significant differences by Tukey test ($p<0.05$).

Following, the remaining energy densities of LC (60 sec, 120 sec and 180 sec) also achieved a statistically significant reduction in microbial viability. When compared, 120 sec and 180 sec irradiation time presented similar results ($p> 0.05$), and both showed better results than 60 sec irradiation time ($p <0.05$) (figure 3). The log reduction results were calculated by subtraction of $\log_{10}$ counts in the negative control ($S^-L^-$), from $\log_{10}$ counts in the other groups ($S^+L^-$, $S^-L^+$, $S^+L^+$), and $\log_{10}$ reductions of up to 2.38 and 2.27 were observed when $S^-L^-$ and $S^+L^+$ groups were compared after 120 and 180 seconds of irradiation, respectively.

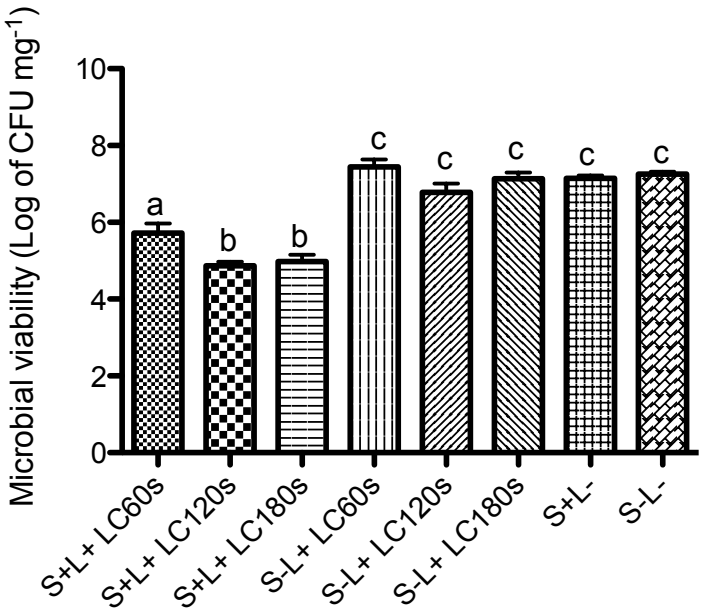


Figure 3. Microbial viability (Log of CFU $mg^{-1}$) after photodynamic therapy performed with LumaCare® 60, 120 and 180 seconds (energy densities 149 $Jcm^{-2}$, 317.05 $Jcm^{-2}$, 475.58 $Jcm^{-2}$ ) compared with negative controls (S-L+ LC60s; S-L+ LC120s, S-L+ LC180s, S+L-, S-L-). Vertical bars denote standard deviation and different letters represent statistically significant differences by Tukey test ($p<0.05$).

**Discussion**

In the present study, we investigated the antimicrobial photodynamic effect of a red light source associated with TBO on *in vitro* biofilms. The choice of a LED light and a non-coherent light source, instead of a laser device, was determined by its physical characteristics that associated with its low cost and portability made it more desirable to be used in PACT. In addition, the lack of collimation and coherence, which result in wider bands of emission, can provide light emission throughout the entire absorption spectrum of photosensitizer [Zanin et al., 2006], which may promote optimisation of photodynamic processes. TBO was chosen as sensitizer due to its characteristics of an optimal photosensitizer including photo-physical, chemical and biological characteristics such as possibility of local delivery into the infected area, selectivity for microorganisms in low concentrations, avoiding damage to host tissue, and diffusion capacity [O'Neill et al., 2002]. This cationic phenothiazine is efficient against both gram positive and gram negative bacteria (Nagata et al., 2005) and Usacheva et al. (2001) suggest

that TBO may bind to the polyphosphates of the membrane and produce molecular damage to lipids and proteins. Besides, Rolim et al., (2012) demonstrated that TBO, associated to a LED, was the most effective photosensitizer against planktonic cultures of *S. mutans* out of other five tested at the same molar concentration and irradiation conditions.

Our results confirmed the antimicrobial activity of photodynamic therapy performed with TBO and red LED in *S. mutans* biofilms, as observed in other studies [Zanin et al., 2005; Zanin et al., 2006; Teixeira et al., 2012]. Although we observed a statistical reduction of microbial viability, the total inactivation was not achieved, in contrast with Rolim et al. (2012) and Paschoal et al. (2013), who tested similar parameters in *S. mutans* planktonic cultures. It has been know that bacteria in biofilm are less sensitive to antibacterial agents than planktonic bacteria, manly due differences in cell wall composition, rate of growth, metabolic activity and gene expression. Furthermore, bacteria in biofilms are embedded in a polysaccharide matrix, which protects them from external threats, including antimicrobial agents [Anwar et al., 1992; Costerton et al., 1999; Svensater et al., 2001].

Zanin et al (2006) conducted a study performing PACT in *S. mutans* biofilms using the red LED of 638.8 nm and output power 40 mW (energy density 85.7 J.cm$^2$) and TBO at the same concentration used in this study, also observed a 2-log reduction in microbial viability, similar results to that obtained in our study using a lower energy density (55 J.cm$^2$). Similar results obtained with different energies doses can be explained by the difference in substrates. The previously cited study had used enamel slabs as substrate and our study used hydroxyapatite discs. Li et al. (2010) reported that the nature of a surface influences biofilm characteristics including biomass accumulation and susceptibility to antimicrobial treatments. Paschoal et al. (2013) used the red probe LC, energy densities 18 J.cm$^2$, 35 J.cm$^2$ and 53 J.cm$^2$, in combination with TBO at 25$\mu$M, 50$\mu$M and 100$\mu$M concentrations, and achieved total inactivation of microbial viability. However, this study used planktonic suspensions of *S. mutans*, unlike our study using mature biofilms of *S. mutans*. To our knowledge, this is the first study that had used this non-coherent red light with (630 nm, 1.68W) against mature biofilms.

In our study, no significant difference was observed in therapy performed with LC irradiation time 120 and 180 seconds, indicating that the increase of irradiation time did not lead

to increased reduction of the bacterial viability. This may have occurred due photosensitizer saturation, or photobleaching process [Metcalf et al., 2006]. On the other hand, both were superior to all other experimental groups of this study. In addition to its greater output power, another advantage of the LC is the possibility to produce the entire spectrum of visible light by changing the specific wavelenghts probes. Thus photosensitizers can be activated using the same device, only changing the probe. The most relevant aspect of this study is that the therapy performed with LB irradiation time 15 minutes had the same performance of LC irradiation time 22 seconds, both reaching a energy density of 56.6 $Jcm^{-2}$. Although this result was expected considering that both treatments used the same density energy, its represent a substantial advance in PACT therapy if we consider a clinical application. The huge difference of irradiation time between the two devices is due to the difference in the output power (LB 40mW and LC 1.68W). Also, the LC device allows the increase of power without significant increase of temperature (data not shown).

As conclusion, PACT significantly decreased the microbial viability of mature *S. mutans* biofilms when compared to the control groups. PACT performed with LB and LC at same energy density, did not statistically differ from each other, demostrating the LC be a better alternative to PACT, due to its reduced irradiation time – from 15 min to 22 sec. Also, PACT using LC irradiation time of 120 sec, seems to be the most suitable for reducing mature *S. mutans in vitro* biofilms when the photosensitizer TBO is used.

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## 3.2 Capítulo 2

# Intracellular Reactive Oxygen Species in *S. mutans* biofilm after Photodynamic Antimicrobial Chemotherapy.

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**Abstract**

Photodynamic Antimicrobial Chemotherapy (PACT) appears as an alternative treatment to dental plaque-related diseases. Products of these photochemical reactions such as reactive oxygen species (ROS) can damage essential components of cells or change their metabolic activities resulting in bacterial death. However, more studies are needed to determine whether reactive oxygen species (ROS) produced are able to penetrate into bacterial cells organized in biofilms and protected by a matrix of polysaccharides. Therefore, the aim of this study was to measure the amount of intracellular ROS found in *S. mutans* biofilms after PACT performed with orto-toluidine blue (TBO) and a non-coherent red light (LumaCare®; 570-690 nm). Biofilms of *Streptococcus mutans* UA159 were formed on saliva-coated hydroxyapatite discs during 5 days and after that the biofilms were exposed to PACT using the energy density of 211.37 $Jcm^{-2}$ (1-minute irradiation) and 422.74 $Jcm^{-2}$ (2-minutes irradiation). Production of ROS in *S. mutans* cells as consequence of PACT was determined by using the oxidative-stress-sensitive probe dihydrorhodamine - DHR 123. The 2-minutes PACT group showed the highest levels of ROS ($p < 0.0001$). PACT performed for 1 minute, despite having a lower production of ROS compared to the 2-minutes group, it was also superior to control groups ($p<0.05$). In conclusion, PACT under tested conditions generates significant levels of intracellular oxidative species in *S. mutans* biofilms.

**Key words:** *Streptococcus mutans*; Biofilm; Photodynamic Antimicrobial Chemotherapy; Reactive oxygen species

## Introduction

Dental caries remains as a major worldwide problem. Although it was observed a decline in the prevalence of this condition, it is one of the most prevalent chronic diseases in the world, reaching about 60% to 90% of school children and almost 100% of the adult population [1] (Petersen *et al*, 2005). *Streptococcus mutans* is acknowledged as the major etiological agent in dental caries [2] and it has a great influence on the formation and composition of pathogenic biofilms. The production of acids by acidogenic and aciduric bacteria in the biofilm is essential for the development of carious lesions and it has been observed that bacteria in biofilms show distinct physiological characteristics that result in increased resistance of biofilms to antimicrobial agents when compared to the same bacteria grown in planktonic form [3].

The current treatment for plaque-related diseases involves the use of traditional antimicrobials in conjunction with the mechanical removal of the biofilm. However, due to the emergence of strains resistant to traditional antimicrobial agents, it has increased the interest of the scientific community to develop alternative antimicrobial therapies that make the emergence of genetically resistant strains unlikely [4].

In this regard, photodynamic antimicrobial chemotherapy (PACT) emerges as an alternative treatment to the use of traditional antimicrobials. During this process, photosensitive cell compounds pass into an excited state when exposed to a light-length complementary wave, which is characterized by the passage of electrons to higher energy levels. In this excited state, the photosensitizer can interact with the molecular oxygen, initiating the formation of highly reactive singlet oxygen (Type II photoprocess) or to interact with other molecules, as electron acceptors, resulting in the production of hydroxyls and other organic radicals (Type I photoprocess) [5]. Products of these photochemical reactions, the reactive oxygen species (ROS), can damage essential components of cells or change their metabolic activities resulting in bacterial death.

Although the PACT mechanism is already well established, and previous studies have showed the ROS production in a large combinations of photosensitizers and lights using planktonic cultures [6,7], more studies are needed in order to evaluate the intracelular ROS levels on *S. mutans* matures biofilms. Therefore, the aim of this study was to measure the amount of

intracellular ROS found in *S. mutans* biofilms as result of PACT treatments using the association of ortho-toluidine blue O (TBO) and a non-coherent red light source, and thus verify the oxidative stress promoted by this therapy, and evaluate the use of dihydrorhodamine 123 dye in biofilms. Our hypothesis was that *S. mutans* biofilms submitted to PACT will present the highest levels of ROS.

**Materials and methods**

*Experimental design:* Hydroxyapatite (HA) sterile discs (63.58mm$^2$) were randomly allocated into 5 groups, with 3 experimental units per set of group (45 discs). To minimize the inherent bias related to microbiological procedures, three independent experiments were performed at different time points. The treatment conditions in which biofilms were exposed are as follows: biofilms were exposed to both TBO and light under different conditions ($S^+L^+$), biofilms were not exposed to sensitizer or light ($S^-L^-$), biofilms were exposed only to sensitizer ($S^+L^-$) or biofilms were exposed only to light ($S^-L^+$) using different irradiation times (energy density) as follows (Figure 1).

| **Group** | **Description** |
|---|---|
| **$S^-L^-$** | Milli-Q Water (5 min) followed by a 2 min-period in the dark |
| **$S^+L^-$** | TBO Sensitization (5 min) followed by a 2 min-period in the dark |
| **$S^-L^+$ 2min** | Milli-Q Water (5 min) followed by a 2 min LED irradiation (422.74 J/cm$^2$) |
| **$S^+L^+$ 1min** | TBO Sensitization (5 min) followed by 1 min LED irradiation (211.37cm$^2$) |
| **$S^+L^+$ 2min** | TBO Sensitization (5 min) followed by a 2 min LED irradiation (422.74cm$^2$) |

Figure 1. Experimental group description

*Inoculum and biofilm model: Streptococcus mutans* UA159 (ATCC 700610) was obtained from single colonies isolated on blood agar plates, inoculated in tryptone yeast-extract broth containing 1 % (w/v) glucose and incubated for 18–24 h at 37 °C under microaerophilic conditions (5 % of $CO_2$). *S. mutans* UA159 biofilms were formed on saliva-coated hydroxyapatite discs (HA) placed in batch cultures at 37 °C in 5 % $CO_2$ for 5 days. The biofilms were grown in tryptone yeast-

extract broth containing 1 % sucrose (w/v) and were kept undisturbed for 24 h to allow initial biofilm formation. The culture medium was replaced once daily.

*Light Sources and Parameters:* The non-coherent red light LumaCare® (Model LC-122, Medical Group, Newport Beach, CA) with predominant wavelength of 630 nm (570 nm – 690 nm), spot size of 113.1 $mm^2$, fixed output power of 2.24W was used and 2 energy densities were tested: 211.37 $J/cm^2$ (1 minute irradiation time, energy dose 134.21 J) and 422.74 $J/cm^2$ (2 minutes irradiation time, energy dose 268.43 J). The distance between the light source tip and the exposed sample was 3mm.

*Photosensitizer:* The photosensitizer (PS) used was toluidine blue ortho (TBO, CI 52040, Sigma Chemicals, Poole, UK), dissolved in deionized water (100µg $mL^{-1}$) and stored at room temperature in the dark. The pre-irradiation time used was 5 minutes.

*PACT treatment and measurement ROS:* Production of ROS in *S. mutans* cells as consequence of PACT was determined by using the oxidative-stress-sensitive probe dihydrorhodamine 123 (DHR 123) (Life Technologies®, Carlsbad, CA, USA). At $5^{th}$ day, the HA discs with *S. mutans* mature biofilms were transferred to 24-well plates containing 1mL of 15$\mu M$ DHR 123. The discs remained at this solution for 30 minutes in the dark. Thereafter, the PACT was performed using different treatments as described in figure 1. Immediately after treatment, the biofilms were dip-washed three times in NaCl 0.89%, in order to remove loosely bound biofilm, placed in 5 ml sterile of the same solution and the HA discs surfaces were gently scraped with a sterile spatula to harvest adherent cells. The removed biofilms were subjected to sonication using three 15-s pulses at an output of 7 W (Fisher Scientific, Sonic Dismembrator model 100; USA). Following, the homogenized suspensions were distributed in 96-well black plates and the oxidation of DHR 123 was measured by fluorimetry (SpectraMax M5, Molecular Devices, USA) using excitation and emission wavelengths of 507 nm and 529 nm respectively. This experiment was performed in triplicates.

*Statistical analyses:* The normality distribution was checked using the Kolmogorov–Smirnov statistical test. One-way analysis of variance test (ANOVA) was used to detect differences

between the groups, followed by a Tukey–Kramer test for pairwise comparisons. Significance level was set at 5% ($p< 0.05$) and the confidence interval at 95%. Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA, USA) was used to perform the analyses.

**Results**

The results were expressed as units of fluorescence, since this means the ROS amount detected. All groups tested emitted some level of fluorescence (Figure 1). Overall, the results showed that ROS production seems to be light dose-dependent once $S^+L^+$ 2min group showed the highest levels of ROS ($p <0.0001$) followed by $S^+L^+$ 1min, ($p<0.05$). The presence of sensitizer in absence of light do not to affect ROS production on *S. mutans* biofilm. However, the presence of 2 min light irradiation increase the ROS production ($p> 0.05$) indicating the stress of environment in this tested condition (Figure 2).

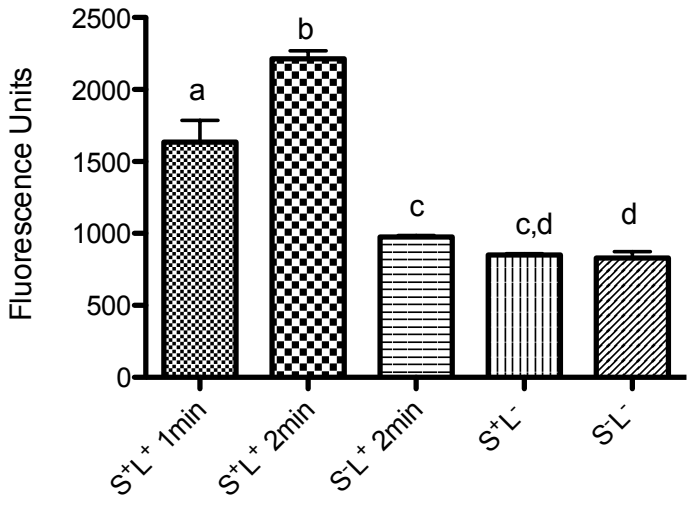


Figure 2. Fluorescence units (arbitrary units) representative of intracellular amount of ROS in *S. mutans* biofilm cells after exposed to experimental groups. Data represent mean values (n = 3) and different letters differ statistically ($p < 0.05$).

**Discussion**

The present study analyzed the production of intracellular ROS in *S. mutans* biofilms exposed to PACT. Since the antimicrobial mechanism of photodynamic therapy is based on the production of reactive oxygen species, it is essential to check whether the proposed therapy produced significant levels of ROS and if these are able to penetrate into bacterial cells organized in biofilms. It is known that *S. mutans* biofilm presents a thick layer of extracellular polysaccharide [8], which could hinder the penetration of these species. Although previous studies have analyzed the ROS [6] and singlet oxygen [7] production generated by a number of photosensitizers and light sources combinations, as far as we know, this is the first one that analyzed the presence of intracellular ROS in *S. mutans* biofilm after PACT exposure.

Dihydrorhodamine 123 (DHR 123) was used as an indicator of intracellular ROS presence. Previous researchers have reported that DRH is no fluorescent, uncharged, and readily taken up by cells, whereas DHR-123, the product of DHR oxidation, exhibits green fluorescence [9,10]. Farrell et al. [11] using the same dye, analyzed the production of intracellular ROS in *Candida albicans* after exposed to pulsed light. In Farrell's study, the light exposure was not directly proportional to ROS production, with fluorescence peaks observed after 20 and 150 pulses. This result contrasts with those observed in our study, where the irradiation time (energy density) was directly proportional to the levels of fluorescence obtained.

All experimental groups showed some levels of ROS production. Oxidative stress is an unavoidable consequence of life in an oxygen-rich atmosphere. Oxygen radicals and other activated oxygen species are generated as products of aerobic metabolism [11]. In addition, biofilms were removed from the culture medium and then exposed to experimental groups. Removal of the discs from their nutritional medium can itself generate a degree of oxidative stress. However, only elevated levels of intracellular ROS can be biologically deleterious, potentially damaging a wide range of macromolecules including nucleic acids, proteins and lipids [11].

The intracelular ROS amount found to $S^+L^+$ 2 min group it was almost 3 times higher than that observed to negative control $S^-L^-$ group. This result was expected since the photodynamic therapy mechanism is based on the production of these species. We emphasize that 2-minutes irradiation (422.74 J/cm$^2$) increased by 69% the ROS production compares to 1-minute irradiation group (211.37 J/cm$^2$). Zanin et al. [12] evaluated the antimicrobial effect of TBO +

LED (energy densities of between 49 and 294 J/cm$^2$) on *S. mutans* biofilms and they verified that the bactericidal effect was light dose-dependent, which may be a reflection of the production of ROS.

Biofilms exposed only to 2 min red light irradiation, had higher levels of intracellular ROS than those exposed only to the water in the dark in negative control group ($p<0.05$). Despite this difference, that amount of ROS detected may not mean reduction of microbial viability. As mentioned above, only elevated ROS levels may be harmful to the cell. Previous studies have demonstrated that only the exposure to red light, or only the exposure to TBO, shows no bactericidal effect in *S. mutans* planktonic cultures [7,13] and biofilms [12, 14-15]. However, the energy density used in this study was 2 to 23 times greater than used in these studies previously cited. The high energy density used in this study was only possible due to the great output power provided by LumaCare® device (2.24W), allowing higher energies doses in shorter irradiation times, without considerable heating (data not shown).

The results of this study demonstrate that photodynamic therapy may be considered an approach to the treatment of dental plaque-related diseases, since it generates significant levels of intracellular oxidative stress by *S. mutans* into biofilms. More studies should be conducted in order to verify the relationship between the levels of intracellular ROS and microbial viability of *S. mutans* embedded in biofilm. The DHR-123, already used as dye for intracellular ROS in planktonic suspensions, can also be used for bacteria in biofilms. We accept the hypothesis since biofilms receiving therapy showed the highest levels of intracellular ROS.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

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## 3.3 Capítulo 3

### Photodynamic Antimicrobial Chemotherapy on *S. mutans* Mature and Forming Biofilms

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**Abstract**

Front the emergence of strains resistant to traditional antimicrobial agents, the photodynamic antimicrobial chemotherapy (PACT) appears as an alternative therapy to killing oral bacteria. The aim of this study was to evaluate the effect of PACT, using the association of sensitizer toluidine blue-ortho (TBO) and non-coherent red light LumaCare® (energy densities 211.37 and 422.74 J/cm$^2$), on the microbial viability, polysaccharide matrix and topography of *S. mutans* biofilm. To analyze the effects of therapy in mature biofilms, PACT was applied in a single dose on 5 days-old biofilms, and to investigate the effects of PACT on biofilm formation, therapy was applied twice/daily over the 5 days. Chlorhexidine digluconate (CHX) 0.12% was used as positive control. Biofilms were analyzed for bacterial viability and extracellular polysaccharides (insoluble and soluble) production. Variable pressure scanning electron microscopy (VPSEM) was used to check biofilm topography. PACT, energy density 422.74 J/cm$^2$, achieved 5 logs reduction when used in single dose and 6.5 log reduction when used twice/daily, being superior to all other groups. No differences were observed in the matrix of polysaccharides after PACT when single-dose treatment was performed on mature biofilms. However, twice/daily PACT treatment dramatically reduced the production of soluble and insoluble polysaccharides in *S. mutans* biofilms. The VPSEM images showed a small number of cell clusters (microcolonies) and lost of the connective EPS structures in the biofilms treated with PACT twice/daily. PACT performed with Red non-coherent light and TBO (100μg/ml) is effective in both kill bacteria in mature biofilms and also statistically reduce the formation of *in vitro S. mutans* biofilms, emerging as an efficient alternative to conventional antimicrobial therapy to dental caries prevention.

## Introduction

The formation of acid ending-products through the metabolism of carbohydrates by acidogenic microorganisms in biofilms is an important factor in the development of dental caries (Svensater *et al*., 2003). Bacteria in biofilms show distinct physiological characteristics that result in increased resistance to antimicrobial agents when compared to the planktonic forms (Svensater *et al*., 2001). Defense mechanisms of biofilm microorganisms may include the formation of a physical barrier (polysaccharides), difficulty in dissemination of antimicrobial agents within the biofilm, as well as the expression of specific phenotypes (Mah and O'Toole, 2001). *Streptococcus mutans* is a key contributor to the formation and composition of cariogenic biofilms mainly due its ability to quickly degrade fermentable carbohydrates and synthesize extracellular polysaccharides (EPS) (Paes Leme *et al*., 2006). The analyses of EPS matrix formation could advance the current understanding of the development process and structural organization of oral biofilms, which would be essential for designing novel and effective anti-biofilm therapies. (Xiao and Koo, 2010)

Front the emergence of strains resistant to traditional antimicrobial agents, the photodynamic antimicrobial chemotherapy (PACT) appears as an alternative therapy to killing oral bacteria. It is based on the use of extrinsic photosensitizers (PS), light-absorbing molecules that initiate a photochemical reaction when exposed to light of a specific wavelength. This photochemistry process leads to reactive oxygen species (ROS) formation, which may cause irreversible damage to essential bacterial cell compounds and may change cell metabolism resulting in bacterial death (Dougherty TJ *et al*. 1998). The use of ortho-toluidine blue (TBO) as PS seems to be interesting due its cationic nature, its ability to inactivate both gram-positive and gram-negative bacteria (Nagata *et al*. 2012), and also by its capacity of diffuse through the bacterial cell membrane due its high transmembrane permeability coefficient (Usacheva *et al*. 2001).

Since *S. mutans* is the major etiological agent in dental caries (Bowen, 1999), and the EPS are essential for the maintenance of cariogenic biofilms (Marsh, 2004), the purposes of this study were to evaluate the effect of PACT, using the association of sensitizer toluidine blue-ortho (TBO) and non-coherent red light (energy densities 211.37 and 422.74 J/cm$^2$), on the microbial viability, polysaccharide matrix and topography of *S. mutans* biofilm, both during biofilms formation and also as a mature biofilm.

**Materials and methods**

*Light Sources:* The light source used in this study was a non-coherent red light (LumaCare® Model LC-122, Medical Group, Newport Beach, CA) with 630 nm predominant wavelength (570-690nm), spot size of 1.13 $cm^2$, and fixed output power of 2.24W. The distance between the light source tip and sample was 3 mm and two energy densities were tested: 211.37 $J/cm^2$ (1 min irradiation time, energy dose 134.21 J) and 422.74 $J/cm^2$ (2 min irradiation time, energy dose 268.43 J).

*Photosensitizer:* The PS used is the toluidine blue-ortho (TBO) (Sigma, CI 52040, Steinheim, Germany) at concentration of 100µg/ml and stored at room temperature in the dark. The pre-irradiation time was 5 minutes.

*Inoculum and biofilm model: Streptococcus mutans* UA159 (ATCC 700610) was obtained from single colonies isolated on blood agar plates, inoculated in tryptone yeast-extract broth containing 1 % glucose (w/v) and incubated for 18–24 h at 37 °C under microaerophilic conditions in partial atmosphere of 5 % $CO_2$. *S. mutans* biofilms were formed on saliva-coated hydroxyapatite discs (HA) placed in batch cultures at 37 °C in 5 % $CO_2$ during 5 days. The biofilms were grown in tryptone yeast-extract broth containing 1 % sucrose (w/v) and were kept undisturbed for 24 h to allow initial biofilm formation. The culture medium was replaced once daily.

*Experimental design and PACT of in vitro biofilms:* For this *in vitro* experiment, 72 hydroxyapatite (HA) sterile discs (0.635 $cm^2$) (144 discs for mature and forming biofilm experiments) were randomly allocated into 8 groups, with 3 experimental units per set of group. To minimize the inherent bias related to microbiological procedures, three independent experiments were performed at different time points. The treatment conditions in which biofilms were exposed are as follows: biofilms were exposed to both TBO and light under different conditions ($S^+L^+$), biofilms were not exposed to sensitizer or light ($S^-L^-$), biofilms were exposed only to sensitizer ($S^+L^-$) or biofilms were exposed only to light ($S^-L^+$), using different energy densities, as described: L+S+ 1min - TBO sensitization (5min) followed by light irradiation 1min (211.37 $J/cm^2$); L+S+ 2min - TBO sensitization (5min) followed by light irradiation 2min

(422.74 J/cm$^2$); CHX - Immersion in 0.12% chlorhexidine digluconate during 1min; L-S- - Milli-Q water (5min) followed by a 1min-period in the dark; L-S+ 1min - TBO sensitization (5min) followed by a 1min-period in the dark; L-S+ 2min - TBO sensitization (5min) followed by a 2 min-period in the dark; L+S- 1min - Milli-Q water (5min) followed by light irradiation 1min (211.37 J/cm$^2$); L+S- 2min - Milli-Q water (5min) followed by light irradiation 2min (422.74 J/cm$^2$).

After 24h initial biofilm formation, the biofilms were exposed to the different treatments twice daily (10 a.m. and 4 p.m.) until the fifth day of the experimental period to analyze the effects of PACT on the biofilm formation. To evaluate the effect of PACT on mature biofilms, treatments were held only once, on the fifth day.

*Biofilm analysis:* At the end of the experimental period, the biofilms were dip-washed three times in order to remove the loosely bound biofilm, the hydroxyapatite surfaces were gently scraped with a sterile spatula to harvest adherent cells and the biofilm collected was placed in 5 ml sterile saline solution. These biofilms were subjected to sonication using three 15-s pulses at an output of 7 W (Fisher Scientific, Sonic Dismembrator model 100; USA). The homogenized suspension was used for dry weight, bacterial viability (colony-forming units – CFU per miligram of dry weight biofilm), and polysaccharide analyses (EPS-soluble, EPS-insoluble) as described in the previous study (Duarte *et al*. 2006).

*Dry weight:* For the dry weight determination, three volumes of cold ethanol (-20°C) were added to 1 ml biofilm suspension, and the resulting precipitate was centrifuged (10,000 g for 10 min at 4°C). The supernatant was discarded, and the pellet was washed with cold ethanol, and then lyophilized and weighed (Duarte *et al*. 2006)

*Bacterial viability:* An aliquot (0.1 mL) of the homogenized suspension was serially diluted (1:10; 1:100; 1:1000; 1:10000; 1:100000; 1:1000000) and plated on blood agar. The plates were incubated in 5% $CO_2$ at 37°C for 48 h, and then the number of CFU/mg of biofilm dry weight was determined.

*Polysaccharide analyses:* Soluble and insoluble extracellular polysaccharides (EPS-soluble and EPS-insoluble, respectively) were analyzed. The polysaccharide content was expressed per μg.

Briefly, an aliquot (3.9 ml) of the suspension was sonicated for 30-s pulses at an output of 7 W and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and the biofilm pellet was resuspended and washed in 5 mL of milli-Q water; this procedure was repeated three times. The supernatant was used for the EPS-soluble assay and biofilm pellet was used for the EPS-insoluble and IPS assays. All of the supernatants were pooled, three volumes of cold ethanol were added, and the resulting precipitate was collected by centrifugation and resuspended in 5 mL Milli-Q water and the total amount of carbohydrate was determined by the phenol–sulfuric acid method (Dubois *et al*., 1956) by espectofotometry. The EPS-insoluble was extracted using 1 N NaOH (1 mg biofilm dry weight/0.3 ml of 1 N NaOH) under agitation for 1 h at 37°C. The supernatant was collected by centrifugation, and the precipitate was resuspended again in 1 N NaOH; this procedure was repeated three times. The total amount of carbohydrate was also determined by the phenol–sulfuric acid method (Dubois *et al*., 1956).

*Variable pressure scanning electron microscopy (VPSEM):* The HA discs were transferred to glass slides, biofilms upwards, and placed on the VPSEM [Zeiss EVO 50 (Carl Zeiss Microscopy, LLC, Thornwood, NY)] chamber. The images were captured at 100 Pa and 15.00 Kv, working distance of 7.5 mm and field width 7.5mm (Weber *et al*., 2014). Based on the results of the quantitative analysis (bacterial viability and soluble and insoluble polysaccharides), the VPSEM analysis for L+S-, L-S+ groups were not performed.

*Statistical analysis:* Prior to analysis, assumptions of equality of variances and normal distribution of errors were checked by Kolmogorov-Sminorv test. Bacterial counts were calculated and the CFU/mg dry weight biofilms were transformed in $\log_{10}$ in order to reduce variance heterogeneity. The mean and the standard deviation were calculated to bacterial viability and polysaccharide analyses and when the variances between the groups were homogeneous, ANOVA test was used, followed by Tukey-Kramer test for pairwise comparisons. When heterogeneous variances between groups were detected, the Kruskal-Wallis test was used, followed by Dunn's test for pairwise comparisons. Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA, USA) was used to perform the analyses. The confidence interval was set at 95%.

## Results

*Bacterial Viability*

Photodynamic therapy showed a significant reduction in bacterial viability in both biofilm models ($p<0.05$). In mature biofilms, L+S+ 2min was different from all other experimental groups and achieved 5-log reduction compared to the L-S- group ($p<0.05$). L+S+ 1min also statistically reduced the number of colony-forming units/mg (CFU/mg) of *S. mutans* biofilm, however, this reduction was less than that achieved by L+S+ 2min group ($p<0.05$). Also, CHX group showed no statistically significant effect on bacterial viability in mature biofilms (Figure 1). Analyzing the effects of therapy in biofilm formation, we found that the group L+S+ 2min was similar to the group L+S+ 1min ($p>0.05$) thought superior to CHX in CFU reduction ($p<0.05$) (Figure 2). The groups that received only LED irradiation, only the application of TBO, or no treatment at all were statistically equal, showing no effect on bacterial viability ($p>0.05$) (Figure 1 and 2).

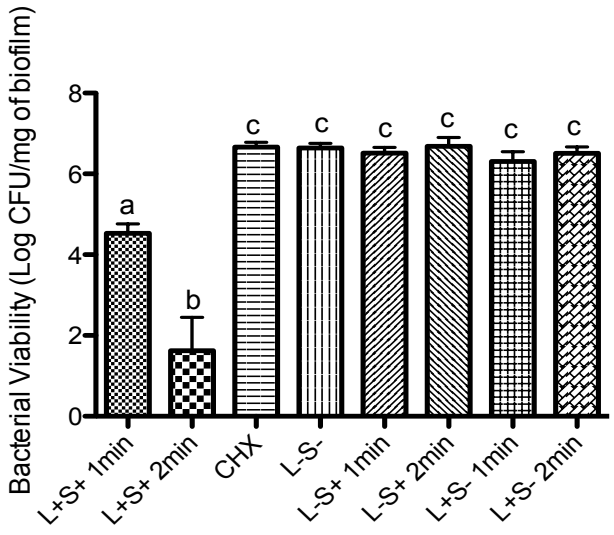


Figure 1. Bacterial viability of *S. mutans* mature biofilms after different treatments. Data represent mean values (n = 3) and error bars represent standard deviations. Data followed by different letters differ statistically ($p < 0.05$)

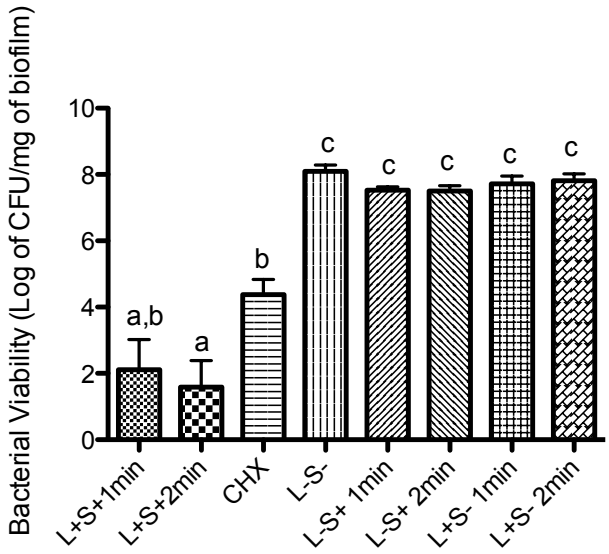


Figure 2. Bacterial viability of *S. mutans* biofilms submitted to different treatments during its formation. Data represent mean values (n = 3) and error bars represent standard deviations. Data followed by different letters differ statistically ($p < 0.05$).

*Polysaccharide analyses*

No differences were observed in the amount of soluble and insoluble EPS in mature biofilms among all experimental groups ($p>0.05$) (Table 1). However, when we analyzed the production of these polysaccharides in forming-biofilms, it was observed that photodynamic therapy statistically reduce the EPS formation. The results demonstrated that both the L+S+ 1min and L+S+ 2min treatments reduce similar amounts of soluble EPS ($p>0.05$), being lower that all other groups, including CHX one. The insoluble EPS analysis demonstrated that L+S+ 1min, L+S+ 2mim and CHX groups were similar among them ($p> 0.05$), and more effective in reducing the formation of this polysaccharide then the other groups tested ($p<0.05$) (Table 2).

Table 1: Total carbohydrate amounts of EPS soluble and insoluble (μg) determined by phenol-sulfuric acid method in *S. mutans* mature biofilms. Data represent mean values (n = 3) and data followed by different letters differ statistically ($p < 0.05$). Statistical analysis refers to data in rows.

| | **L+S+ 1min** | **L+S+ 2min** | **CHX** | **L-S-** | **L-S+ 1min** | **L-S+ 2min** | **L+S- 1min** | **L+S- 2min** |
|---|---|---|---|---|---|---|---|---|
| *EPS-soluble* | $350.6^{a}$ | $310.5^{a}$ | $342.2^{a}$ | $291.9^{a}$ | $263.9^{a}$ | $357.1^{a}$ | $269.6^{a}$ | $253.2^{a}$ |
| *EPS-insoluble* | $225.1^{A}$ | $212.0^{A}$ | $229.3^{A}$ | $226.2^{A}$ | $263.0^{A}$ | $287.8^{A}$ | $208.0^{A}$ | $277.2^{A}$ |

Table 2: Total carbohydrate amounts of EPS soluble and insoluble (ug) determined by phenol-sulfuric acid method in *S. mutans* forming-biofilms. Data represent mean values (n = 3) and data followed by different letters differ statistically ($p < 0.05$). Statistical analysis refers to data in rows.

| | **L+S+ 1min** | **L+S+ 2min** | **CHX** | **L-S-** | **L-S+ 1min** | **L-S+ 2min** | **L+S- 1min** | **L+S- 2min** |
|---|---|---|---|---|---|---|---|---|
| *EPS-soluble* | $57.33^{a}$ | $61.06^{a}$ | $287.2^{b}$ | $427.6^{b}$ | $286.7^{b}$ | $379.4^{b}$ | $373.9^{b}$ | $375.8^{b}$ |
| *EPS-insoluble* | $13.64^{A}$ | $33.18^{A}$ | $90.71^{A}$ | $460.3^{B}$ | $447.1^{B}$ | $368.6^{B}$ | $490.5^{B}$ | $389.9^{B}$ |

*Variable pressure scanning electron microscopy (VPSEM)*

The VPSEM images illustrate the effects of PACT on *S. mutans* biofilm topography. We visualized a small number of cell clusters (microcolonies) and lost of the connective EPS structures in the biofilms treated with PACT twice/daily (forming-biofilms) (figures 3a and 3b). No great differences between the groups were observed in mature biofilms submitted to different treatments (figure 4).

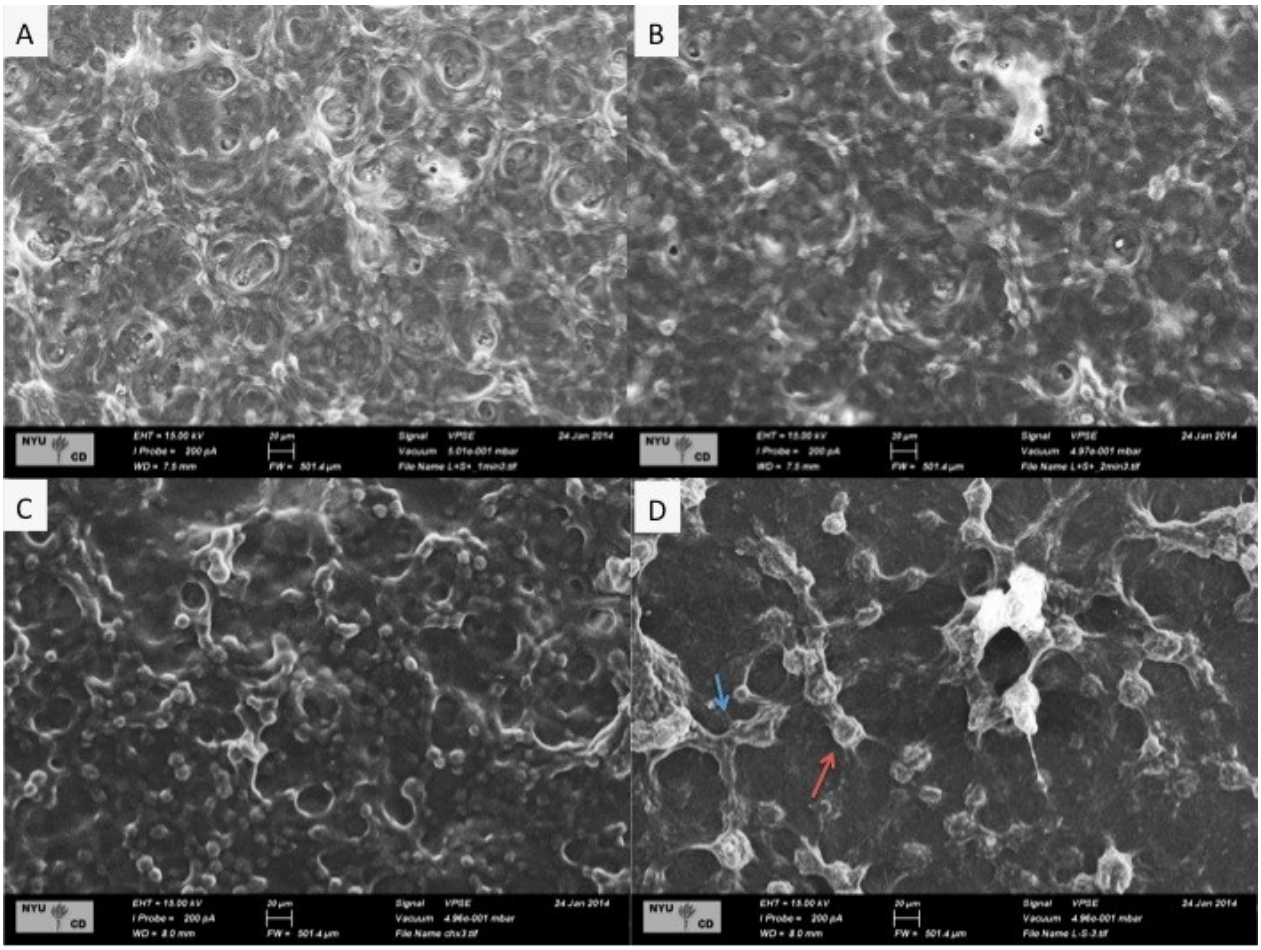


Figure 3. VPSEM images of forming-biofilms after exposure to different treatments (field widht 500$\mu$m). A= refers to L+S+ 1min; B refers to L+S+ 2min; C refers to CHX and D referes to L-S- treatment. The red arrow indicates a cell cluster and the blue arrow indicates the connective structures of the polysaccharide matrix.

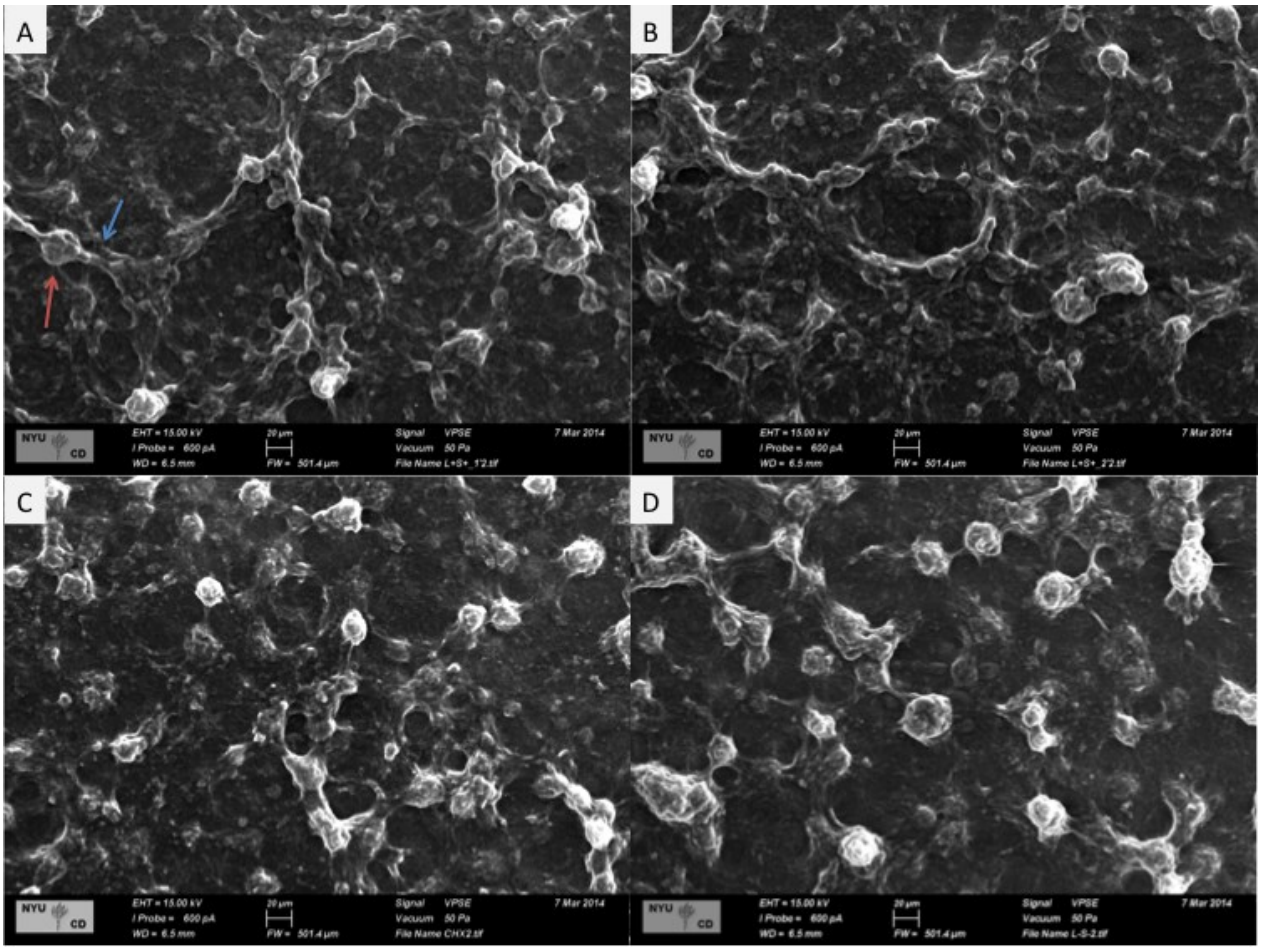


Figure 4. VPSEM images of mature biofilms after exposure to different treatments (field widht 500$\mu$m). A refers to L+S+ 1min, B refers to L+S+ 2min; C referes to CHX and D refers to L-S- treatment. The red arrow indicates a cell cluster and the blue arrow indicates the connective structures of the polysaccharide matrix.

## Discussion

Initially, the purpose of this study was to analyze the PACT effect in inactivating mature cariogenic biofilms using a unique treatment at the end of experimental period. Thus, this study aimed to analyze the PACT effect on *S. mutans* biofilm using twice/daily treatment in order to also investigate the potential use of this therapy in the prevention of biofilms formation.

PACT performed with the non-coherent red light (630 nm predominant wavelength, 2.24W output power) and the photosensitizer TBO showed great effect on bacterial viability, polysaccharide production and topography of *S. mutans* forming-biofilms, in both energy densities tested (211.37 and 422.74 J/cm$^2$). The choice for a non-coherent light, instead of a laser

device, was determined by its lower cost and portability, making it a better option for PACT. In addition, it does not emit any considerable heat, which may cause additional tissue damage (Nagata *et al*. 2012). Previous studies have also reported similar reductions ranging from 2-5 logs using light emitting diodes (Zanin *et al.,* 2005; Zanin *et al.,* 2006, Teixeira *et al.,* 2012), but with irradiation times ranging from 5 to 30 minutes what is far away distant from what is clinically acceptable. The great output power provided by LumaCare® device (2.24W) allows higher energies doses in shorter irradiation times, without considerable heating. The proposed therapy using 2 minutes irradiation (422.74 J/cm$^2$), achieved 5-log reduction of bacterial viability, and 2-log reduction with 1 minute irradiation (211.37 J/cm$^2$). It is noteworthy that these reductions were obtained after 1 single dose irradiation in a 5 days old mature biofilm.

The twice/daily PACT treatment reduced 6-6.5 logs in the microbial viability after 1 and 2 minutes irradiation times, respectively. As far as we know, this is the first study that evaluated the PACT (Red Light + TBO) in a daily treatment on *S. mutans* biofilm formation. Chlorhexidine digluconate was the positive control used in this study, and it is considered the gold standard in inhibiting plaque formation due to its antimicrobial effects, substantivity and ability to inhibit bacterial adhesion (Autio-Gold, 2008). Chlorhexidine achieved a 4-logs reduction in a twice/daily treatment, a reduction statistically inferior to that obtained by PACT irradiation time 2 minutes. Also, chlorhexidine did not show any bactericidal effect in mature biofilms when a unique treatment was performed under mature biofilms.

PACT performed in a single dose in mature biofilm showed no effect on the soluble and insoluble polysaccharides dosages. However, PACT held twice/daily reduced significantly EPS formation, with no difference between the irradiation time 1 and 2 minutes. EPS plays a major role in the pathogenesis of dental caries, by promoting bacterial accumulation to the tooth surface and influencing the physical and biochemical properties of biofilms (Paes Leme *et al.*, 2006). The EPS-soluble may be readily digested and used as a reserve source of energy and contribute in part at least to the low pH values observed in cariogenic plaque (Bowen and Koo, 2011; Paes Leme *et al.*, 2006). Also, elevated amounts of EPS-insoluble significantly reduced the inorganic concentration in the matrix, particularly Ca, P and F (Cury *et al*., 2000; Paes Leme *et al*., 2006) increasing the cariogenic potential of dental plaque. In this way, the significant reduction on soluble and insoluble EPS formation promoted by PACT in the twice/daily treatment is extremely relevant and may be in part explained by the fact that PACT is mediated

by singlet oxygen production which may has a direct effect on extracellular molecules leading the polysaccharides present in the extracellular matrix in the biofilm to be also susceptible to photodamage (Konopka and Goslinski, 2007). Such dual activity (antibacterial and antiplaque) is not exhibited by antibiotics and may represent a significant advantage of PACT. To our knowledge, this is the first study that analyzed PACT effects performed with non-coherent light and TBO on polysaccharide matrix of *S. mutans* biofilms.

The effects of PACT performed twice/daily in the biofilm matrix can also be verified by the remarkable differences observed in the VPSEM images. The choice of VPSEM technique, instead of conventional SEM, was due the VPSEM provided a more accurate image of the *S. mutans* biofilm morphology with respect to topography and EPS content. Our images suggest that PACT performed twice/daily might have altered the biofilm organization and promoted disorganization and disaggregation of the microorganisms in the biofilm. The presence of EPS is essential for the initial formation and in maintaining the three-dimensional structure of the microcolony (Xiao and Koo, 2010). Bevilacqua *et al*. (2007) analyzed SEM images of *S. mutans* biofilms submitted to PACT performed with red LED and TBO. Unlike our study, the authors applied the therapy in planktonic suspensions and then these bacteria previously exposed were used in the biofilm formation. They verified no biofilm formation in samples submitted to PACT and only slight differences were observed when just TBO or just LED irradiation were used.

Finally, neither red light alone, nor TBO alone had significant effects in reducing bacterial viability and production of polysaccharides in *S. mutans* biofilm, in accordance with previous studies (Zanin *et al*., 2005; Zanin *et al*. 2006, Schneider *et al*., 2012; Teixeira et a*l.* 2012). Our results suggest that the combined action of red light and photosensitizer TBO is essential to get the desirable effects of photodynamic therapy in *S. mutans* biofilms.

**Conclusion**

The effects in mature biofilms show that PACT performed with non-coherent red light (630 nm) and TBO (100μg/ml) may represent an auxiliary therapy in the inactivation of established biofilms. Thus, under tested conditions, PACT was effective in reducing the formation of *S. mutans* biofilm *in vitro* and it may represents an efficient alternative to conventional antimicrobial therapy using chlorhexidine digluconate. Also, PACT seems also inhibits the matrix-rich biofilm formation and prevent the clustering of *S. mutans*, by decreasing

the number of microcolonies. The energy density of 422.74 J/cm$^2$ seems to be the most suitable to achieve the desired effects of photodynamic therapy in *S. mutans* biofilms. Further studies are needed to assess the PACT effects on multispecies *in situ* and *in vivo* biofilms.

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## 4 DISCUSSÃO GERAL

As pesquisas apresentadas neste trabalho analizaram os efeitos da terapia fotodinâmica antimicrobiana em biofilmes de *S. mutans* formados *in vitro*. O tratamento atual das doenças relacionadas a presença de biofilmes dentais envolve a utilização de agentes antimicrobianos tradicionais em conjunto com a remoção mecânica do biofilme. No entanto, devido ao surgimento de cepas resistentes aos antimicrobianos tradicionais, tem aumentado o interesse da comunidade científica em desenvolver terapias antimicrobianas alternativas que tornem o surgimento de cepas resistentes geneticamente improváveis (HAMBLIN e HASAN, 2004). Neste sentido, a terapia fotodinâmica antimicrobiana surge como uma alternativa ao uso de antimicrobianos convencionais.

No capítulo 1, a ação antimicrobiana de dois diodos emissores de luz (LEDs) (Laserbeam® ou LumaCare®) combinados com o fotossensibilizador azul de orto-toluidina (TBO) em biofilmes de *S. mutans* foi avaliada. A escolha de um LED e uma luz não-coerente, em vez de um dispositivo de laser, foi determinada pelas características físicas das luzes, associadas ao seu baixo custo e portabilidade, tornado-o mais desejável para ser utilizado na terapia fotodinâmica antimicrobiana (ZANIN et al., 2006). O azul de orto-toluidina (TBO), uma fenotiazina catiônica, foi escolhido como fotosensibilizador, devido às suas características de um fotossensibilizador ideal, incluindo suas propriedades químicas, biológicas e foto-físicas, tais como a possibilidade da seleção do local a ser aplicado, a seletividade à microrganismos em baixas concentrações, evitando danos ao tecido hospedeiro, e capacidade de difusão (O'NEILL et al., 2002). O TBO é eficiente tanto contra bactérias Gram-positivo e Gram-negativo (NAGATA et al., 2005) e Usacheva et al. (2001) sugerem que TBO pode ligar-se aos polifosfatos da membrana e produzir danos moleculares para lípidos e proteínas. Além disso, Rolim et al. (2012) demonstraram que TBO, associado a um LED, foi o fotossensibilizador mais eficaz contra culturas planctônicas de *S. mutans*, dentre outros cinco testados nas mesmas condições de concentração e de irradiação.

Os nossos resultados confirmaram a atividade antimicrobiana da terapia realizada com TBO e luz vermelha em biofilmes de *S. mutans*, conforme observado em outros estudos (ZANIN et al., 2005; ZANIN et al., 2006; TEIXEIRA et al., 2012). Nenhuma diferença

significativa foi observada entre os tempos de irradiação 120 segundos e 180 segundos na terapia realizada com a luz não coerente. Por outro lado, estes tratamentos foram superiores a todos os outros grupos experimentais. Além da sua maior potência, outra vantagem do equipamento LumaCare® é a presença de sondas cambiáveis que emitem luz em todo o espectro de luz visível. Dessa forma, diferentes fotossensibilizadores podem ser ativados utilizando o mesmo dispositivo. O aspecto mais relevante deste estudo é que a terapia realizada com LumaCare®, tempo de irradiação 22 segundos, teve o mesmo desempenho de Laserbeam® tempo de irradiação 900 segundos, ambos com a densidade de energia de 56,6 J/cm$^2$. Embora este resultado fosse esperado, uma vez que ambos os tratamentos utilizaram a mesma dose de energia, isto representa um avanço significativo na aplicabilidade clínica da terapia fotodinâmica.

Uma vez que o principal mecanismo de ação da terapia fotodinâmica é baseado na produção de espécies reativas de oxigênio (ERO), e apesar de estudos anteriores terem analisado a produção de ERO (BOUILLAGUET et al. 2010) e oxigênio singleto (ROLIM et al. 2012) por esta terapia em culturas planctônicas, era essencial verificar se estas espécies efetivamente penetravam no interior da célula bacteriana, especialmente quando as bactérias estavam organizadas na forma de biofilmes. No capítulo 2 analisamos a quantidade de EROs presentes em nível intracelular em *S. mutans* inseridos em biofilmes uma vez que os biofilmes de *S. mutans* apresentam uma espessa camada de polissacarídeos extracelulares que poderia dificultar a penetração de ERO (BOWEN e KOO, 2011). A dihidrorodamina 123, já utilizada como marcador da presença de ERO intracelular em células eucariontes (PASSOS et al., 2013) e em suspensões planctônicas (FARRELL et al., 2011 e RISTIC et al., 2014), se mostrou eficiente também em biofilmes de *S. mutans* organizados. A quantidade de ERO intracelular do grupo S+L+ 2min foi quase 3 vezes maior do que a observada para o controle grupo negativo S-L-. Embora esse resultado fosse esperado, vale ressaltar que 2 minutos de irradiação (422,74 J/cm$^2$) aumentou em 69% a produção de ROS comparada ao grupo de irradiação de 1 minuto (211,37 J/cm$^2$). Zanin et al. (2005) avaliaram o efeito antimicrobiano do TBO + LED vermelho (densidades de energia de entre 49 e 294 J/cm$^2$) sobre os biofilmes de *S. mutans* e verificaram que o efeito bactericida é dependente da dose, o que pode ser um reflexo da produção de ERO.

No capítulo 3 analisamos o efeito da terapia fotodinâmica antimicrobiana (TBO + luz vermelha) na inativação de biofilmes maduros de *S. mutans* utilizando uma única aplicação da

PACT ao fim do período experimental; bem os efeitos dessa terapia na prevenção da formação de biofilms com 2 tratamentos ao longo de 5 dias. A terapia proposta mostrou grandes efeitos na viabilidade bacteriana, na produção de polissacarídeos e na topografia dos biofilmes em formação. Com relação a viabilidade bacteriana, observamos uma redução de 2 a 5 logs quando o tratamento pontual dos biofilmes maduros foi realizado. Estudos anteriores verificaram reduções semelhantes (ZANIN et al*.,* 2005; ZANIN et al*.,* 2006, TEIXEIRA et al*.,* 2012) quando tempos de exposição a luz de 5 a 30 minutos foram utilizados. O tratamento diário (2x/dia) com PACT reduziu em 6 - 6,5 logs a viabilidade bacteriana após 1 e 2 minutos de irradiação, respectivamente. Até onde sabemos, este é o primeiro estudo que avaliou os efeitos do tratamento diário, utilizando a combinação TBO e luz vermelha, na formação de biofilmes de *S. mutans*. Ressaltamos que o digluconato de clorexidina 0,12%, considerado o agente antimicrobiano de escolha para inibição da formação de biofilmes cariogênicos (AUTIO-GOLD, 2008) e utilizado como controle positivo desse estudo, reduziu a viabilidade bacteriana em 4 logs quando utilizado diariamente e não apresentou nenhum efeito quando utilizado no tratamento de biofilmes maduros.

A terapia realizada em dose única em biofilmes maduros não mostrou nenhum efeito na quantidade de polissacarídeos extracelulares (PECs) solúveis e insolúveis produzidos. Entretanto, o tratamento diário 2x/dia com a PACT, reduziu drasticamente a sua produção. Os PECs desempenham um papel importante na patogênese de cáries dentária e a dupla-atividade (antibacteriana e anti-placa) da terapia que não é exibida por antibióticos pode representar uma vantagem significativa da utilização clínica da terapia fotodinâmica antimicrobiana. Os efeitos da terapia na matriz de polissacarídeos também podem ser observados nas imagens obtidas através de microscopia electronica de varredura com pressão variada. As imagens mostram apenas um pequeno número de aglomerado de microcolônias, e perda de estruturas de conexão dos PECs, nos biofilmes onde a terapia fotodinâmica foi utilizada diariamente (2x/dia).

Por meio dos resultados obtidos nesses três estudos, torna-se evidente o efeito antimicrobiano da terapia fotodinâmica utilizando a combinação fotossensibilizador TBO e luz vermelha, tantos em biofilmes maduros, como na inibição da formação de biofilmes cariogênicos. Além disso, a obtenção de resultados significativos (com reduções de ate 6,5 logs nas contagens) obtitidos após tempos de exposição a luz tão reduzidos torna as possibilidades de utilização

clínica dessa terapia evidente. No entanto, novas pesquisas são necessárias a fim de analisar os efeitos da terapia proposta em biofilmes multispecies formados *in situ* e *in vivo,* além dos efeitos em células humanas (citotoxicidade).

## 5 CONCLUSÃO GERAL

Com base nos resultados desta tese, pode-se concluir que:

I. Ambas as fontes de luz testadas (Laserbeam® e LumaCare®) reduziram significantemente a viabilidade bacteriana de biofilmes de *S. mutans* quando combinadas ao fotossensibilizador TBO (100µg/ml). Ambas as fontes luminosas têm desempenho igual na redução da viabilidade bacteriana, quando utilizadas na mesma densidade de energia. No entando, a luz não coerente testada (1.68W) mostrou ser uma melhor alternativa para a realização da terapia fotodinâmica antimicrobiana, devido o seu reduzido tempo de irradiação (de 900 segundos para 22 segundos), além de mostrar superior efeito antimicrobiano quando os tempos de irradiação 120 e 180 segundos foram utilizados.

II. A terapia fotodinâmica antimicrobiana nos parâmetros testados mostrou ser capaz de gerar altos níveis de stress oxidativo intracelular, mesmo em *S. mutans* inseridos em biofilmes.

III. A terapia fotodinâmica antimicrobiana realizada com TBO (100µg/ml) e luz vermelha não coerente (2.24W) é efetiva na inibição da formação de biofilmes de *S. mutans* e pode representar uma alternativa à terapia antimicrobiana convencional que utiliza o digluconato de clorexidina. Os efeitos em biofilme maduro mostram que esta terapia pode representar uma terapia auxiliar na inativação de biofilmes maduros já estabelecidos.

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