Universidade Estadual Paulista
Júlio de Mesquita Filho"
Faculdade de Odontologia de Araraquara
Programa de Pós-Graduação em Odontologia

Gabriel Garcia de Carvalho

Terapia Fotodinâmica Antimicrobiana com Utilização da Clorina-e6 sobre Biofilme Periodontopatogênico

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Orientadora: Profa. Dra. Daniela Leal Zandim-Barcelos

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Dissertação para obtenção do grau de Mestre em Odontologia

Comissão Examinadora

Profa. Dra. Daniela Leal Zandim-Barcelos (Orientadora)
Departamento de Diagnóstico e Cirurgia / Faculdade de Odontologia de Araraquara - UNESP

Profa. Dra. Carla Raquel Fontana Mendonça
Departamento de Análises Clínicas / Faculdade de Ciências Farmacêuticas de Araraquara - UNESP

Prof. Dra. Juliana Rico Pires
Centro Universitária da Fundação Educacional de Barretos

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DADOS CURRICULARES

Gabriel Garcia de Carvalho

NASCIMENTO	15 de Abril de 1992 – João Pessoa – PB – Brasil
FILIAÇÃO	Adriana Coeli Garcia de Carvalho Arlan Giovanni Duarte Carvalho
2010 – 2017	Graduação em Odontologia Universidade Federal da Paraíba
2017	Atualização em Cirurgia Oral Associação Brasileira de Odontologia – Secção Paraíba
2018 – Atual	Curso de Pós-Graduação em Odontologia, Área de Concentração em Periodontia – Nível Mestrado Faculdade de Odontologia de Araraquara

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Meu Nordeste

Nasci na terra seca, dura e sofrida
Onde não se chora a dor deixada pela ferida
Vi o sol queimar o verde e a flor
Deixando o cinza, como principal cor

Pedi a Deus todos os dias
Pra água cair do céu e me dar alegria
Sofri com a fome e a dor
Mas sigo, singelo, com fé e amor

A cicatriz na pele é fruto do trabalho
De onde tirei o sustento
Dia e noite, sem nenhum atrapalho

Minha terra amada, de beleza pura e clara
Cheia de glória e lamento
Lugar do meu nascimento
Minha joia rara*

* Diogo Carneiro, Meu Nordeste. Riacho dos Cavalos – PB, Dia do Nordestino, 08 de outubro de 2019.

Carvalho GG. Terapia fotodinâmica antimicrobiana com utilização da clorina-e6 sobre biofilme periodontopatogênico [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2020.

RESUMO

A resistência bacteriana é uma ameaça real alertada pela OMS. A terapia fotodinâmica antimicrobiana (TFDA) pode ser uma das soluções para superar este desafio, uma vez que já demonstra ação antimicrobiana frente a inúmeros patógenos. A clorina-e6 (Ce6) provou ser um fotossensibilizador (FS) com potente efeito quando irradiada pela luz vermelha, contra diferentes biofilmes. No entanto, o principal pico de absorção deste FS está no espectro visível de luz azul, ainda insuficientemente investigado. Este estudo teve como objetivo avaliar o efeito da TFDA com utilização da Ce6 irradiada a 450 e 660 nm contra biofilmes relacionados à doença periodontal. Biofilmes monoespécie de *Streptococcus oralis, Fusobacterium nucleatum, Porphyromonas gingivalis e Aggregatibacter actinomycetemcomitans* foram desenvolvidos em condições adequadas por cinco dias. A TFDA foi realizada em diferentes concentrações de fotossensibilizador (100 e 200 μM) e comprimentos de onda de luz (450 e 660 nm), e comparada ao controle negativo (DMSO a 1%) e positivo (clorexidina a 0,2%) por análise de unidades formadoras de colônias (UFC) e microscopia confocal. O uso de luz e FS também foram testados individualmente. A maior redução bacteriana foi observada no grupo em que a TFDA foi realizada com utilização da Ce6 a 200 μM e aplicação de luz azul para todas as cepas (redução de 4,01 $\log_{10}$ para *A. actinomycetemcomitans* e redução total para *P. gingivalis* e *S. oralis*), exceto para *F. nucleatum*, onde o melhor resultado foi obtido com a TFDA utilizando irradiação por luz vermelha (redução de 3,36 $\log_{10}$) ($p < 0,05$). O efeito antimicrobiano da TFDA usando Ce6 contra os biofilmes monoespécie testado foi confirmado por meio de microscopia confocal. FS na ausência de luz reduziu significativamente o número de células viáveis quando comparado ao grupo não tratado, em todos os biofilmes testados, exceto para *F. nucleatum*. A correlação entre redução bacteriana e concentração do FS foi observada apenas em biofilmes de algumas cepas testadas. O uso de TFDA mediado por Ce6 promoveu redução significativa de células bacterianas viáveis, com redução mais evidente nos grupos tratados com luz azul para *S. oralis, P. gingivalis e A. actinomycetemcomitans*.

Palavras-chave: Fotoquimioterapia. Fármacos fotossensibilizadores. Periodontite.

Carvalho GG. Chlorin-e6-mediated antimicrobial photodynamic therapy against periodontitis-related pathogens [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2020.

ABSTRACT

Antimicrobial photodynamic therapy (aPDT) has been used in the treatment of many infection diseases as main or adjuvant therapy. Chlorin-e6 (Ce6), as a photosensitizer (PS), has demonstrated significant reduction of microorganisms' viability when irradiated by red light. However, the main absorption peak of this PS is located at blue light visible spectrum, which has been poorly investigated. This study aimed at evaluating the effect of pure-chlorin-e6-mediated aPDT and different light sources (450 or 660 nm) against biofilms related to periodontitis. *Streptococcus oralis, Fusobacterium nucleatum, Porphyromonas gingivalis,* and *Aggregatibacter actinomycetemcomitans* single-species biofilms were developed under proper conditions for five days. aPDT was performed using different concentrations of Ce6 (100 and 200 μM), wavelengths (450 and 660 nm) and compared to negative and positive (0.2% Chlorhexidine) control after colony forming unit and confocal laser scanning microscopy (CLSM) analysis. The use of light and PS were also individually tested. The greatest bacterial elimination was observed in the group where aPDT was employed with blue light and concentration of 200 μM for all bacterial strains tested (reduction of 4.01 $\log_{10}$ for *A. actinomycetemcomitans*, and total elimination for *P. gingivalis* and *S. oralis*), except for *F. nucleatum*, where the lowest mean value was observed in the group that received aPDT with red light and 200 μM Ce6 (3.36 $\log_{10}$ reduction) ($p<0.05$). The antimicrobial effects of aPDT mediated by Ce6 for all single pathogenic biofilms were confirmed by live/dead staining under CLSM analysis. For all single-species biofilms, the use of aPDT mediated by chlorin-e6 photosensitizer under blue or red-light irradiation (450 and 660 nm) promoted a significant reduction in bacterial viability, especially in the groups treated with blue light for *S. oralis, P. gingivalis,* and *A. actinomycetemcomitans*.

Keywords: Photodynamic Therapy. Photochemotherapy. Photosensitizing Agents. Periodontitis.

SUMÁRIO

1 INTRODUÇÃO

A doença periodontal afeta grande parte da população mundial, encontrando-se no grupo das patologias bucais mais recorrentes, sendo uma das principais causas da perda dentária. Essa doença tem sua origem relacionada a fatores microbiológicos, e de maneira geral são divididas em gengivite e periodontite, dependendo do tecido comprometido pela inflamação. Trata-se de uma doença infecciosa dependente da interação de bactérias com seu hospedeiro, estas apresentam fatores de virulência que modificam a resposta imunoinflamatória do hospedeiro, especialmente quando organizadas em forma de biofilme e localizadas no sulco gengival[1,2].

A remoção e alteração da microbiota patogênica do biofilme é imprescindível para a prevenção e controle da doença periodontal, sendo a Raspagem e o Alisamento Radicular (RAR) a terapia não-cirúrgica de escolha para tratamento da doença e manutenção da homeostasia periodontal. Apesar dos resultados clínicos positivos obtidos com RAR[3-6], esta terapia pode apresentar limitações e insucessos quando abordada isoladamente em determinados quadros clínicos. Assim, terapias alternativas têm sido investigadas em virtude do limitado acesso à superfície radicular durante RAR[7-9], da presença de periodontopatógenos nas bolsas mais profundas[10] e da habilidade de algumas bactérias em invadir tecido adjacente[11]. Quando consideramos o tratamento de doença periodontal instalada, o uso de antibióticos locais ou sistêmicos é apresentado como alternativa, mas seu uso como coadjuvante pode induzir efeitos sistêmicos indesejáveis e formação de espécies bacterianas resistentes à terapia medicamentosa[12-14]. Além disso, a eficácia da terapia com uso de antibióticos sistêmicos depende da colaboração do paciente em seguir corretamente o protocolo medicamentoso indicado.

Compreendendo o crescimento da resistência das cepas bacterianas pelo uso indiscriminado de antibióticos e o interesse de pesquisadores em buscar estratégias terapêuticas que apresentem pouco ou nenhum efeito colateral, nota-se o crescimento exponencial da utilização dos lasers e de outras fontes de luz no tratamento de diversas patologias, bem como as periodontais[15,16]. Neste contexto, a terapia fotodinâmica antimicrobiana (TFDA) tem se destacado como um procedimento adjunto no combate de infecções microbianas. Diversos estudos comprovam a eficácia da TFDA, tanto in vitro como in vivo, em animais e humanos, no tratamento das doenças periodontais[17-28].

A TFDA consiste na excitação do fotossensibilizador (FS, em seu estado fundamental) por uma luz no espectro visível (laser de diodo semicondutores de baixa potência), com a energia absorvida, esta molécula pode sofrer transição ao estado singleto ou tripleto excitado, que poderá

desencadear três reações. Na reação de tipo I, ocorre a transferência de elétron entre o FS e oxigênio molecular, permitindo o seu retorno ao estado fundamental e a formação de espécies reativas de oxigênio. Na reação tipo II, o FS transfere sua energia de excitação para o oxigênio molecular no estado fundamental, resultando na produção do oxigênio singleto, um poderoso agente oxidante altamente tóxico para as células[29]. Acredita-se que os processos do Tipo II preservem melhor a estrutura molecular do FS em um estado fotoativável e, em algumas circunstâncias, uma única molécula de FS pode gerar 10 000 moléculas de oxigênio singleto. Uma terceira via (Tipo III), pouco comum, ocorre na ausência de oxigênio, quando a molécula do FS interage diretamente com uma biomolécula, provocando um dano celular (efeito citotóxico) e consequente destruição do FS[29-31]. A TFDA não induz a inviabilidade celular de áreas distantes à área tratada, pois seus produtos citotóxicos (ex. oxigênio singleto e hidroxilas) não migram mais que 0,02 mm após sua formação[32].

Dentre as vantagens do emprego desta nova terapia, encontram-se: a diminuição da potência e intensidade da fonte de luz utilizada, viabilizando financeiramente o seu uso e reduzindo o risco de danos aos tecidos e microbiota adjacentes; a morte bacteriana rápida e localizada, evitando a manutenção de altas concentrações de fármacos por longo período (antibióticos e antissépticos); o fato do efeito bactericida não estar ligado à mediação de radicais químicos, reduzindo o risco à resistência bacteriana; o acesso facilitado a regiões de difícil acesso pela terapia convencional (RAR), como bolsas profundas e bi- ou trifurcação de raízes[33].

A TFDA tem sido indicada como terapia coadjuvante no tratamento da periodontite, retratamento de bolsas residuais, descontaminação para terapia cirúrgica, terapia de manutenção, dentre outros[34-36]. Sua indicação na terapia de manutenção tem como vantagem o menor desgaste da superfície radicular, que seria um aspecto negativo da utilização da técnica convencional (RAR). Neste caso a PDT seria utilizada isoladamente, reduzindo a incidência de hipersensibilidade causada pela remoção de cemento nas múltiplas sessões de RAR[37].

Um fotossensibilizador ideal deve ser atóxico, demonstrar toxicidade local apenas após excitação por luz e ter alto rendimento quântico para produção de espécies reativas de oxigênio (ROS)[12,38]. Diversos fotossensibilizadores estão descritos na literatura, sendo as fenotiazinas azul de metileno e azul de toluidina os mais referidos[21,39-44], porém alguns estudos não têm demonstrado benefícios clínicos adicionais no tratamento periodontal[21,40,45,46]. Desta forma, novos fotossensibilizadores estão surgindo na estratégia de superar as deficiências destes citados

anteriormente.

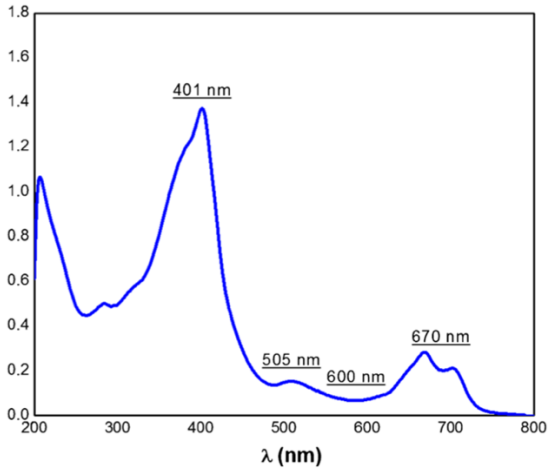
A clorina-e6 (Ce6) surgiu em meados dos anos 90 como um FS de segunda geração bastante promissor inicialmente em terapias oncológicas[47]. A Ce6 e seus derivados estão sendo utilizados em diversos tratamentos com utilização de luz, como cânceres[48,49], leucoplasias[50], infecções endodônticas[51], e contra cepas de *Streptococcus mutans*[52]. Algumas formulações a base de clorina-e6 estão disponíveis no mercado para tratamento antibacteriano, antienvelhecimento e antineoplásico, como Revixan®, Photolon® e Photodithazine®. Este FS também demonstrou atividade antimicrobiana, in vitro, quando utilizado sobre bactérias presentes na doença periodontal na forma planctônica, apresentando redução das unidades formadoras de colônia[53-55]. Os estudos citados não testaram a utilização desse FS contra microrganismos organizados na forma de biofilme. Os biofilmes são estruturas biológicas constituídas por microrganismos envoltos por uma matriz extracelular de polissacarídeos, que assegura a sobrevivência dos mesmos[56,57], agindo como uma barreira protetora a agentes físicos e químicos externos, o que pode limitar a penetração de agentes antimicrobianos[58]. Particularmente na cavidade oral, o biofilme é responsável pelo desenvolvimento de diversas patologias, incluindo a doença periodontal. Também não foram encontrados na literatura estudos clínicos avaliando a eficácia do uso deste FS diretamente no tratamento da doença periodontal.

Fotossensibilizadores a base de clorina-e6 apresentam dois picos de absorção de luz, na luz vermelha e azul (Gráfico 1). Tradicionalmente, a luz vermelha é preferida por se acreditar em sua maior capacidade de penetração nos tecidos. Porém, comprimentos de onda em torno de 412 nm (azul) apresentaram 30-33% maior coeficiente de absorção de energia e efeito bactericida que comprimentos de onda equivalentes a luz visível vermelha[59]. Estes resultados demonstram que fotossensibilizadores excitados em comprimentos de onda na luz visível azul podem ser preferidos em terapias que visem tratar superfícies, como é o caso dos protocolos antimicrobianos sobre biofilmes.[60] Um estudo recente avaliou a ativação deste FS nos dois comprimentos de onda, 405 nm (luz azul) e 660 nm (luz vermelha). Para a pele, os valores de saturação foram de aproximadamente 0,2 J/mm^2 para ambos os comprimentos de onda, indicando que cerca de 40% do incidente da energia é absorvida dentro do tecido. No entanto, para a mucosa, o valor de saturação para comprimento de onda de 405 nm foi de 0,28 J/mm^2 (56% da intensidade do incidente é absorvida); e para o de 660 nm, a energia absorvida foi muito menor, aproximando-se de 0,03 J/mm^2 (6% da intensidade do incidente) para a espessura da camada de 4 mm. Por este motivo a

luz azul tem atraído a atenção de pesquisadores e indústrias que sugerem a utilização da luz azul em tratamentos dermatológicos. Em um estudo de optimização de solventes utilizados na diluição de fotossensibilizadores, foi possível observar maior espectro de absorção quando a Ce6 foi diluída em solventes polares e excitada sobre comprimentos de onda de luz azul[61].

É importante levantar que a luz azul está comumente presente na prática odontológica, onde é utilizada na polimerização de materiais restauradores ou fotoativação de adesivos e clareadores, portanto, estes equipamentos já disponíveis no consultório podem ser empregados na terapia fotodinâmica quando fotossensibilizadores ativados na luz azul são escolhidos, reduzindo eventuais custos relativos à equipamentos necessários para realização da TFDA.

Gráfico 1- Espectro de absorção da clorina-e6



Fonte: Elaboração própria.

De maneira geral, poucos estudos avaliaram o efeito antimicrobiano da TFDA com utilização da Ce6 como fotossensibilizador contra patógenos orais[53-58,62]. Um estudo desenvolvido recentemente avaliou a efetividade de um fotossensibilizador a base de clorina-e6 (Photodithazine®) contra biofilme monoespécie de microrganismos periodontopatogênicos, onde a TFDA com uso da clorina-e6 apresentou redução significante da viabilidade microbiana (1,12 e 2,66 $\log_{10}$)[63]. O Photodithazine® é um fotossensibilizador composto por Ce6 (~60%) e outros derivados do extrato bruto da clorofila. No presente estudo, foi testado o extrato puro da Ce6 (100%), composto ainda não patenteado, desenvolvido pelo Laboratório de Química Bio-orgânica, UFSCar – São Carlos. Não há registro de estudos que tenham avaliado a excitação de fotossensibilizadores a base de Ce6 em comprimentos de onda na luz azul contra microrganismos.

Além disto, até o presente momento, não existem relatos na literatura do emprego da TFDA com uso da clorina-e6 como terapia adjunta no tratamento da doença periodontal. Assim, desenvolvemos o presente estudo com a seguinte hipótese principal: a terapia fotodinâmica com utilização da Ce6 apresenta efeito antimicrobiano sobre biofilme de periodontopatógenos.

2 PROPOSIÇÃO

O objetivo deste estudo foi testar o efeito antimicrobiano da terapia fotodinâmica com utilização da Ce6 irradiada por luz azul ou vermelha sobre biofilme de periodontopatógenos, logo consideramos a seguinte hipótese nula: A terapia fotodinâmica antimicrobiano com utilização da clorina-e6 irradiada por luz azul ou vermelha não elimina biofilme periodontopatogênico.

3 PUBLICAÇÃO*

O presente estudo representa os primeiros resultados de uma série de experimentos desenvolvidos para testar a terapia fotodinâmica com utilização da clorina-e6 como possível terapia adjunta no tratamento da doença periodontal. Neste primeiro artigo, buscamos testar o efeito antimicrobiano da terapia citada sobre biofilme monoespécie de espécies com papel importante e reconhecido no desenvolvimento da doença periodontal.

* Artigo formatado segundo as normas do periódico Photodiagnosis and Photodynamic Therapy para o qual foi submetido.

RESEARCH ARTICLE

Photodynamic Inactivation using a Chlorin-Based Photosensitizer with Blue or Red-Light Irradiation against Single-Species Biofilms Related to Periodontitis

Running title: Chlorin-mediated photodynamic inactivation against periodontal pathogens

Gabriel **Garcia de Carvalho**[a]

Julio Cesar **Sanchez-Puetate**[a]

Maria Carolina **Donatoni**[b]

Patricia **Milagros Maquera Huacho**[c]

Alessandra Nara **de Souza Rastelli**[d]

Kleber Thiago **de Oliveira**[b]

Denise Madalena **Palomari Spolidorio**[c]

Daniela **Leal Zandim-Barcelos***[a]

Email address: garcia.carvalho@unesp.br, julio.sanchez@unesp.br, carolinadonatoni@yahoo.com.br, pattymi_6@hotmail.com, alessandra.nara-souza-rastelli@unesp.br, kleber.oliveira@ufscar.br, dmps@foar.unesp.br, daniela.zandim-barcelos@unesp.br.

[a]*Department of Diagnosis and Surgery, São Paulo State University (Unesp), School of Dentistry, Rua Humaitá, 1680, 14801-903, Araraquara, SP, Brazil.*

[b]*Department of Chemistry, Federal University of São Carlos (UFSCar), São Carlos, SP, 13565-905, Brazil*

[c]*Department of Physiology and Pathology, São Paulo State University (Unesp), School of Dentistry, Rua Humaitá, 1680, 14801-903, Araraquara, SP, Brazil.*

[d]*Department of Restorative Dentistry, São Paulo State University (Unesp), School of Dentistry, Rua Humaitá, 1680, 14801-903, Araraquara, SP, Brazil.*

***Corresponding author:**

Daniela Leal Zandim-Barcelos
Department of Diagnostic and Surgery, Araraquara School of Dentistry, São Paulo State University, Humaitá, 680, 14801-385, Araraquara, São Paulo, Brazil.
Tel: +55-16- 3301-6508
E-mail: daniela.zandim-barcelos@unesp.br

Author role
All authors contributed to the manuscript.

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Abstract

Antimicrobial photodynamic inactivation (PDI) has been used in the treatment of many infection diseases as main or adjuvant therapy. Chlorin-e6 (Ce6), as a photosensitizer (PS), has demonstrated significant reduction of microorganisms' viability when irradiated by red light. However, the main absorption peak of this PS is located at blue light visible spectrum, which has been poorly investigated. This study aimed to evaluate the effect of pure-chlorin-e6-mediated PDI using different light sources (450 or 660 nm) against biofilms related to periodontitis. *Streptococcus oralis, Fusobacterium nucleatum, Porphyromonas gingivalis,* and *Aggregatibacter actinomycetemcomitans* single-species biofilms were developed under proper conditions for five days. PDI was performed using different concentrations of Ce6 (100 and 200 μM), wavelengths (450 and 660 nm) and compared to negative and positive (0.2% Chlorhexidine) control after colony forming unit and confocal laser scanning microscopy (CLSM) analysis. The use of light and PS were also individually tested. The greatest bacterial elimination was observed in the group where PDI was employed with blue light and concentration of 200 μM for all bacterial strains tested (reduction of 4.01 $\log_{10}$ for *A. actinomycetemcomitans*, and total elimination for *P. gingivalis* and *S. oralis*), except for *F. nucleatum*, where the lowest mean value was observed in the group that received PDI with red light and 200 μM Ce6 (3.46 $\log_{10}$ reduction) ($p<0.05$). A correlation between bacterial elimination and PS concentration was not observed. The antimicrobial effects of PDI mediated by Ce6 for all single pathogenic biofilms were confirmed by live/dead staining under CLSM analysis. For all single-species biofilms, the use of PDI mediated by chlorin-e6 photosensitizer under blue or red-light irradiation (450 and 660 nm) demonstrated a significant reduction in bacterial viability, especially in the groups treated with blue light for *S. oralis, P. gingivalis,* and *A. actinomycetemcomitans*.

Key Words: Photodynamic Therapy, Photochemotherapy, Photosensitizing Agents, Periodontitis.

1 – Introduction

Periodontitis along with dental caries are the most common oral infection disease and, both can lead to tooth loss. The prevalence of periodontitis is reported to range from 31% to 76%[1], and its severe form affects 11% of the global population[2]. Oral biofilms, composed of pathogenic bacterial species and their virulence factors, are well-stablished as the key etiological agent of periodontal diseases[3]. Microorganisms such as *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*, are often found in periodontal sites with clinical signs of disease[4]. These bacteria are thought to trigger an exacerbated inflammatory response and consequent progression of disease severity[5].

The excessive, neglected and uncontrolled use of antibiotics led the scientific community to an endless exploration of new therapeutic approaches, since bacterial resistance development to conventional antimicrobial agents is just a matter of time. Trivial infections will soon become untreatable for current available drugs[6]. The 2014 World Health Organization (WHO) report warns for the post antibiotic era followed by an increased and urgent demand of coordinated action against microbial infections[7]. Antimicrobial photodynamic inactivation (PDI) emerges as a promising alternative against this humanity threat[8].

The first PDI report dates back to the very beginning of 20th century, latterly this therapy was suppressed by the antibiotic era[9]. Under light excitation, a photosensitizer (PS) increases its energy, and from this point the PDI's mechanism can head towards two major pathways: (I) the energy of the PS induces the formation of radicals in the presence of electron-rich substrates; (II) the excited PS transfers its energy directly to the molecular oxygen that surrounds it, forming singlet oxygen ($^1O_2$)[10, 11]. PDI has proved to be effective for cancer and different infection diseases[12]. Compared to conventional antimicrobial therapies, PDI shows some advantages: effect on multiple cellular targets, immediate onset of action, local delivery, minimal invasiveness, and weak systemic repercussion (ensure organ preservation), limited diffusion distance, and quick washout[13-16]. In terms of periodontitis, PDI has proved to be safe and effective as an adjunct to conventional periodontal disease treatment in various studies[17-19].

The high complex multispecies subgingival biofilm, which might be up to 1000 times more difficult to arrest[20], the gingival crevicular fluid drainage and saliva fluid turnover[21], and the difficult access to affected sites are the main challenges for chemical control of the subgingival biofilm. Therefore, mechanical removal remains the essential treatment for periodontitis while chemical/photochemical alternatives need improvements. PDI advancements have been studied mainly through the addition of nanoparticle vehicle[14, 21-23], enhancement with efflux pump inhibitor[24], synergistic effect with antimicrobial peptides[25], and light

regimen optimization[26]. This last is one of the targets in the present study.

Chlorin-e6 (Ce6) emerged in the mid-1990s as a very promising second-generation PS initially tested in cancer therapies[27]. Ce6 and its derivatives have been tested in several light-based treatments for oral bacterial infections related to caries lesion[28], endodontic contamination[29], and periodontitis[30,31], proving their efficacy against biofilms of gram positive and negative bacteria. Chlorin-e6 act predominantly by type II mechanism with singlet oxygen quantum yield between 0.5 and 0.8, while traditional PSs, such as methylene blue shows coefficients below 0.5[32]. Among other PSs, Ce6 is a promising PS agent with high purity and sensitizing efficacy, low toxicity, enough solubility, and rapid elimination from the body[32,33].

Two pronounced absorption peaks, at 401 and 670 nm, in the blue and red regions of the visible range, can be observed for Ce6. Traditionally, red light is employed because of its greater ability to penetrate tissues. However, wavelengths around 412 nm showed 30-33% higher energy absorption coefficient and greater bacterial killing effect than wavelengths equivalent to the red light[22]. Later, Shakhova and collaborators[26], showed that saturation value of absorption for 405 nm wavelength in the mucosa is 0.28 J/mm^2 (56% of incident intensity is absorbed); and at 660 nm, the absorbed energy is much lower, approaching 0.03 J/mm^2 (6% of incident intensity) through 4 mm layer thickness. Evidences regarding biomodulation and toxicity at different wavelengths might support modifications in light regimen when PDI is employed.

From the best of our knowledge, there are only two studies evaluating PDI reaction or antimicrobial effect depending on the wavelength employed[26,34]. There are no studies evaluating the use of chlorin-mediated PDI for periodontitis treatment *in vivo*, and only few studies investigating the antimicrobial effect of Ce6 against periodontal pathogens *in vitro*[21,23,25], none of them using blue light activation. Therefore, this study aimed at testing the following main hypothesis: “photodynamic inactivation using pure-chlorin-e6 irradiated by blue and red light has antimicrobial effect on periodontal-related biofilm”.

2 – Material and Methods

2.1 Photosensitizer and Light Source

Chlorin-e6, a chlorophyll derivative, was semi-synthesized as described by Uliana and co-workers at the Bioorganic Chemistry Laboratory – UFSCar – São Carlos-SP[36]. Stock solution was prepared by dissolving 0.01193 g of Ce6 (dye) in 1 mL DMSO (Dimethyl sulfoxide, Sigma-Aldrich, St. Louis, MO, USA) to reach 20 mM concentration, mixed (VX-200 Vortex Mixer, Edison, NJ, USA), and stored in the dark at -20°C until use. Working solution was prepared 30 min before each experiment by dissolving stock

solution in distilled water to 100 μM (59.7 mg/L) and 200 μM (119.34 mg/L) concentration and stored in the dark at room temperature until use.

Blue and red light-emitting diode (LED) irradiation was used at a fluence of 30 J/cm^2 (22 mW/cm^2 under 450 nm excitation wavelength for 22 min and 40 s, and 19 mW/cm^2 under 660 nm for 26 min and 20 s), irradiation was performed from underneath. Both light source (Biotable, MMOptics, São Carlos, SP, Brazil) consisted of 24 LED as a continuous and homogeneous illumination system under water cooling. PS concentrations and light dose were determined based on a pilot study previously developed by our research group.

2.2 Human Saliva Preparation

Before human saliva collection, the protocol was approved by the Research Ethics Committee of the School of Dentistry at Araraquara, São Paulo State University (CAAE 23396619.5.0000.5416). Unstimulated saliva was obtained from a 27-years-old healthy male with no active caries or periodontal disease, no orthodontic appliance, no systemic disease, no teeth brushing or food/beverage intake in the 12 h prior to donation, and no systemic antimicrobial therapy for at least six months before collection. Saliva was processed by centrifugation at 10,000 g for 15 min at 4°C (Centrifuge 5430R, Eppendorf, Hamburg, Germany), and then filtered with a Sterile Syringe Membrane Filter (0.22 μm pore size, Kasvi, São Jose do Pinhais, PR, Brazil), and stored at -80ºC until use (Thermo Scientific 88400D, Massachusetts, EUA) (35).

2.3 Bacterial Strain and growth conditions

The bacterial strains used in this study were *Streptococcus oralis* (American Type Culture Collection - ATCC 35037), *Fusobacterium nucleatum* (ATCC 25586), *Porphyromonas gingivalis* (ATCC 33277) and *Aggregatibacter actinomycetemcomitans* (JP2, kindly provided by Prof. Mario Júlio Ávila-Campos from University of Sao Paulo – USP, São Paulo, Brazil). All bacterial strains were cultured in enriched brucella agar Petri dishes (Brucella Agar, Neogen, Idaiatuba, SP, Brazil; Petri dish 90 x 10 cm, Kasvi, São Jose do Pinhais, PR, Brazil) with 5% defibrinated sheep blood (Microlab, Campinas, SP, Brazil) at 37°C under proper atmosphere condition for each bacterial strain; anaerobic condition for *F. nucleatum* and *P. gingivalis* (85% N_2, 10% H_2 e 5% CO_2, White Martins, Rio de Janeiro, RJ, Brazil) maintained in anaerobic workstation (VA 500, Don Whitley Scientific, England); and incubation in a 5% CO_2 environment (Incubator, GE Healthcare Life Sciences, Chicago, IL, USA) for *S. oralis and A. actinomycetemcomitans*.

After 48 h, bacterial colonies were transferred to 10 mL Brain Heart Infusion (BHI – Difco Laboratories Inc, MI, USA) broth medium supplemented with yeast extract (6 g/L), hemin (10 mg/mL) and

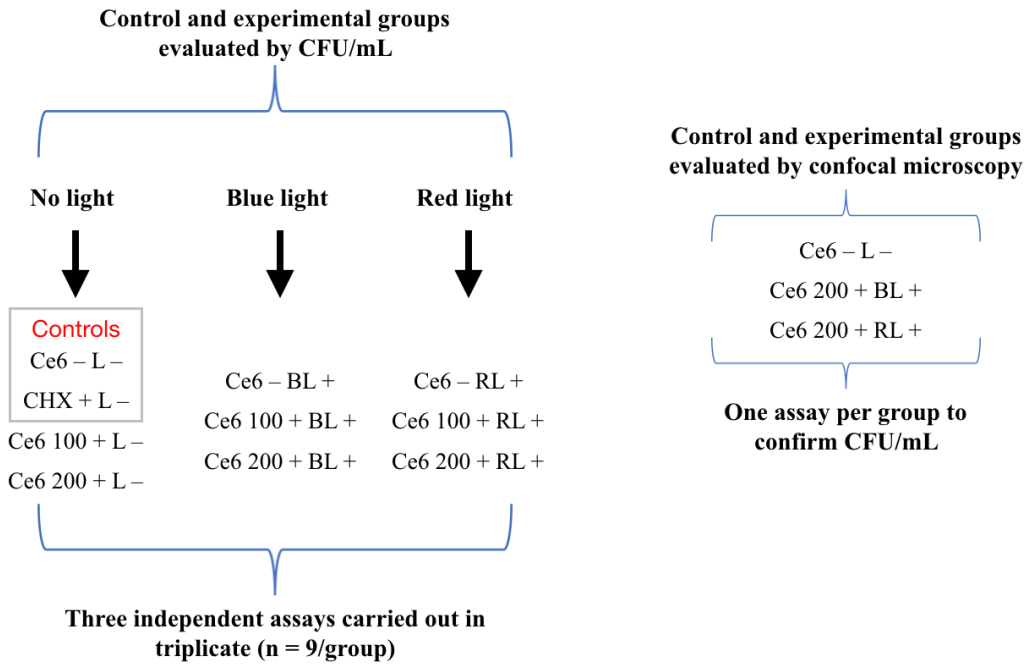
menadione (5 mg/mL), followed by incubation in the conditions previously described for 24 h. Finally, bacterial cells were adjusted in fresh medium according to the mid-exponential growth phase established previously, by optical density and colony-forming units (CFU)/mL (*P. gingivalis,* 15 h, OD600 nm = 0.95 ± 0.01, CFU/mL = 4.3 x 10^9; *F. nucleatum,* 10 h, OD600 nm = 0.45 ± 0.01, CFU/mL = 1 x 10^8*, S. oralis,* 4 h, OD600 nm = 0.55 ± 0.01, CFU/mL = 1.5 x 10^9*, A. actinomycetemcomitans,* 8 h, OD600 nm = 0.18 ± 0.01, CFU/mL = 5.66 x 10^8) until reaching 10^7 CFU/mL using a spectrophotometer (BioSpectrometer® D30, Eppendorf, Hamburg, Germany) under 600 nm[37].

2.4 Biofilm Formation

All bacterial strains were grown as a single-species biofilm model as previously described[35]. Salivary pellicle formation was carried out at the bottom of each well of a 96-well plate (Kasvi, São Jose do Pinhais, PR, Brazil) for *P. gingivalis* biofilm, in order to promote an irreversible initial attachment. Fifty microliter of sterilized saliva (protocol described earlier) was add to each well and the plate was kept in an orbital shaker (430 RD incubator, Nova Etica, Brazil), 75 rpm at 37°C for 4 h[38]. In sequence, the saliva was removed, and the wells were gently washed twice with 200 μL of sterile phosphate-buffered saline (PBS). A 200 μL aliquot of the standardized suspension (10^7 CFU/mL) of each bacterial species was dispensed into each well (only *P. gingivalis* plates were pretreated with a conditioned saliva-derived film), plates were incubated under proper condition. After 24h of the adhesion phase, nonadherent cells were removed by adding and aspirating 200 μL of PBS, and 200 μL of fresh medium was added to continue biofilm formation. Culture medium was replaced daily, for 5 days, very slowly, to avoid biofilm disruption. For all experimental groups, the wells were washed twice at the end of incubation period and before further analyses.

2.5 Control and experimental groups

Ten groups were divided in three independent plates (no light, blue light and red light) for analysis of viable bacteria, as follows: 1) Negative control, treated with 1% DMSO (Ce6 – L –); 2) Positive control, treated with 0.2% Chlorhexidine for 5 min (CHX + L –); 3) Ce6 100 μM in the dark (Ce6 100 + L –); 4) Ce6 200 μM in the dark (Ce6 200 + L –); 5) treated with blue light without Ce6 (Ce6 – BL +); 6) Ce6 100 μM with blue light (Ce6 100 + BL +); 7) Ce6 200 μM with blue light (Ce6 200 + BL +); 8) treated with red light without Ce6 (Ce6 – RL +); 9) Ce6 100 μM with red light (Ce6 100 + RL +); 10) Ce6 200 μM with red light (Ce6 200 + RL +). Two experimental groups that indicated the greatest reduction in the analysis of viable bacteria were selected to be analyzed at confocal microscopy, and also the negative control group. The groups and methods investigated in this study are represented in **Scheme 1**.



Scheme 1. A schematic design of the experimental and control groups conducted in this investigation. Ce6: chlorin-e6; CHX: chlorhexidine (0.2%); L: light; BL: blue light; RL: red light; 100 and 200: concentrations of Ce6 in millimolar (mM). Positive and negative symbols indicate the presence and absence of substance or light, respectively.

2.6 Photodynamic Inactivation

In summary, after the steps described in biofilm formation section, 200 µL of treatment solution (1% DMSO, CHX and Ce6 under both concentrations) were added according to experimental groups (section 2.5). All groups with Ce6 were incubated in the dark at room temperature for 5 min, as a preirradiation time. Subsequently, the groups P – BL +, Ce6 100 + BL +, Ce6 200 + BL + were irradiated by blue LED, just as groups P – RL +, Ce6 100 + RL +, Ce6 200 + RL + were irradiated by red LED, for the time described in the section 2.1. The plate with no light regimen were maintained in the dark for the same time as red-light groups (26 min and 20 s), to analyze any possible effect of PS against biofilm without light.

2.7 Viable bacteria by colony forming unit (CFU) analysis

Following treatment protocols, 200 µL of PBS were added to all of the wells. Biofilms were collected from the polystyrene surface by mechanical scraping with a sterile pipette tip and re-suspended several times to disperse cells. Serial dilution was carried out and 25 µL of each dilution sample was plated onto petri dish with the culture medium previously described. The petri dishes were incubated for 1 to 3 days depending on the bacterial species. After incubation, images of all samples were taken (6D Mark II, Canon, Tokyo, Japan), colony counts were performed using a software of image analysis (ImageJ, NIH, Bethesda, Maryland, USA) and expressed as CFU/mL[35].

2.8 Confocal laser scanning microscopy analysis (CLSM)

Biofilms were grown in 48-well plates and treated as described in Sections 2.4 and 2.6. The biofilm thickness and bacterial cell viability were evaluated after staining with Live/Dead BacLightTM Bacterial Viability staining assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After PBS washing, biofilms were analyzed with LSM 800 confocal laser-scanning microscope (Zeiss, Jena, Germany). Images were taken through 10× dry (EC Plan-Neofluar NA 0.3 air) objective. Excitation wavelength of 488 nm (Ar laser) and 561 nm (HeNe laser) were employed to visualize the distribution of live and dead bacteria[35].

2.9 Statistical Analyses

Three experiments were performed independently in triplicate at different times for CFU analysis. Thus, a total of nine wells were used per group (n = 9). Data distribution was assessed considering descriptive statistical analysis and Shapiro Wilk test. The CFU/mL counts were $\log_{10}$ transformed and subjected to one-way analysis of variance (ANOVA) with Welch's correction and Games-Howell post hoc test for pairwise comparison ($p < 0.05$). Data presented are plotted as the mean ± standard deviation (SD). Statistical analyses were performed using IBM® SPSS® Statistics Grad Pack 26.0 (New York, USA), a significance level of 5% was considered for all analyses. For confocal analysis, experiments were performed twice in duplicate to ensure reproducibility.

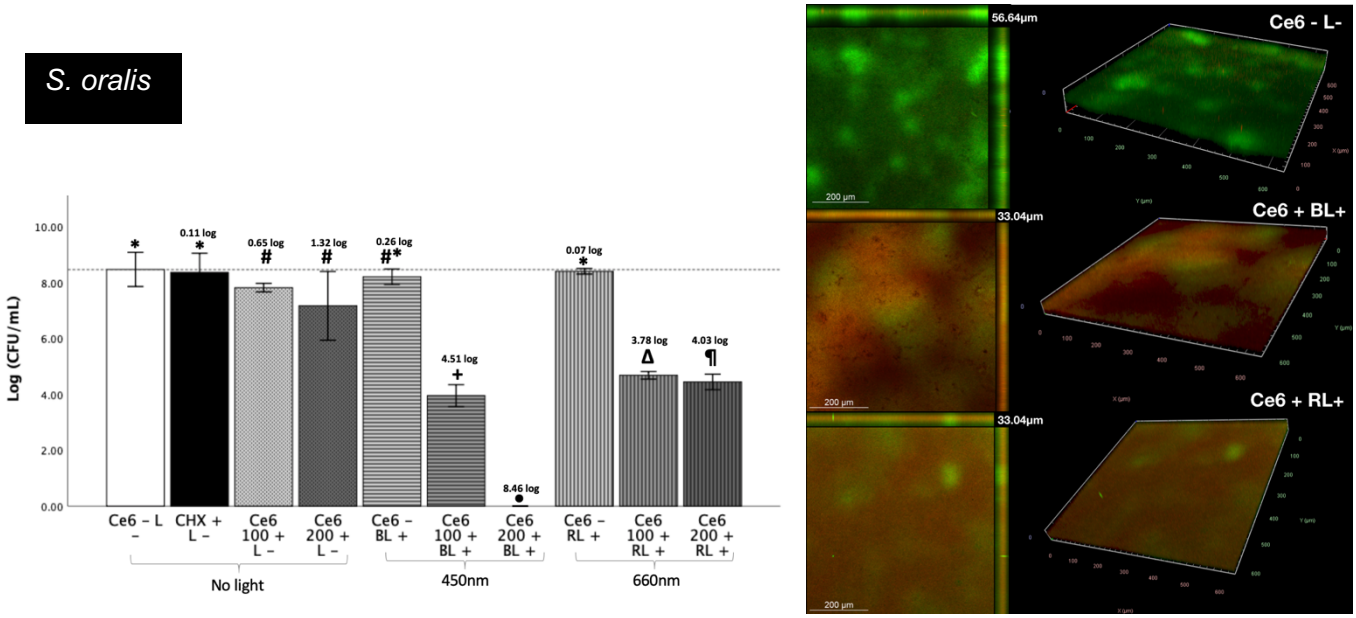
3 – Results

The CFU/mL counts showed that all bacterial strains formed large amounts of mature biofilm on the surface of the wells, ranging from 7.84 to 9.17 $\log_{10}$ in negative control groups. Negative controls were treated with DMSO at a noninhibitory concentration (1%), as great solvent vehicle[39]. The experiment was designed to identify any effect of light and photosensitizer individually, and the combination of both (PDI). All groups were submitted to a pairwise comparison. The numbers of viable colonies from single-species biofilms after treatments are shown in the **Figure 1**.

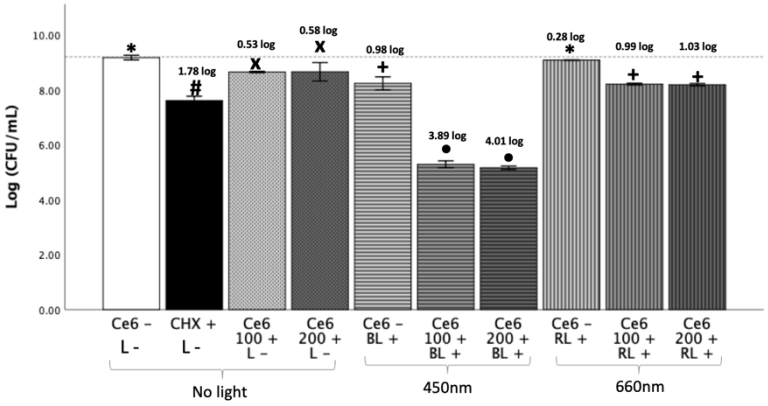
The positive control (Chlorexidine 0,2%, for 5 min) demonstrate statistically significant effect only against *A. actinomycetemcomitans* strain. For all bacterial strains, except *F. nucleatum*, the PS in the absence of light irradiation demonstrated some reduction of the number of viable bacteria when compared to the untreated group. Briefly, the greatest bacterial elimination was observed in the groups where PDI was performed using 200 μM of Ce6 and blue light was applied for all bacterial strains (reduction of 4.01 $\log_{10}$ for *A. actinomycetemcomitans*, and total elimination for both, *P. gingivalis* and *S. oralis*), except for *F.*

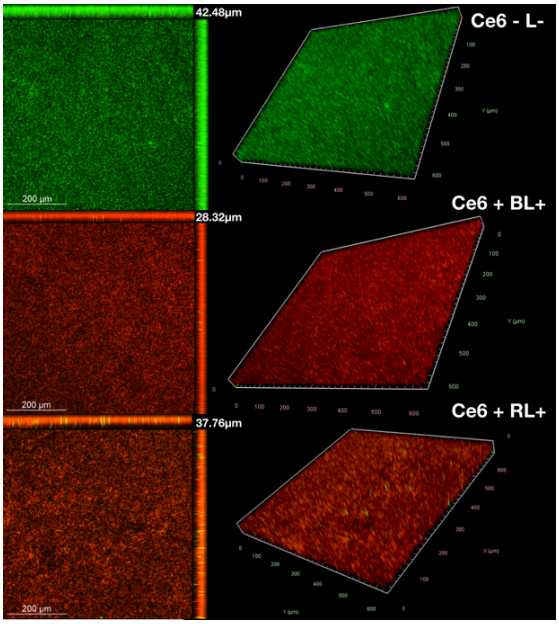
nucleatum, where the lowest mean was observed in the group treated with 200 μM of Ce6 and red light (3.46 log_{10} reduction).

In general, the LED irradiation alone, blue or red, did not show antimicrobial activity. For all bacterial strains, the use of PDI mediated by Ce6 photosensitizer demonstrated significant reduction of viable bacteria, regardless of the PS concentration used. The groups where Ce6 at 200 μM were irradiated by blue or red light presented a great decrease in the CFU counts. For this reason, only these groups were selected for confocal analyses. In addition, the reduction of viability was more evident in the groups treated with blue light for *A. actinomycetemcomitans*, *P. gingivalis,* and *S. oralis*. The distribution of stained bacteria and biofilm thickness under confocal analysis confirmed the CFU/mL results previously obtained, with a viewable number of dead bacteria after PDI treatment **(Figure 1**).

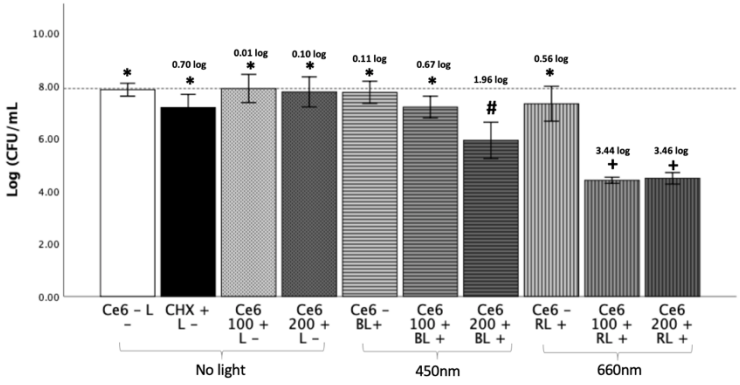


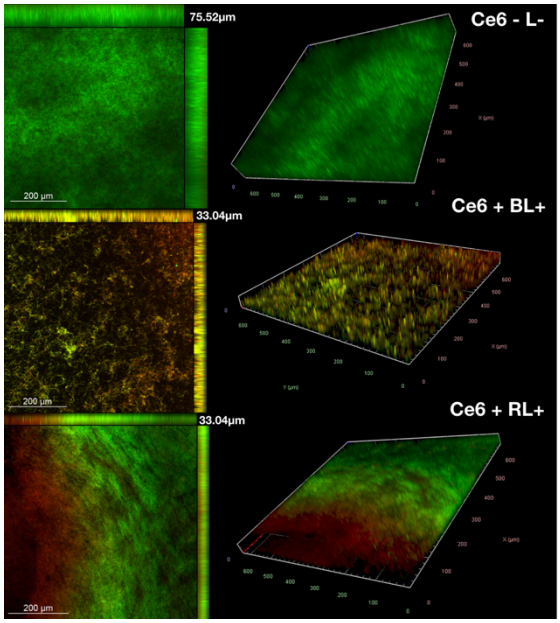
A. *actinomycetemcomitans*



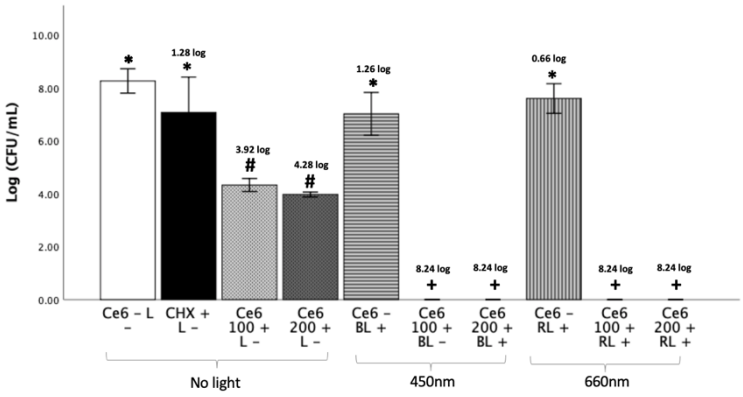


F. *nucleatum*





P. gingivalis



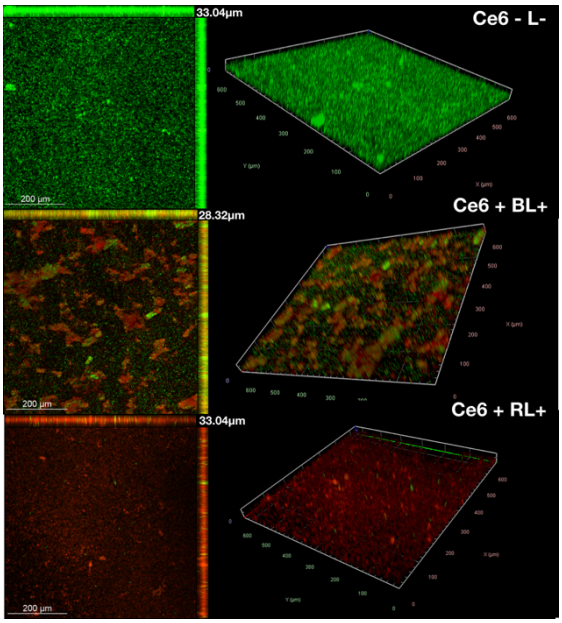


Fig 1. Effect of light and two concentrations (100μM and 200 μM) of chlorin-e6 irradiated by 450 and 660nm on *S. oralis,* A. *actinomycetemcomitans,* F. *nucleatum, P. gingivalis* biofilms. Bacterial counts after treatments are showed in logarithm of colony-forming units (CFU) per milliliter. Different symbols (*#+•Δ¶) indicate significant differences compared to the negative control ($p < 0.05$). Data represent the mean and ± SD of three assays performed independently in triplicate at different times. Images illustrating the effect of PDI in the groups Ce6 + BL+ and Ce6 + RL+ besides the negative groups, under confocal laser-scanning microscopy. Images reveal the live and dead bacterial population and biofilm thickness.

4 – Discussion

The main hypothesis of our study was confirmed since pure-Ce6-mediated PDI promoted a significant decrease of microorganisms' viability in all single-species biofilms. However, PDI irradiated with blue light showed the highest bacterial elimination against *S. oralis*, *P. gingivalis* and *A. actinomycetemcomitans*, while red light was more effective against *F. nucleatum*.

Bacterial elimination of at least 3 $\log_{10}$ is considered biologically relevant according to general guidelines of the Americana Society for Microbiology[39]. Our data revealed that both 100 and 200 μM Ce6-mediated PDI were able to reduce more than 3 $\log_{10}$ for *A. actinomycetemcomitans*, *P. gingivalis*, and *S. oralis* (100 μM – *A. actinomycetemcomitans* 3.89 $\log_{10}$, *S. oralis* 4.51 $\log_{10}$ and total elimination for *P. gingivalis*; 200 μM – *A. actinomycetemcomitans* 4.01 $\log_{10}$, and total elimination for *P. gingivalis* and *S. oralis*) irradiated by blue light. For *F. nucleatum*, blue light promoted 1.96 $\log_{10}$ at 200 μM Ce6 concentration, and 3.44 and 3.46 when exposed to red light under 100 and 200 μM of Ce6, respectively. It is important to note that a higher elimination was achieved in PDI groups than CHX, this last is considered the gold standard oral antimicrobial agent at the moment. However, the impressive effect of CHX is due to its substantivity leading to a prolonged bacteriostatic and bactericidal effects. In our study, biofilm was washed, and dilution was incubated immediately after treatment. CHX might have a higher effect in a re-growth biofilm analysis[40].

Our findings are coherent with previous reports that showed 3 $\log_{10}$ reduction after Ce6-mediated photodynamic inactivation using visible light plus water-filtered infrared-A (vis+wIRA) against *F. nucleatum* biofilm[41], and 4-5 log against *F. nucleatum* and *P. gingivalis* biofilms[21]. On the other hand, a smaller reduction was observed for *F. nucleatum* (1.12 $\log_{10}$) and P. gingivalis (2.66 $\log_{10}$) in biofilms using a commercially available chlorin-type PS (Photodithazine® - PDZ) irradiated by red light (660nm)[35]. These results might be explained by the PDZ composition that is not clearly disclosed. In our study, the bactericidal activity against all four single biofilms was confirmed by live/dead viability assays via confocal microscopy, again coherent with images obtained in a previous study[35]. Chlorin-type photosensitizer has also proved to be effective against *Streptococcus mutans* and Candida species[42,43].

S. oralis, *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans* were selected as representatives bacteria that contribute to the development of a high virulence subgingival biofilm considered at the early, middle, and late stages, respectively[3]. A pre-coat salivary pellicle was developed previously to *P. gingivalis* formation (section 2.2), since it was observed in our pilot studies that this late colonizer needs a salivary pellicle to constitute a safe biofilm able to tolerate aspiration and rinsing of the wells. At least two hours immersion in saliva was considered adequate for forming salivary pellicle on surfaces[44]. Four hours was the period used in this study[38].

The effect of a PS in the absence of light might be interpreted as a possible sign of dark toxicity. Some dark toxicity was observed in studies using chlorin-mediated PDI[28,35], as detected in our study. This finding may be further investigated in studies with proper designs (e.g. co-culture system) to mimic even more an *in vivo* condition. A wholly non-toxic photosensitizer might not exist[8]. Dark toxicity and bactericidal effect under light exposure appear to be directly proportional to drug concentration. A correlation between bacterial elimination and PS concentration was not observed for all bacterial strains. This occurrence was also observed in a recent study[35]. The use of 200 μM concentration is not fully supported, once there was no statistical difference between the highest and the lowest PS dose for *A. actinomycetemcomitans* when exposed to blue or red light, what was also observed for *S. oralis* and *F. nucleatum* irradiated by red light. A dose dependent interaction has to be further investigated, since the energy transferred in the mechanism or the available oxygen molecules surrounding the PS might be fully spent, or light dose has to be increased concomitantly with PS concentration.

Shakhova and collaborators affirm that wavelengths around 405 nm might be beneficial for superficial impact, especially in case of antibacterial or antiaging photodynamic therapy. Over the mucosa, differently of the skin, where the high scattering in stratum corneum resulting in high backscatter from the tissue, blue light is more intensely absorbed[26]. This evidence supports the use of blue light against infection in the oral cavity, which is fully covered by mucosa. The light doses ranging from 20 to 50 J/cm^2 is usually employed for PDI[14,24,26,28,35]. The presence of two peaks (around 405 and 670 nm) in chlorin-e6 absorption spectrum provides additional choice of irradiation regimen.

Blue LED under 400–500 nm is used in dentistry for detecting early caries lesion[45] and matured dental plaque[46], resin restoration, and tooth whitening[47]. A recent study reported the susceptibility of different species of oral bacteria to blue light[48], what provides evidence that light around 450 nm wavelength (widely used in general dentistry and available at any dental office) could be easily added as a choice of irradiation regimen for PDI in the oral cavity.

Red light is rather chosen for PDI in the oral cavity (endodontic and periodontal treatment) once its

wavelength range matches the absorption band of the most used photosensitizers in dentistry, and its ability to penetrate deeply into the tissues always draws attention. It is worth mentioning that fiber optics are available in the market for many purposes of light exposure, including for periodontal pocket and root canal applications. These devices are able to access the most complicated and deep sites without damaging surrounding tissue. If blue light is not able to reach the desirable application site, it could be easily solved by the use of fiber optics.

PDI acts essentially in the cell wall or membrane and the damage seems to be proportional to the reactive species produced (by type I and II mechanism), particularly singlet oxygen[49,50]. Since the cell wall and membrane of Gram-positive and Gram-negative are different, once this last has an additional lipopolysaccharide layer to its single peptidoglycan sheet, the efficacy of PDI against Gram-negative is thought to be reduced compared to Gram-positive[51,52]. The porous cell wall in Gram-positive bacteria represents a facilitated pathway to penetration. In the present study, a large effect was observed in the *S. oralis*, the only Gram-positive strain tested. Although such an expressive effect was not observed for *A. actinomycetemcomitans* and *F. nucleatum*, *P. gingivalis* demonstrated entire elimination.

The huge bacterial elimination detected for *P. gingivalis* strain could be a result of a limitation in the formation of *P. gingivalis* single-specie biofilm or its intrinsic photosensitizer dye. *P. gingivalis* is a late colonizer that requires the previous presence of receptors provided by middle stage colonizers to stablish a strong adhesion to biofilm in development[37]. The polystyrene substrates are different to the tooth surface on which periodontal biofilms development occurs. Additionally, a potential photodynamic effect of an intracellular pigment of *P. gingivalis* was recently discovered when irradiated at 460 nm. Researchers affirmed that blue-light irradiation on *P. gingivalis* is potentially bactericidal[53].

This study was designed to test the efficacy of PDI against periodontal pathogens organized in biofilms, since it is known that biofilms are much more difficult to eradicate than planktonic bacteria. Although multispecies biofilms would reflect a mimetic model, especially when developed in a dynamic flow system, static single-species are economical, practical, and widely used for testing anti-biofilm purposes[14,21,28,35,34]. Additionally, multispecies are even harder to arrest, what could constitute an unbeatable challenge for the early experiments with Ce6 PS, which still needs some improvements. Although, traditional method was adopted for the current study, the optimization of Ce6 has been under current analysis by our group. Even if this *in vitro* model of biofilm has limitation and the results obtained are still far from the clinical setting, the present data are relevant to support further experiments and follow the way of clinical use. Furthermore, to the best of our knowledge, our study was the first to investigate the effect of pure-chlorin-e6-mediated PDI irradiated by red and blue light against biofilms related to periodontitis, although

Ce6 has been under current extensively exploration.

5 – Conclusion

The present study proved that PDI with chlorin-e6 PS is able to produce a significant antimicrobial effect against *Streptococcus oralis*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans* single species biofilms when exposed to blue or red light, especially for S. oralis and P. gingivalis where an entire elimination was observed. Since a higher antimicrobial effect was observed in the groups treated with blue light, for three of the four tested bacterial strains, researchers should consider the use of blue light when chlorin-type photodynamic inactivation is tested.

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Conflicts of Interest

The authors declare no conflicts of interest.

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4 CONCLUSÃO

A terapia fotodinâmica antimicrobiana com utilização da clorina-e6 apresentou efeito antimicrobiano significativo quando irradiada por luz azul ou vermelha sobre biofilme periodontopatogênico, com redução mais evidente nos grupos tratados com luz azul para *S. oralis, P. gingivalis e A. actinomycetemcomitans*.

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APÊNDICE – METODOLOGIA

ESTUDO IN VITRO

Microrganismos

Quatro cepas de referência foram utilizadas, *Streptococcus oralis* (ATCC 35037), *Fusobacterium nucleatum* (ATCC 25586), *Porphyromonas gingivalis* (ATCC 33277) e *Aggregatibacter actinomycetemcomitans* (JP2).

Reativação das Espécies

As cepas de referência foram cultivadas individualmente em Ágar Sangue (Probac do Brasil Produtos Bacteriológicos Ltda, São Paulo, Brasil) durante 5 dias a 37°C em câmara de anaerobiose (85% N_2, H_2 10%, 5% de CO_2, Don Whitley - Inglaterra) ou estufa. Quatro colônias bacterianas foram transferidas a um tubo contendo caldo BHI (Brain Heart Infusion, Acumedia, Sigma-Aldrich) suplementado com extrato de levedura (6 g/l), hemina (10 mg/mL) e menadiona (5mg/mL), e mantidas durante 48 horas em atmosfera apropriada. Após crescimento, a concentração do microrganismo foi ajustada de acordo com sua fase exponencial de crescimento, a partir da densidade óptica e contagem de unidades formadoras de colônia definidas previamente pela curva de crescimento de cada microrganismo.

Coleta de Saliva Humana

Após aprovação do Comitê de Ética em Pesquisa em Seres Humanos da Faculdade de Odontologia de Araraquara – UNESP (CAAE 23396619.5.0000.5416), saliva foi coletada sem estimulação de um doador, homem, 27 anos, saudável. O critério de inclusão foi: ausência de lesões de cárie ou doença periodontal, inexistência de doenças crônicas, nenhum relato de administração de antibióticos nos últimos 6 meses que antecedem a coleta. Após coleta, saliva foi centrifugada (Spin Lab SL-5M, São Carlos, Brasil), 5000 g por 15 min. Após centrifugação, saliva foi purificada em filtro de 0,22μm (Merck Millipore Group, Massachusetts, EUA) e armazenada em freezer -80ºC (Thermo Scientific 88400D, Massachusetts, EUA) até sua utilização[63].

Formação do Biofilme

Biofilmes monoespécie de cada bactéria foram desenvolvidos em placas de microtitulação de 96 poços estéreis. Cada poço recebeu 200 μL de cada suspensão bacteriana correspondendo a

$1x10^7$ UFC/mL, obtida após a incubação em caldo de BHI. As placas de cultura celular contendo os biofilmes foram incubadas em atmosfera apropriada a 37°C durante 5 dias, o meio foi trocado a cada 24 h por aspiração do meio consumido e a adição de meio fresco (200 μL)[64].

Tratamento dos Biofilmes e Análise Quantitativa

Quadro 1- Grupos e seus respectivos tratamentos

	GRUPOS	**CLORINA-e6 (μM)**	**LUZ (J/cm²)**
1	Controle Negativo (DMSO 1%)	0	0
2	Controle Positivo (CHX 0,2%)	0	0
3	Clorina-e6-1	100	0
4	Clorina-e6-2	200	0
5	Led Azul – 450 nm (a)	0	30
6	TFDAa-1	100	30
7	TFDA a-2	200	30
8	Led Vermelha – 660 nm (v)	0	30
9	TFDA v-1	100	30
10	TFDA v-2	200	30

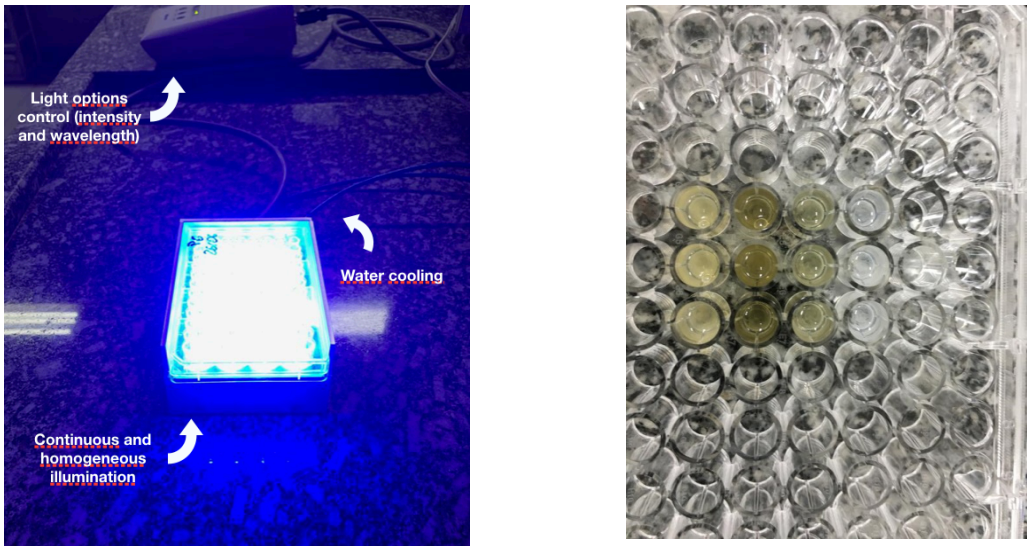
Fonte: Elaboração própria.

Após o crescimento dos biofilmes cada poço foi lavado duas vezes com PBS estéril, e os biofilmes tratados de acordo com os grupos estabelecidos (Quadro 1).

No grupo controle positivo, foi utilizada clorexidina (CHX) na concentração de 0,2% durante 5 min. No caso dos grupos tratados com clorina-e6, 200 μL das diferentes concentrações do fotossensibilizador foi adicionado aos poços e as placas foram incubados no escuro por 5 minutos (tempo pré-irradiação). Uma solução stock de Ce6 ($C_{34}H_{36}N_4O_6$. Peso molecular: 596,67) desenvolvida no Laboratório de Química Bioorgânica - LQBO, UFSCAr – São Carlos), sob coordenação do Prof. Dr. Kleber Thiago de Oliveira, foi diluída em Dimetilsulfóxido absoluto (DMSO, Sigma Aldrich) na concentração de 20 mM. Soluções de trabalho foram obtidas a partir da diluição da solução stock em água destilada, nas concentrações de 100 μM (0,5% DMSO) e 200 μM (1% DMSO). Foi utilizado um tempo de irradiação de 22min e 40 s para a luz azul (22 mW/cm²), e 26 min e 20 s para a luz vermelha (19 mW/cm²), ambos correspondentes a 30 J/cm². A concentração do fotossensibilizador e dose de luz foram determinadas com base em estudo piloto

desenvolvido recentemente por nosso grupo de pesquisa. Os biofilmes foram irradiados uniformemente com sistema de iluminação a LED (**Figura 1.** BioTable, Laboratório de Apoio Tecnológico, IFSC-USP, São Carlos, SP, Brasil) em comprimento de onda de 450 nm ou 660 nm.

Figura 1 – Aplicação da terapia fotodinâmica com utilização da clorina sobre biofilme de microrganismo periodontopatogênico cultivado em placa de 96 poços

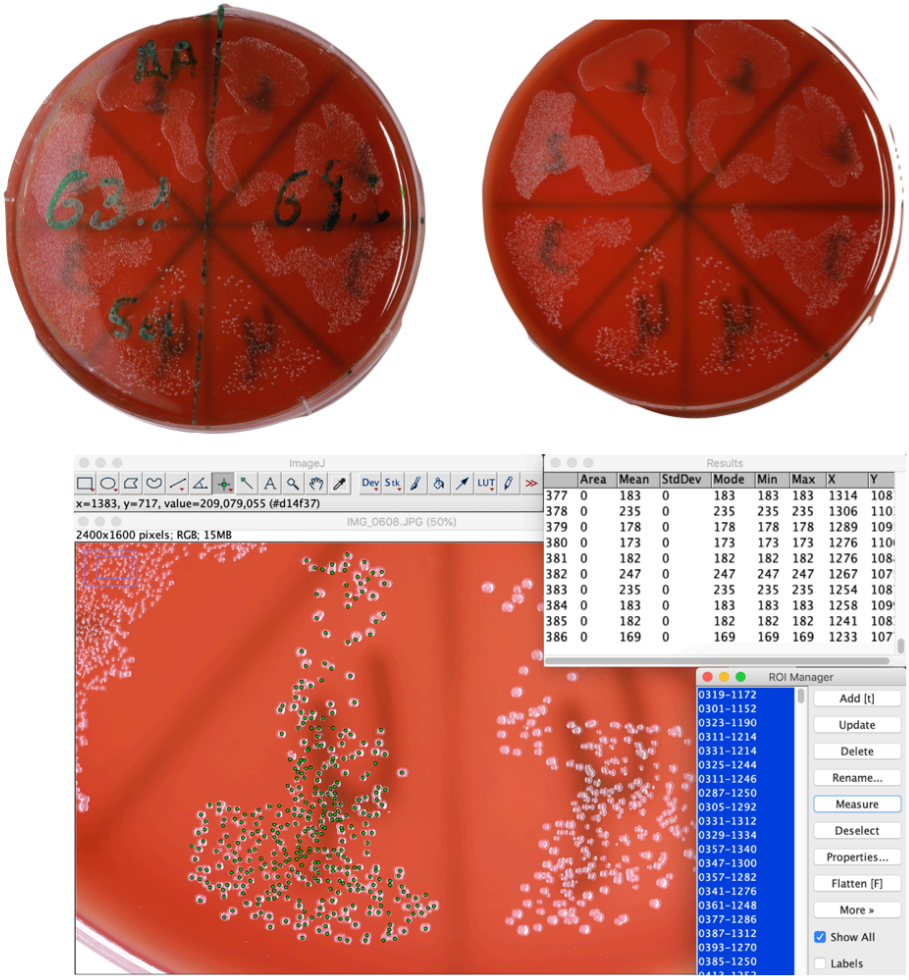


Fonte: Elaboração própria.

Análise de Unidades Formadoras de Colônias (UFC)

Na avaliação quantitativa de células bacterianas viáveis de biofilmes monoespécie, o método utilizado para contagem foi o de diluições seriadas, onde a partir da suspensão final de cada poço, suspensão considerada inicial (100) para o procedimento de contagem, foram feitas 8 diluições, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} para cada grupo. De cada diluição foi pipetado 25 µL em cada octante sobre meio de cultura apropriado (ágar sangue) contido em placas de Petri. As placas foram incubadas a 37ºC, em atmosfera de anaerobiose, pelo período necessário para ocorrer a multiplicação bacteriana a ponto de surgirem colônias visíveis a olho nu, sendo que cada colônia visível corresponde a uma UFC. Segundo esta técnica de contagem, deve-se escolher as placas que apresentam de 30 a 300 UFC para cálculo do número de UFC/mL (**Figura 2**). Este número é multiplicado pelo fator da diluição para se obter o valor de UFC/mL presentes na solução inicial a partir da qual foram feitas as demais diluições em série.

Figura 2 – Método utilizado para contagem de colônias utilizando o Software Image J (ImageJ, NIH, Bethesda, Maryland, USA)



Fonte: Elaboração própria.

Análise dos Resultados

Os dados obtidos após análise das unidades formados de colônia foram convertidos em função log de base 10, agrupados em tabelas e encaminhados para avaliação estatística, de forma a permitir a comparação entre os diferentes grupos experimentais. A dados foram analisados quanto a sua distribuição considerando a estatística descritiva e teste de Shapiro Wilk. Após verificar a normalidade da distribuição dos dados e a heterocedasticidade das variâncias (Teste de Levene), utilizou-se a análise de variâncias (ANOVA) na estatística inferencial e o teste post-hoc Games-Howell para identificar a diferença entre os grupos. A análise estatística foi realizada no software

IBM® SPSS® Statistics Grad Pack 26.0 (New York, USA), um nível de significância de 5% foi adotado para todas as análises.

ANEXO - CERTIFICADO DO COMITÊ DE ÉTICA

UNESP - FACULDADE DE ODONTOLOGIA - CAMPUS ARARAQUARA

Continuação do Parecer: 3.719.002

Assentimento / Justificativa de Ausência	TCLEClorinaFINAL.pdf	09:32:14	Zandim-Barcelos	Aceito
Cronograma	CronogramaFINAL.pdf	17/10/2019 09:32:07	Daniela Leal Zandim-Barcelos	Aceito
Folha de Rosto	FolhaRosto.pdf	09/10/2019 08:36:38	Daniela Leal Zandim-Barcelos	Aceito
Orçamento	Orcamento.pdf	09/10/2019 08:36:28	Daniela Leal Zandim-Barcelos	Aceito
Projeto Detalhado / Brochura Investigador	ProjetoCEPFINAL.pdf	07/10/2019 11:01:47	Daniela Leal Zandim-Barcelos	Aceito
Outros	Cartacumprimentonormascomite.pdf	07/10/2019 11:00:53	Daniela Leal Zandim-Barcelos	Aceito
Declaração de Instituição e Infraestrutura	Carta_Denise_CEP.pdf	07/10/2019 10:05:41	GABRIEL GARCIA DE CARVALHO	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

ARARAQUARA, 22 de Novembro de 2019

Assinado por:
Andréa Gonçalves
(Coordenador(a))

Endereço: HUMAITA 1680
Bairro: CENTRO **CEP:** 14.801-903
UF: SP **Município:** ARARAQUARA
Telefone: (16)3301-6459 **E-mail:** cep@foar.unesp.br

Não autorizo a publicação deste trabalho pelo prazo de até 2 anos após a data de defesa (28/02/2022)

Araraquara, 02 de março de 2020.

Gabriel Garcia de Carvalho