



**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 e LL-37 em  
nanoemulsão no tratamento da doença periodontal: estudo *in vitro* e *in vivo***

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Tese apresentada à Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara, para obtenção do título de Doutor em Odontologia, na Área de Periodontia

**Orientadora:** Profa. Dra. Denise Madalena  
Palomari Spolidorio

**Coorientadora:** Profa. Dra. Daniela Leal Zandim-  
Barcelos

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**Gabriel Garcia de Carvalho**

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**Comissão Examinadora**

**Tese para obtenção do grau de Doutor em Odontologia**

Presidente e orientador Profa. Dra. Denise Madalena Palomari Spolidorio

2º Examinador Profa. Dra. Telma Blanca Lombardo Bedran

3º Examinador Profa. Dra. Cristina Kurachi

4º Examinador Profa. Dra. Juliana Campos Junqueira

Araraquara, 27 de março de 2024.

## **DADOS CURRICULARES**

### **Gabriel Garcia de Carvalho**

|              |                                                                                                                                           |
|--------------|-------------------------------------------------------------------------------------------------------------------------------------------|
| NASCIMENTO   | 15 de Abril de 1992 – João Pessoa – PB – Brasil                                                                                           |
| FILIAÇÃO     | Adriana Coeli Garcia de Carvalho<br>Arlan Giovanni Duarte Carvalho                                                                        |
| 2010 – 2017  | Graduação em Odontologia<br>Universidade Federal da Paraíba                                                                               |
| 2017         | Atualização em Cirurgia Oral<br>Associação Brasileira de Odontologia – Secção Paraíba                                                     |
| 2018 – 2020  | Curso de Pós-Graduação em Odontologia, Área de<br>Concentração em Periodontia – Nível Mestrado<br>Faculdade de Odontologia de Araraquara  |
| 2019 – 2020  | Especialização em Reabilitação Oral<br>Associação Paulista de Cirurgiões Dentistas – APCD<br>Araraquara                                   |
| 2020 – 2023  | Especialização em Periodontia<br>Associação Paulista de Cirurgiões Dentistas – APCD<br>Araraquara                                         |
| 2020 – 2023  | Especialização em Implantodontia<br>Associação Paulista de Cirurgiões Dentistas – APCD<br>Araraquara                                      |
| 2020 – Atual | Curso de Pós-Graduação em Odontologia, Área de<br>Concentração em Periodontia – Nível Doutorado<br>Faculdade de Odontologia de Araraquara |

À Telma Ribeiro Garcia (tia – *In memoriam*),  
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## RESUMO

A Raspagem e Alisamento Radicular (RAR) é considerada a terapia não-cirúrgica de escolha para tratamento da doença periodontal (DP). No entanto, esta terapia pode apresentar limitações e insucessos quando utilizada isoladamente em determinados casos clínicos. Assim, terapias alternativas têm sido investigadas para suprir deficiências da RAR que podem estar relacionadas ao limitado acesso à superfície radicular durante o tratamento, a presença persistente de periodontopatógenos nas bolsas mais profundas e defesa imunológica deficiente. Dentre estas terapias, destaca-se a terapia fotodinâmica antimicrobiana (aPDT) e o uso de peptídeos antimicrobianos (PAM). O objetivo geral deste estudo foi investigar a otimização de clorina-e6 (Ce6) na terapia fotodinâmica e seu efeito como terapia coadjuvante no tratamento não cirúrgico da DP. Os objetivos específicos foram: (1) testar o efeito antimicrobiano da Ce6 irradiada por luz azul ou vermelha sobre biofilme multiespécie de periodontopatógenos; (2) otimizar o Ce6 em nanoemulsão e testar o efeito sinérgico do LL-37 com aPDT sobre biofilme multiespécie de periodontopatógenos; (3) testar o efeito de aplicações múltiplas da aPDT utilizando nanoemulsão carregada com Ce6 e LL-37 no tratamento de ratos com DP induzida. Biofilme multiespécie com bactérias periodontopatogênicas foi desenvolvido em condições ideais para avaliação antimicrobiana nos estudos 1 e 2, e a mesma foi avaliada por meio de ensaio qPCR. No estudo 1, a maior eliminação bacteriana foi observada nos grupos onde a luz azul foi aplicada. No estudo 2, após otimização e caracterização da Ce6 em nanoemulsão, foi observado que a combinação Ce6 e LL-37 em nanoemulsão foi efetiva na eliminação de todas as bactérias testadas. No estudo *in vivo*, sessões únicas ou múltiplas de terapia fotodinâmica com clorina-e6 conjugada com peptídeo antimicrobiano LL-37 em nanoemulsão como adjuvante da RAR no tratamento da DP não proporcionaram benefícios em comparação à terapia convencional isoladamente.

**Palavras-chave:** Fotoquimioterapia. Fármacos fotossensibilizantes. Periodontite.

Carvalho GG. Antimicrobial photodynamic therapy with the use of chlorine-e6 and LL-37 in nanoemulsion for the treatment of periodontal disease: an *in vitro* and *in vivo* study. [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2024.

## **ABSTRACT**

Scaling and root planning (SRP) is considered the first choice as the non-surgical therapy for the treatment of periodontal disease (PD). However, this therapy might present limitations and failures. Thus, alternative therapies have been investigated to overcome SRP deficiencies that may be related to limited access to the root surface, the persistent presence of periodontopathogens in deeper pockets and poor immune defense. Among these therapies, antimicrobial photodynamic therapy (aPDT) and the use of antimicrobial peptides (AMP) stand out. The general purpose of this study was to investigate the optimization of chlorin-e6 (Ce6) in photodynamic therapy and its effect as an adjuvant therapy in the non-surgical treatment of PD. The specific objectives are: (1) to test the antimicrobial effect of Ce6 irradiated by blue or red light against multispecies biofilm of periodontopathogens; (2) to optimize the Ce6 in a nanoemulsion and test the synergistic effect of LL-37 with aPDT against multispecies biofilm of periodontopathogens; (3) to test the effect of multiple applications of aPDT using a nanoemulsion loaded with Ce6 and LL-37 in the treatment of rats with induced periodontal disease. Multispecies biofilm with periodontopathogenic bacteria was developed under ideal conditions for antimicrobial evaluation in studies 1 and 2, antimicrobial effect was evaluated using qPCR assay. In study 1, the highest bacterial elimination was observed in the groups where blue light was applied. In study 2, after optimization and characterization of Ce6 in nanoemulsion, it was observed that the combination of Ce6 and LL-37 in nanoemulsion was effective in eliminating all tested bacteria. In the *in vivo* study, single or multiple sessions of aPDT with chlorin-e6 conjugated to the antimicrobial peptide LL-37 loaded nanoemulsion as adjunct to SRP in the treatment of periodontitis provided no benefits compared to SRP alone in rats.

**Keywords:** Photochemotherapy. Photosensitizing agents. Periodontitis.

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## 1 INTRODUÇÃO

A doença periodontal afeta grande parte da população mundial, encontrando-se no grupo das doenças 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. Apresentam fatores de virulência que modificam a resposta imunoinflamatória do hospedeiro, especialmente quando organizadas em forma de biofilme e localizadas no sulco gengival<sup>1,2</sup>.

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<sup>3-6</sup>, 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 a RAR<sup>7-9</sup>, da presença de periodontopatógenos nas bolsas mais profundas<sup>10</sup> e da habilidade de algumas bactérias em invadir tecido adjacente<sup>11</sup>. Quando se considera o tratamento, em caso de doença periodontal estágio 3, 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<sup>12-14</sup>. 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 doenças, bem como as periodontais<sup>15,16</sup>. Neste contexto, a terapia fotodinâmica antimicrobiana (*antimicrobial photodynamic therapy* - aPDT) tem se destacado como um procedimento adjunto no combate de infecções microbianas. Diversos estudos comprovam a eficácia da aPDT, tanto in vitro como in vivo, em animais e humanos, no tratamento das doenças periodontais<sup>17-28</sup>.

A aPDT consiste na excitação do fotossensibilizador (FS) em seu estado fundamental por uma luz no espectro visível. 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 (ROS – *reactive oxygen species*). 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<sup>29</sup>. 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 a biomolécula, provocando um dano celular (toxicidade no escuro) e consequente destruição do FS<sup>29-31</sup>. A aPDT 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<sup>32</sup>.

Dentre as vantagens do emprego desta terapia, encontram-se: 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; e o acesso facilitado a regiões de difícil acesso pela terapia convencional (RAR), como bolsas profundas e bi- ou trifurcação de raízes<sup>33</sup>.

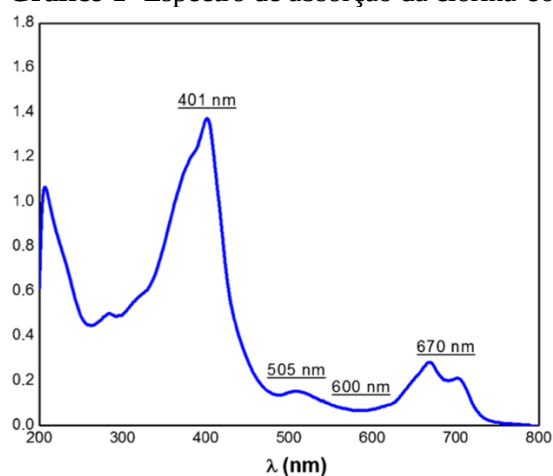
A aPDT tem sido indicada como terapia coadjuvante no tratamento da periodontite estágio 3, retratamento de bolsas residuais, descontaminação para terapia cirúrgica, terapia de manutenção, dentre outros<sup>34-36</sup>. Sua indicação na terapia de manutenção tem como vantagem o menor desgaste da superfície radicular, que seria um aspecto negativo da utilização da técnica convencional (RAR) e neste caso, a aPDT seria utilizada isoladamente, reduzindo a incidência de hipersensibilidade causada pela remoção de cimento inerente às múltiplas sessões de RAR<sup>37</sup>.

A clorina e6 (Ce6) surgiu em meados dos anos 90 como um FS de segunda geração bastante promissor em terapias oncológicas<sup>38</sup>. A clorina e seus derivados estão sendo utilizados em diversos tratamentos com utilização de luz, como cânceres<sup>39,40</sup>, leucoplasias<sup>41</sup>, infecções endodônticas<sup>42</sup>, e contra biofilmes formados por *Streptococcus mutans*<sup>43</sup>. Trata-se de um composto natural extraído da clorofila. Algumas formulações a base de clorina estão disponíveis no mercado para tratamento antibacteriano, antienvelhecimento e antineoplásico, como Revixan® e Photolon®. 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<sup>44-46</sup>.

Fotossensibilizadores a base de clorina apresentam dois picos de absorção de luz, nas

luzes 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<sup>47</sup>. 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. O estudo de Shakhova e colaboradores<sup>48</sup> 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<sup>2</sup> para ambos os comprimentos de onda, indicando que cerca de 40% do incidente da energia é absorvida dentro do tecido; para a mucosa, o valor de saturação para comprimento de onda de 405 nm foi de 0,28 J/mm<sup>2</sup> (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<sup>2</sup> (6% da intensidade do incidente) para a espessura de 4 mm.

**Gráfico 1-** Espectro de absorção da clorina-e6



Fonte: Elaboração própria.

É importante ressaltar que a luz azul está comumente presente na prática odontológica, onde é utilizada na polimerização de materiais restauradores ou fotoativação de adesivos e clareadores. Esses 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 aPDT.

Estudos anteriores do nosso grupo de pesquisa testou, de maneira pioneira, a utilização da Ce6 ativada por luz azul contra biofilme periodontopatogênico monoespécie, obtendo

resultados de eliminação microbiana de até 3 log<sub>10</sub>, para *Streptococcus oralis*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* e *Fusobacterium nucleatum*<sup>49</sup>.

Para biofilmes multiespécies, acredita-se que a aplicação tradicional da aPDT pode não produzir efeitos antimicrobianos significativos, uma vez que os mecanismos de defesa aprimorados nestes casos, tornam sua erradicação dificultada. Desta forma, as nanopartículas<sup>50,51</sup> e nanoemulsões<sup>52</sup> surgem como uma estratégia que podem superar limitações no emprego de fotossensibilizadores na aPDT.

Peptídeos antimicrobianos (PAM) são componentes indispensáveis da resposta imune inata de animais e plantas e são considerados a primeira linha de defesa contra patógenos<sup>53</sup>. Os PAMs interagem com a membrana celular de bactérias por meio de interações eletrostáticas, sofrem adsorção e alteração conformacional, promovendo danos de membrana<sup>54</sup>. Um possível efeito sinérgico da aplicação de aPDT + PAM, poderia ser explicado pela maior internalização das moléculas do FS a partir dos danos de membrana produzidos pela ação do PAM, ou mesmo, aumento da suscetibilidade da bactéria afetada pelo PAM à presença de espécies reativas dispersas na região (produzidas pela aPDT)<sup>55,56</sup>.

De maneira geral, poucos estudos avaliaram o efeito antimicrobiano da aPDT com utilização da clorina como fotossensibilizador contra patógenos orais<sup>57-64</sup>. Estudos pregressos, desenvolvidos pelo nosso grupo de pesquisa, demonstram que a Ce6 é um FS promissor no tratamento da doença periodontal, e a formulação deste em nanoemulsão pode garantir maior estabilidade, substantividade e solubilidade. Ainda, investigar um possível efeito sinérgico deste FS conjugado ao PAM, pode representar efeito biológico clinicamente expressivo. No presente estudo foi testado o extrato puro da Ce6, composto ainda não patenteado, desenvolvido pelo Laboratório de Química Bio-orgânica, UFSCar – São Carlos. Assim, o presente estudo investigou a otimização de Ce6 na terapia fotodinâmica e sua contribuição na resolubilidade da doença periodontal.

## **2 PROPOSIÇÃO**

Avaliar o efeito da terapia fotodinâmica com utilização da Ce6 no tratamento da doença periodontal.

### **2.1 Objetivos Específicos**

Artigo 1: Avaliar o efeito antimicrobiano da terapia fotodinâmica com utilização da Ce6 irradiada por luz azul ou vermelha sobre biofilme multiespécie de periodontopatógenos. Foi considerada a seguinte hipótese nula: A terapia fotodinâmica antimicrobiana com utilização da clorina-e6 irradiada por luz azul ou vermelha não elimina biofilme periodontopatogênico.

Artigo 2: Avaliar o efeito antimicrobiano da terapia fotodinâmica utilizando nanoemulsão carregada com Ce6 e LL-37 contra biofilme multiespécie de periodontopatógenos. Foi considerada a seguinte hipótese nula: A terapia fotodinâmica antimicrobiana com utilização da clorina-e6 e o peptídeo antimicrobiano LL-37 não elimina biofilme periodontopatogênico.

Artigo 3: O objetivo deste estudo foi avaliar o efeito de aplicações múltiplas de terapia fotodinâmica utilizando nanoemulsão carregada com Ce6 e LL-37 no tratamento de doença periodontal induzida em ratos. Foi considerada a seguinte hipótese nula: A terapia fotodinâmica antimicrobiana com utilização da clorina-e6 e o peptídeo antimicrobiano LL-37 não oferece benefício adicionais como adjuvante no tratamento da doença periodontal em ratos.

### 3 PUBLICAÇÕES

Nesta sessão serão apresentadas as publicações originadas do projeto de pesquisa que resulta na presente tese de doutorado.

#### 3.1 Artigo 1\*

##### **Chlorin-Based Photosensitizer under Blue or Red-Light Irradiation against Multi-species Biofilms related to Periodontitis**

**Running title:** Chlorin-mediated photodynamic therapy against periodontal pathogens

##### **Author role**

All authors contributed in all aspects of the study.

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##### **Declaration of Competing Interest**

None of the authors has any conflict of interest to be disclosed.

##### **Conflict of interest statement**

This manuscript has been read and approved in final form by all authors and the authors declare no conflicts of interest.

#### RESEARCH ARTICLE

##### **Chlorin-Based Photosensitizer under Blue or Red-Light Irradiation against multi-species Biofilms related to Periodontitis**

##### **Abstract**

In our previous study, Chlorin-e6 (Ce6) demonstrated a significant reduction of microorganisms' viability against single-species biofilm related to periodontitis once irradiated by red light (660 nm). Also, higher bacteria elimination was observed under blue light (450 nm) irradiation. However, the use of blue light irradiation of Ce6 for antimicrobial administration is poorly explored. This study evaluated the effect of chlorin-e6-mediated antimicrobial photodynamic therapy (aPDT) using different wavelengths (450 or 660 nm) against multi-species biofilms related to periodontitis. *Streptococcus oralis*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans* composed the mature

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\* Artigo formatado segundo as normas do periódico *Photodiagnosis and Photodynamic Therapy* no qual foi publicado.

Garcia de Carvalho G, Pacheco Mateo R, Costa E Silva R, Maquera Huacho PM, de Souza Rastelli AN, de Oliveira KT, Chierici Marcantonio RA, Zandim-Barcelos DL, Palomari Spolidorio DM. Chlorin-based photosensitizer under blue or red-light irradiation against multi-species biofilms related to periodontitis. *Photodiagnosis Photodyn Ther.* 2023; 41: 103219. doi: 10.1016/j.pdpdt.2022.103219.

biofilm developed under proper conditions for five days. aPDT was performed using different concentrations of Ce6 (100 and 200  $\mu\text{M}$ ), wavelengths (450 or 660 nm), and comparisons were made after qPCR assay and confocal laser scanning microscopy (CLSM) analysis. The greatest bacterial elimination was observed in the groups where Ce6 was used with blue light, for *S. orallis* ( $2.05 \text{ Log}_{10} \text{ GeQ mL}^{-1}$ ,  $p < 0.0001$ ) and *P. gingivalis* ( $1.4 \text{ Log}_{10} \text{ GeQ mL}^{-1}$ ,  $p < 0.0001$ ), aPDT with red light showed significant bacteria reduction only for *S. orallis*. aPDT with blue light demonstrated statistically higher elimination in comparison with aPDT with red light. The aPDT did not show a statistically significant effect when tested against *A. actinomycetemcomitans* and *F. nucleatum* ( $p = 0.776$  and  $0.988$ , respectively). The aPDT using blue light showed a promising higher photobiological effect, encouraging researchers to consider it in the irradiation of Ce6 for further investigations.

**Key Words:** Photodynamic Therapy, Chlorin e6, Photochemotherapy, Photosensitizing Agents, Periodontitis.

## 1 – Introduction

Just as brief as possible, periodontitis is an oral infection triggered by pathogenic bacterial species and exacerbated host inflammatory response (1). Leading scientists in the field of periodontal disease concentrate their efforts on two arms: controlling host response and bacterial biofilm. Considering this latter strategy, chemical and mechanical plaque disarrangement is the only evidence-based method to reach disease resolution (2). The professional mechanical removal of the pathogenic biofilm is named scaling and root planning (SRP) and, although it is sufficient to restore periodontal health in the majority of the cases, this therapy might not be enough when complicating factors (difficult access to instrumentation, incompetent host response, poorly informed or unmotivated patient regarding oral hygiene,) are present, or patient is systemically compromised (3).

Although the principle of photodynamic therapy (PDT) was discovered during the early 1900s (4), its use for antimicrobial purposes in periodontal disease only starts in the early 1990s. Perhaps surprisingly, it has not been considered a clinical reality in periodontal treatment yet due to questionable outcomes in randomized clinical trials (5,6,7,8). Apparently, multiple applications of antimicrobial photodynamic therapy (aPDT) tend to favor clinical outcomes (9).

Different challenges limit the use of aPDT in periodontal treatment: few light delivery devices were developed precisely to oral application reasons, low oxygen concentrations in

deep periodontal sites (10), hard and soft tissues dye staining around local application (11), ineffective uptake of photosensitizer (PS) by certain bacterial strains (12), the low amount of light reaching deep into periodontal pockets. It seems that the main reasons for insufficient aPDT function rely on the PS effectiveness and light delivery and absorption, this study is focused on this latter strategy.

Red light is present in the vast majority of studies with photodynamic therapy for antimicrobial elimination. In a previous study, our group provided evidence regarding the aPDT effect at different wavelengths using chlorin-e6 (Ce6), blue light showed a promising higher antimicrobial effect than the Ce6 irradiated by red light, encouraging further investigation on this topic (13). At that time, there were only a few studies investigating the antimicrobial effect of Ce6 against periodontal pathogens in vitro (14,15,16). From the best of our knowledge, our study was the first to report the use of Ce6 activated by blue light against single-species biofilms related to periodontitis. Afterward, other investigations confirmed the Ce6 antimicrobial effect against biofilms related to periodontitis (17,18). It is important to highlight that Ce6 absorption spectrum (**Figure 1**) is strictly higher at the solet band (~400nm, blue visible light), controversially, almost all studies investigating Ce6 prefer red light from among these two choices, only two studies have taken the advantage of blue light to intensify bacterial reduction during Ce6-mediated aPDT (13,19) Traditionally, the choice for the red light is based on the belief of its greater ability to penetrate tissues, which might not be relevant to treat oral biofilms, generally formed on dental surfaces. Now it is shown below the use of Ce6 exposed to blue or red light against multi-species biofilms related to periodontal disease, is this combination able to reduce bacterial viability in this condition?

## 2 – Material and Methods

### 2.1 Photosensitizer and Light Source

Chlorin-e6 was semi-synthesized in the Bioorganic Chemistry Laboratory at Federal University of São Carlos - UFSCar (Sao Paulo, Brazil), as previously described (20). Chlorine e6 structure and absorption spectrum is shown in **Figure 1**. Stock solution was prepared by dissolving Ce6 in DMSO (Dimethyl sulfoxide, Sigma-Aldrich, St. Louis, MO, USA) to reach 20 mM concentration, and stored in the dark at -20 °C until use. Later, a working solution was prepared by diluting stock solution in distilled water to 100 µM (59.7 mg/L) and 200 µM (119.34 mg/L) concentration right at the time of application, pH ~7.6, 30 min before each

experiment.

The light was delivered by a 24 LED source as a continuous and homogeneous illumination system under water cooling (Biotable, MMOptics, Sao Carlos, SP, Brazil). Irradiation was performed from underneath of culture well-plates. A fluency of 30 J/cm<sup>2</sup> (22 mW/cm<sup>2</sup> under 450 nm excitation wavelength for 22 min and 40 s, and 19 mW/cm<sup>2</sup> under 660 nm for 26 min and 20 s) was achieved for both blue and red wavelength and the temperature was frequently checked to avoid overheating (13), an increase of 3-4 degrees is expected for blue light, 2-3 degrees for red light. The same PS concentration and light dose used in the previous study investigating single-species biofilm related to periodontitis was considered for the present study (13).

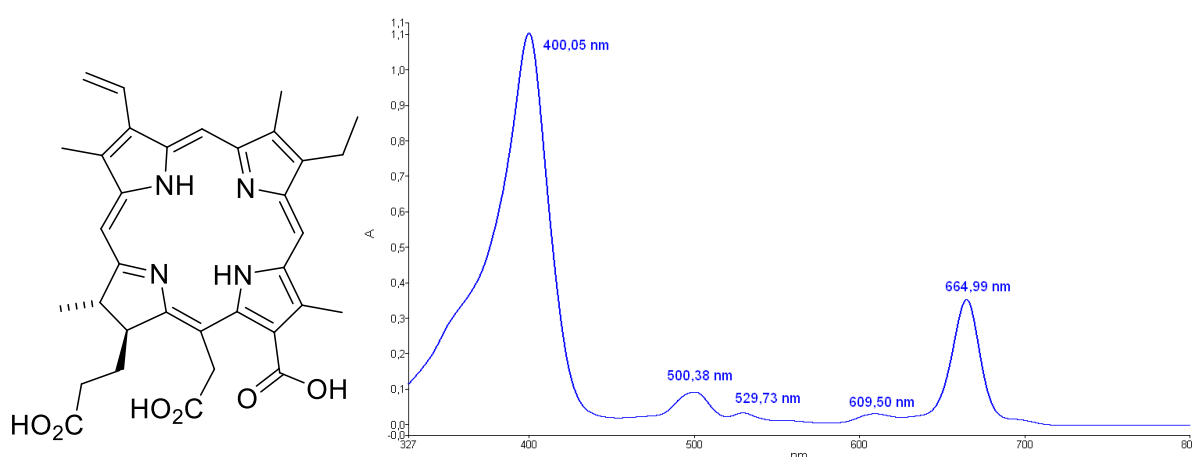


**Figure 1** - Chlorine e6 structure and absorption spectrum

## 2.2 Human Saliva Preparation

Human saliva composed the first layer of the multi-species biofilm described latterly. After Research Ethics Committee approval (School of Dentistry at Araraquara, Sao Paulo State University-UNESP, CAAE 23396619.5.0000.5416), unstimulated saliva was obtained from 5 volunteers, aging from 25 to 30 years old, healthy, non-smokers, 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 collection, and no use of systemic antibiotics for at least six months before collection. After collection, the pool of saliva was centrifuged at 10,000 g for 15 min at 4 °C (Centrifuge 5430R, Eppendorf, Hamburg, Germany) and filtered with a sterile syringe membrane filter (0.22 µm pore size, Kasvi, São Jose do Pinhais, PR, Brazil).

Saliva was stored at -80 °C until use (Thermo Scientific 88400D, Massachusetts, EUA) (16).

### 2.3 Bacterial strain, growth conditions, and biofilm formation

*Streptococcus oralis* (American Type Culture Collection - ATCC 35037), *Fusobacterium nucleatum* (ATCC 25586), *Porphyromonas gingivalis* (ATCC 33277) and *Aggregatibacter actinomycetemcomitans* (JP2) were cultured in Brucella agar Petri dishes (Brucella Agar, Neogen, Idaiatuba, SP, Brazil; Petri dish 90 x 10 cm, Kasvi, Sao Jose do Pinhais, PR, Brazil) with 5% defibrinated sheep blood (Microlab, Campinas, SP, Brazil) supplemented with yeast extract (6 g/L), hemin (10 mg/mL) and menadione (5 mg/mL) at 37°C. Atmosphere condition was considered for each bacterial strain; anaerobic condition for *F. nucleatum* and *P. gingivalis* (85% N<sub>2</sub>, 10% H<sub>2</sub> e 5% CO<sub>2</sub>, White Martins, Rio de Janeiro, RJ, Brazil; anaerobic workstation, VA 500, Don Whitley Scientific, England), and incubation in a 5% CO<sub>2</sub> environment (Incubator, GE Healthcare Life Sciences, Chicago, IL, USA) for *S. oralis* and *A. actinomycetemcomitans*.

After growth, bacterial colonies were transferred to 10 mL Brain Heart Infusion (BHI – Difco Laboratories Inc, MI, USA) broth medium supplemented as described before, followed by incubation in the same conditions for 24 h. Finally, bacterial suspensions were adjusted according to the mid-exponential growth phase established previously, by optical density (OD600 nm, BioSpectrometer® D30, Eppendorf, Hamburg, Germany) and colony-forming units CFU mL<sup>-1</sup> (*P. gingivalis*, 15 h, OD600 nm = 0.95 ± 0.01, CFU mL<sup>-1</sup> = 4.3 x 10<sup>9</sup>; *F. nucleatum*, 10 h, OD600 nm = 0.45 ± 0.01, CFU mL<sup>-1</sup> = 1 x 10<sup>8</sup>, *S. oralis*, 4 h, OD600 nm = 0.55 ± 0.01, CFU mL<sup>-1</sup> = 1.5 x 10<sup>9</sup>, *A. actinomycetemcomitans*, 8 h, OD600 nm = 0.18 ± 0.01, CFU mL<sup>-1</sup> = 5.66 x 10<sup>8</sup>) until reaching 10<sup>7</sup> CFU mL<sup>-1</sup> (21).

Fifty microliters of sterilized saliva were placed at the bottom of each well of a 96-well plate (Kasvi, São Jose do Pinhais, PR, Brazil) and kept in an orbital shaker (430 RD incubator, Nova Etica, Brazil), 75 rpm at 37 °C for 4 h in order to promote bacterial initial attachment (21). A 200 µL aliquot of the standardized suspension (10<sup>7</sup> CFU mL<sup>-1</sup>) of *S. oralis* was added first to each well (pretreated with a saliva-derived film) and incubated in aerobic conditions, at 37 °C. After 24 h, nonadherent bacteria and medium were removed and 200 µL of the same concentration of *F. nucleatum* was added in fresh medium to each well, then plates were incubated in anaerobic conditions. After 24 h, the suspension was removed and 100 µL of *P. gingivalis* and 100 µL of *A. actinomycetemcomitans* (both 10<sup>7</sup> CFU mL<sup>-1</sup>) were added to each well to continue biofilm formation (anaerobic condition). The culture medium was replaced

daily, for 3 more days, very slowly, to avoid biofilm disruption. For all groups, the wells were washed twice at the end of the incubation period and before further analyses.

## 2.4 Control and experimental groups

The investigated groups were divided considering the wavelength used, in three independent plates for blue (450 nm), red (660 nm), and no light, to isolate from refracted light as a confounding factor. The groups were divided as follow: 1) Negative control, no Ce6 (distilled water was applied to avoid biofilm dryness) and no light (Ce6 – L –); 2) Positive control, treated with 0.2 % Chlorhexidine for 1 min and no light (CHX + L –); 3) Ce6 100  $\mu$ M and no light (Ce6 100 + L –); 4) Ce6 200  $\mu$ M and no light (Ce6 200 + L –); 5) no Ce6 (growth medium) with blue light (Ce6 – BL +); 6) Ce6 100  $\mu$ M with blue light (Ce6 100 + BL +); 7) Ce6 200  $\mu$ M with blue light (Ce6 200 + BL +); 8) no Ce6 (growth medium) with red light (Ce6 – RL +); 9) Ce6 100  $\mu$ M with red light (Ce6 100 + RL +); 10) Ce6 200  $\mu$ M with red light (Ce6 200 + RL +). Confocal microscopy analysis was carried out in the groups with the greatest reduction in the previous analysis of viable bacteria.

## 2.5 Photodynamic Inactivation

After biofilm formation, the growth medium was removed and 200  $\mu$ L of treatment solution (fresh growth medium, CHX and Ce6 in both concentrations) were added according to the description (**section 2.4**). All groups with Ce6 were incubated in the dark at room temperature for 5 min (pre-irradiation time defined previously) (13). Subsequently, the 96-well plate with blue light groups was irradiated with blue light (450 nm), just as the red light groups were exposed to 660 nm light, fluency was described previously (**section 2.1**). The plate with no light regimen was kept in the dark for the same time as red-light groups (26 min and 20 s), to analyze any possible dark toxicity effect of Ce6 against the biofilm.

## 2.6 DNA extraction

After aPDT application, biofilms were carefully washed twice with phosphate buffer saline (PBS), removed (ultrasonic generator, Cristofoli, Parana, Brazil), and transferred to microtubes for subsequent DNA extraction. Before DNA extraction, samples were exposed to PMA (propidium monoazide) (Biotium, USA), a photoreactive DNA binding dye used to detect viable microorganisms, preventing from the amplification of dead cells in qPCR reactions.

Then, DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The purified sample was stored at -80°C (Thermo Scientific 88400D, Massachusetts, EUA) until qPCR reaction.

## 2.7 qPCR assay

The qPCR quantification reactions were performed on the CFX96 real-time system equipment (BioRad, Hercules, CA). The Taqman system with specific primers and probes was used (22). For the Taqman reactions, 12.5 µL of Mastermix (Eurogentec, Seraing, Belgium), 4.5 µL of sterile H<sub>2</sub>O, 1 µL of each primer and probe and 5 µL of each sample were used. Primers and probes were used at different concentrations depending on the bacteria. The amplification conditions were: 2 minutes at 50 °C, 10 minutes at 95 °C, followed by 45 cycles of 95 °C for 15 s and 60 s at 60 °C. This experiment was carried out in triplicate, in 3 independent replications (n=9).

## 2.8 Confocal laser scanning microscopy analysis (CLSM)

For this experiment, biofilms were grown in 48-well plates and treated as described in Sections 2.3 and 2.5, selected groups for CLSM analysis are shown in Table 1. The bacterial viability was evaluated after staining with Live/Dead BacLight™ Bacterial Viability staining assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After staining, biofilms were washed and 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 (16).

## 2.9 Statistical Analyses

Three independent experiments were performed in triplicate at different times for qPCR analysis. Thus, a total of nine wells were obtained for each group (n = 9). Data distribution was checked using descriptive statistical analysis and the Shapiro Wilk test. Data were submitted to one-way analysis of variance (ANOVA), Dunn's and Tukey's post hoc test for comparisons between groups (p<0.05). Statistical analyzes were performed using IBM® SPSS® Statistics Grad Pack 26.0 (New York, USA), with a significance level of 5%.

### 3 – Results

The qPCR assay showed that all bacterial strains were similarly present in the mature multi-species biofilm on the surface of the wells. *S. oralis* was found in the highest concentration ( $9.10 \text{ Log}_{10} \text{ GeQ mL}^{-1}$ ) and *P. gingivalis* was the least present ( $4.54 \text{ Log}_{10} \text{ GeQ mL}^{-1}$ ) in the negative controls (**Figure 2**). The photosensitizer and both types of light irradiation were tested separately to check the impact of all variables in the aPDT treatment and also check any possible dark toxicity. The combination of Ce6 and blue or red light may either be seen in **Figure 2**.

No significant effect was found by the use of chlorhexidine 0.2 %. The photosensitizer in the absence of light did not demonstrate any impact on bacteria viability, so no dark toxicity was identified for Ce6 in this experiment. Briefly, the greatest bacterial elimination was observed in the groups where Ce6 was applied with blue light, for *S. orallis* ( $2.05 \text{ Log}_{10} \text{ GeQ mL}^{-1}$  for Ce6200+ BL+,  $p < 0.0001$ ) and *P. gingivalis* ( $1.4 \text{ Log}_{10} \text{ GeQ mL}^{-1}$  for Ce6100+ BL+,  $p < 0.0001$ ). For the two bacterial strains just mentioned before, a statistical difference was found between the results of aPDT with blue light and red light. Ce6100+ BL+ presented a higher reduction of  $1.35 \text{ Log}_{10} \text{ GeQ mL}^{-1}$  in comparison with Ce6100+ RL+. The aPDT application did not demonstrate a statistically significant effect against *A. actinomycetemcomitans* and *F. nucleatum*. Some bacterial reduction may be noted with 450 nm ( $1.13$  for *A. actinomycetemcomitans*,  $p = 0.776$ , and  $0.8 \text{ Log}_{10} \text{ GeQ mL}^{-1}$  for *F. nucleatum*,  $p = 0.988$ ). The LED irradiation alone, blue or red, did not show antimicrobial activity.

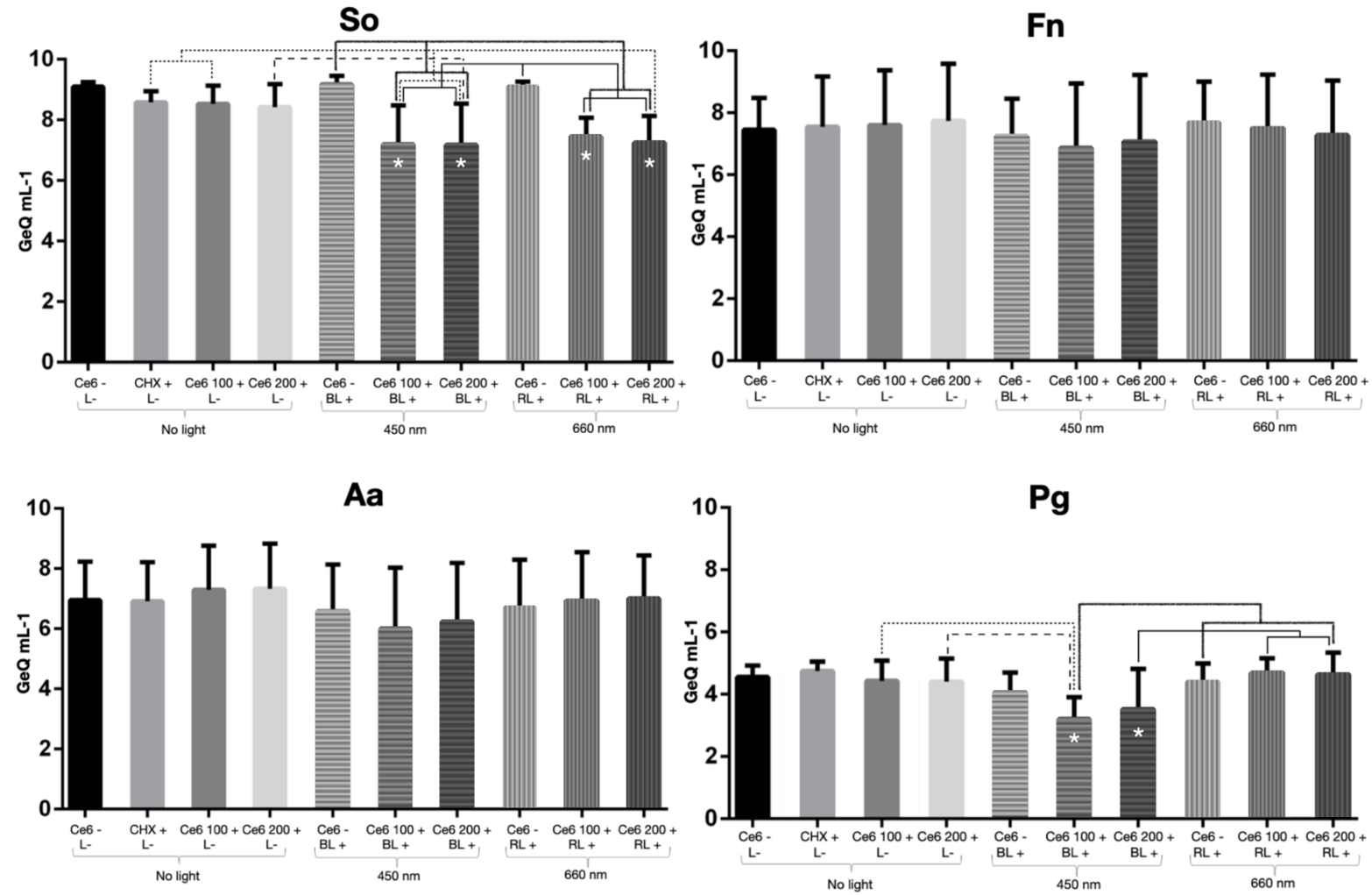


**Figure 2.** Effect of light and two concentrations (100  $\mu$ M and 200  $\mu$ M) of chlorin-e6 irradiated by 450 and 660 nm against a multi-species biofilm composed by *S. oralis*, *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis* biofilms. Quantitative data of bacteria viability after treatments is shown in ( $\text{Log}_{10}$  GeQ mL<sup>-1</sup>). Linked lines mean differences between groups. White asterisks represent that statistical difference was found in comparison with negative control. Data shows the mean of three independent experiments performed in triplicate at different times ( $p < 0.05$ ).

The PS concentration did not impact the results since the increase in concentration did not mean an increase in the antimicrobial effect. For *P. gingivalis* the greatest reduction on bacteria viability was found when Ce6 under the lowest concentration (100  $\mu$ M) was applied with blue light. For this reason, the groups including Ce6 under 100  $\mu$ M and light (blue and red) were considered for confocal images. A negative control group was included in the confocal analysis for comparison and validation. The reduction of bacteria viability was evident in the aPDT groups. These findings were confirmed in the confocal images (**Figure 3**). The distribution of green (live) and red (dead) fluorescence from stained bacteria under confocal analysis confirmed the biofilm formation and bacteria reduction previously mentioned. A slight difference in red fluorescence can be observed in the aPDT groups, the blue light group showed and intense red marking in the merged image.

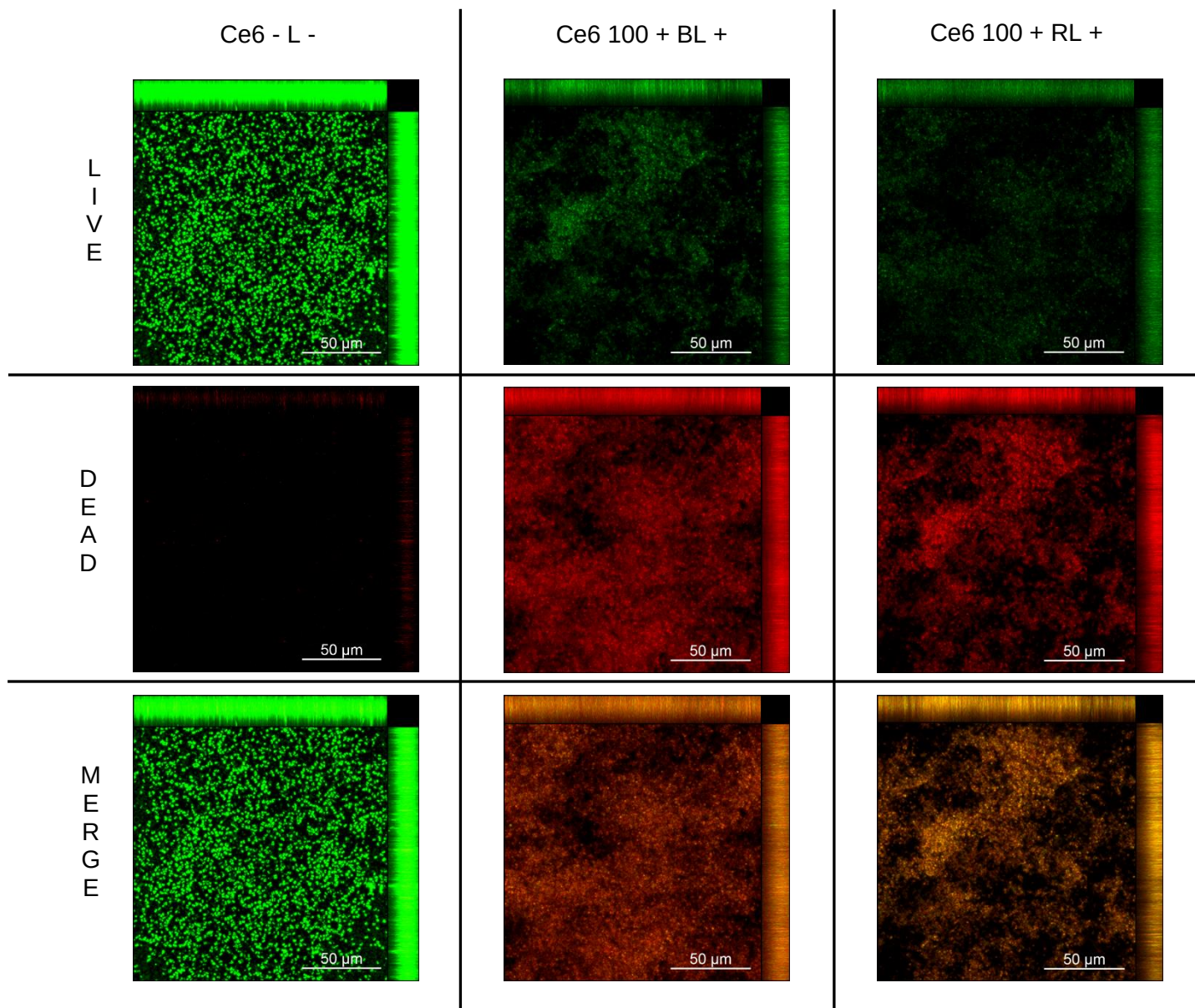


**Figure 3.** Images illustrating the effect of aPDT in the groups Ce6 100 + BL+ and Ce6 100 + RL+ besides the negative groups, under confocal laser-scanning microscopy. Images reveal the live and dead bacterial population.

#### 4 – Discussion

A multi-species biofilm was formed to mimic the challenger clinical condition of bacterial colonization on dental surfaces. The hypothesis that Ce6 combined with blue or red light would be able to reduce bacterial viability against the mentioned biofilm model was partially confirmed. This combination promoted a significant decrease of *S. orallis* and *P. gingivalis* viability, but no difference was found for *F. nucleatum* and *A. actinomycetemcomitans*. Although, there is a trend of bacterial reduction for Ce6 under blue light for these two strains, it was not enough to reach statistically significant difference.

In our results, both 100 and 200 µM Ce6-mediated aPDT were able to reduce around 2

$\log_{10}$  GeQ mL<sup>-1</sup> for *S. oralis* with blue light irradiation, while 1.6 and 1.9 were found for the red light, respectively. For *P. gingivalis*, only aPDT with blue light revealed some difference in comparison with negative control. Antimicrobial photodynamic therapy promoted higher bacteria elimination than CHX in our study. Although the use of CHX as a positive control in aPDT *in vitro* studies has been questioned, the authors suggest the use of a neutralizing solution to minimize CHX substantive effect and permit a fair comparison. In our study, DNA extraction started immediately after all treatments of the biofilm and PMA staining. Then, chlorhexidine substantivity did not relevantly impact the results and this may explain the low efficacy of CHX in our study (23).

It is important to highlight the bacteria species included in our biofilm model. *A. actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis* are considered key microorganisms in the development of the so-called periodontal pathogenic biofilm, not always directly involved in tissue damage and host's inflammatory response, but also in biofilm maturation by allowing colonization of other bacteria (24). The first is part of the green complex established by Socransky in 1998 (25). It is responsible for initial colonization of the subgingival biofilm, bacterial growth, and exacerbated inflammatory response by the host. For a long time, it was associated with rapid disease development and considered to be the crucial pathogen of the disease previously recognized as aggressive periodontitis (26). *F. nucleatum* is included in the orange complex. It is the one that appears lying right in the middle of periodontal biofilm in educational illustrations, it is also noted for its ability to connect to numerous microorganisms in the biofilm (providing specific binding sites for subsequent coaggregation), and that is one of the reasons why this species is difficult to eradicate and important in the transition to a complex and highly pathogenic biofilm (24). The last one is part of the red complex and it is considered the "bad guy" strongly associated with periodontitis, responsible for the production of important inflammatory growth factors directly related to tissue damage and disease progression (27). *S. orallis* is a genus of Gram-positive coccus easily found in the whole oral cavity. It was added to the biofilm model as a pioneer microorganism that allows the safe colonization of the others. Usually, it is present in the incipient aerobic plaque which will be further converted into anaerobic as the biofilm gets deep into the periodontal pocket and becomes complex.

Single species biofilms have been tested in similar prior studies with Ce6 (13,14,15,16). In spite of being worthy as an initial investigation, this biofilm model still falls short in mimicking the real disease situation (28). Multispecies biofilms are well-known for their ability

to resist the varying strategies of elimination due to the symbiosis and coaggregation among species, hence the choice of biofilm model in the present study (29). As far as we know, few studies have already tested the Ce6 against multi-species biofilm related to periodontitis (17,18), but only one considered the blue wavelength for light exposure (19). One study testing red light found a reduction of 3.2 and 4.8  $\log_{10}$  over *P.gingivalis* at 1110 nmol/cm<sup>2</sup> concentration of Ce6 and fluence of 32.2 J/cm<sup>2</sup> and 71.6 J/cm<sup>2</sup>, respectively (18). Another investigation found a 1.47  $\log_{10}$  reduction in the whole biofilm, considering a significantly high photosensitizer concentration (100  $\mu$ g/mL) and fluence of 150 J/cm<sup>2</sup> under red light exposure (17). These data corroborate the result found in the present study, since a similar reduction was found, although comparisons may not be fair due to different light source used, photosensitizer parameters and microbiological analysis.

Recently, Min Nie et al. evaluated the antimicrobial efficacy of Ce6 in combination with H<sub>2</sub>O<sub>2</sub> using both 450 nm (blue) and 660 nm (red) LEDs on multi-species biofilms (19). They found an expressive higher bacterial reduction compared to our study. The combination of 33.3 mM H<sub>2</sub>O<sub>2</sub> and Ce6-aPDT resulted in a 3-log reduction under red light and a 6.6 log reduction under blue light (19). These results demonstrate the research trend in new strategies to maximize the antimicrobial effect of Ce6, altering the photosensitizer formulation or delivery vehicle or testing synergistic effect with other solutions (18,19), as the blue light regimen may already be accepted as the first choice for the discussed condition.

In the case of anti-tumor therapy, another promising application that Ce6 has been tested, obviously, red-light irradiation is preferable once the region of the lesion is usually deep. However, for antimicrobial reasons, Ce6-mediated a PDT involves topical PS administration, and blue light has already proved to improve bacterial reduction in this condition (13, 19, 30). Additionally, one study has already reported that blue-light irradiation is potentially bactericidal for *P. gingivalis* (31) and *A. actinomycetemcomitans* (32) due to their endogenous intracellular pigment which works as a photosensitizer in the aPDT biological process. The highest reduction of *P.gingivalis* in the present study might be explained by the additional effect coming from the endogenous pigment.

Kurakina and collaborators affirm that light doses delivered with red light induce weaker tissue reaction as compared to the same doses delivered with blue light using chlorin-based photosensitizers in an animal study. Blue light showed a better impact on microcirculation and edema (33). The same group of researchers has affirmed before that wavelength around 405 nm might be beneficial for superficial impact, especially in the case of

antibacterial or antiaging photodynamic therapy (34). Blue light is already present in dental offices in the curing-light equipment, it could be easily used for aPDT administration if randomized clinical trials prove its efficiency.

As far as it is known, there is no studies evaluating ROS production of Ce6 under blue light in comparison with red light. The more pronounced effects of blue light observed in the present study might be correlated with a greater efficiency of quantum yield in the ROS generation. Although we cannot affirm this supported by quantitative data, this presumable phenomenon needs to be mentioned.

An in vitro model of investigation of the antimicrobial effect of Ce6 in aPDT was tested. Although a complex biofilm with a significative number of bacteria strains related to periodontitis was formed, in the clinical setting, other bacteria present in the oral environment may play a role in biofilm pathogenicity and a host response should never be left unconsidered, these details may constitute limitations of the present study, and extrapolation should not be made. Our data are relevant to encourage other researchers to apply the blue light in Ce6 experiments, and also support animal studies as the next steps. The optimization of Ce6 has been under current analysis by our group in a new delivery vehicle. In addition, the effect of aPDT with Ce6 as an adjuvant therapy to the mechanical disruption of the biofilm should also be evaluated.

## 5 – Conclusion

This study confirmed the antimicrobial effect of chlorin-e6 against periodontal pathogens, for a multi-species biofilm. The combination of Ce6 and blue light showed significant bacteria reduction especially for *Streptococcus oralis* and *Porphyromonas gingivalis*. Lower reduction was found with red light and only statically significant against *S. orallis*. Our results suggest other researchers to consider the blue light in the irradiation of chlorin-e6 for further investigations.

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## Conflicts of Interest

The authors declare no conflicts of interest.

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### 3.2 Artigo 2\*

#### **Chlorin-e6 conjugated to the antimicrobial peptide LL-37 loaded nanoemulsion enhances photodynamic therapy against multi-species biofilms related to periodontitis**

**Running title:** Chlorin-mediated photodynamic therapy against periodontal pathogens

#### **Author role**

All authors contributed in all aspects of the study.

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#### **Declaration of Competing Interest**

None of the authors has any conflict of interest to be disclosed.

#### **Conflict of interest statement**

This manuscript has been read and approved in final form by all authors and the authors declare no conflicts of interest.

#### RESEARCH ARTICLE

#### **Chlorin-e6 conjugated to the antimicrobial peptide LL-37 loaded nanoemulsion enhances photodynamic therapy against multi-species biofilms related to periodontitis**

### **Abstract**

In our previous studies, Chlorin-e6 (Ce6) demonstrated a significant reduction of microorganisms' viability against multi-species biofilm related to periodontitis while irradiated with blue light. However, the conjugation of Ce6 and antimicrobial peptides, and the incorporation of this photosensitizer in a nanocarrier, is still poorly explored. We hypothesized that chlorin-e6 conjugated to the antimicrobial peptide LL-37 loaded nanoemulsion could inhibit a multi-species biofilm related to periodontitis during photodynamic therapy. The nanoemulsion (NE) incorporated with Ce6 was characterized regarding the physiochemical parameters. Images were obtained by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Later, the Ce6 and LL-37 incorporated in NE was submitted to UV-Vis analysis and Reactive Oxygen Species (ROS) assay. Finally, the combined formulation (Ce6+LL-37 in nanoemulsion) was tested against multi-species biofilm related to periodontitis.

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The formed nanoformulation was kinetically stable, optically transparent with a relatively small droplet diameter (134.2 unloaded and 146.9 loaded), and weak light scattering. The NE system did not impact the standard UV-VIS spectra of Ce6, and the ROS production was improved while Ce6 was incorporated in the NE. The combination of Ce6 and LL-37 in NE was effective to reduce the viability of all bacteria tested. The current aPDT regimen was the best already tested against periodontal biofilm by our research team. Our results suggest that this combined protocol must be exploited for clinical applications in localized infections such as periodontal disease.

Keywords: Photodynamic Therapy, Chlorin e6, Photochemotherapy, Antimicrobial Peptides, LL-37 peptide.

## **1 – Introduction**

The covid-19 pandemic has further alerted the world to the increased risk of infections caused by viruses, fungi, and bacteria. There is an unpleasant worldwide consensus that humanity is losing this battle against microorganisms; lifestyle changes and scientific advances are required. Considering bacterial infections, antimicrobial photodynamic therapy (aPDT; also called photodynamic antimicrobial chemotherapy, PACT) is a promising alternative to traditional drugs, particularly for treating localized infectious diseases, such as periodontal disease [1]. Just as brief as possible, aPDT process is basically the interaction between light and photosensitizer (PS) in the presence of oxygen, generating reactive oxygen species which can damage bacteria [2,3]. The aPDT broad-spectrum activity and its multitarget mechanisms of action favor the lower likelihood of eliciting bacterial resistance [4,5].

Periodontitis is an oral infection triggered by a pathogenic bacterial biofilm developed in an anaerobic microenvironment [6], potentially reducing the efficacy of aPDT since it is known as an oxygen-based reaction. In our previous studies, our research team has demonstrated the effectiveness of the photodynamic therapy with chlorin-e6 (Ce6) as a PS under blue light against different bacteria related to periodontitis [7,8]. Until now, there are only a few studies investigating the antimicrobial effect of Ce6 against periodontal pathogens in vitro [9,10,11,12]. To the best of our knowledge, our team was the first to report the use of Ce6 activated by blue light against biofilms related to periodontitis. Afterward, Nie and collaborators reported the synergetic antimicrobial effect of Ce6, under blue light; and hydrogen

peroxide ( $\text{H}_2\text{O}_2$ ) against multi-species biofilms [12]; with this strategy,  $\text{H}_2\text{O}_2$  is expected to provide oxygen for the aPDT process in an oxygen-depleted environment.

However, the aPDT function relies on PS effectiveness, light delivery and absorption. When the transition to a clinical application is desired, the PS formulation is a concern. Photosensitizers developed for aPDT can be hydrophobic molecules that aggregate easily in water, impairing clinical administration due to lower bioavailability and therapeutic efficiency [13]. Then, nanoemulsion (NE) appears to be a convenient carrier for these water poorly-soluble drugs. NE is a biphasic nanosystem composed of oil-in-water and an appropriate stabilizing surfactant, a high solubilizing capacity for lipophilic drugs is expected for NEs [14]. NEs exhibit kinetic stability during storage; extended contact area to promote intracellular delivery; increased circulation time in the body, maximizing bioavailability; and improved drug biodistribution, consequently minimizing dosage [15, 16, 17, 18, 19].

The conjugation of a PS and an antimicrobial peptide (AMP) has been investigated as a strategy to enhance microorganism inactivation in the aPDT application. These small peptides produced by most eukaryotic organisms play an important role during the non-specific innate immune response [20]. Usually, AMPs bind to pathogens' membranes allowing permeabilization, disruption of the membrane's physical integrity takes place, and the leakage of intracellular components leads to cell death [21,22]. The breakdown of the bacteria membrane, especially the complex and hard-to-be defeated outer membrane of Gram-negative bacteria, permits the penetration of the PS and subsequent damage closer to DNA. In line with this, the combination of PS and AMP for aPDT increases antibacterial activity and target selectivity, and decreases the time of application [23,24,25].

The last three paragraphs support the rationale of the present study. Several strategies to enhance aPDT efficacy have been explored recently, such as: AMP conjugation, nanocarrier loading, synergetic effect with different solutions, and improved light regimen, to name a few. Our team takes some of these strategies together in the present study based on our previous results [7,8] and the recent literature [12, 16, 20, 22, 23, 24], aiming at exploring the best properties of each one and quantify the impact over the altogether treatment. We hypothesized that chlorin-e6 conjugated to the antimicrobial peptide LL-37 loaded nanoemulsion could inhibit a multi-species biofilm related to periodontitis during photodynamic therapy.

## 2 – Material and Methods

### 2.1 Photosensitizer, Antimicrobial Peptide, Hydrogen Peroxide, and Light Source

Chlorin-e6 was semi-synthesized in the Bioorganic Chemistry Laboratory at Federal University of São Carlos - UFSCar (São Carlos-SP, Brazil), from the extraction of chlorophyll present in the *Spirulina maxima*, a seaweed used in food supplementation (rich in vitamins and proteins), as previously described [26]. Later, 200  $\mu\text{M}$  (119,34 mg/L) of Ce6 was added to the nanosystem after the first sonication to reach the working concentration determined by previous studies [7,8].

The antimicrobial peptide (AMP) LL-37 (1 mg) was purchased from SmartBioscience (Saint Egrève, France). Considering a previous study that determined the mean minimum inhibitory concentration of LL-37 against periodontopathogenic microorganisms at 56.51  $\mu\text{g/ml}$  [27], and LL-37 was incorporated into a nanoemulsion system conjugated to Ce6 in our study, a lower concentration is expected to obtain some level of bacteria inactivation, so concentration was set at 8  $\mu\text{M}$  (35.92  $\mu\text{g/ml}$ ) in the present study.

The hydrogen peroxide (Sigma-Aldrich, Darmstadt, Germany) was tested alone or in combination with Ce6 and AMP. When aPDT was performed with  $\text{H}_2\text{O}_2$ , 33.3mM of  $\text{H}_2\text{O}_2$  was placed in contact with the biofilm first and Ce6 + AMP was added later, there was no washout between them.  $\text{H}_2\text{O}_2$  concentration was set according to a previous study [12].

As a pioneering initiative, our group tested the use of the gold-standard dental curing-light (VALO™ Cordless-LED Curing Light, Ultradent, South Jordan, UT, USA) as a light source for aPDT application. It is a blue light source equipment daily used at the dental office for restorative treatments and cementation of prosthetic parts, therefore, safe on oral tissues. The blue light is generated at wavelengths between 385-515 nm, producing high-intensity light with two peaks at  $\sim 410$  (right at the Soret absorption band of Ce6) and  $\sim 470$  nm. The light intensity of the equipment was set at 1000 mW/cm<sup>2</sup>, and 60 seconds was required to reach a light dose of 60 J/cm<sup>2</sup>, in 3 intermittent cycles of 20 seconds of application [8].

### 2.2 Obtention of nanoemulsion

The association of Ce6 and LL-37 to the nanoemulsion system was carried out as described in a previous study [28]. The nanostructured lipid system (nanoemulsion) was prepared with 10% cholesterol (oil phase), 10% polyoxyethylene 20-cetyl ether (Brij 35®,

Sigma-Aldrich), and phosphatidylcholine (Epikuron<sup>®</sup> 200 – Surfactant 2:1, Lecithin) and 80% phosphate buffered saline solution (PBS – aqueous phase) [28]. The nanoemulsion was prepared under low temperature in ultrasound (Q500 – Qsonica, Newtown, CT, USA), with a power of 500 W, 13% amplitude, in a discontinuous mode for 10 min (2 min on, 30 sec off). Afterward, Ce6 was added at the concentration mentioned before, and the same cycle was repeated at 17% amplitude.

## 2.3 Physicochemical characterization

### 2.3.1 Average hydrodynamic diameter, Polydispersion Index and Zeta Potential

The determination of average hydrodynamic diameter and polydispersion index (PDI) of the pure NE or associated with Ce6 was performed by dynamic light scattering (DLS) at 20 °C. The pure NE or associated with Ce6 was also characterized in terms of zeta potential using electrophoretic mobility. The nanoparticles were diluted in ultrapure water (10 µL of the pure NE or Ce6 NE + 990 µL of water) and evaluated in a Zetasizer Nano ZS equipment (Malvern Instruments, Worcestershire, United Kingdom) [29].

### 2.3.2 Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)

The Ce6-loaded nanoemulsion was diluted (1:20) in deionized water, 3 µL was added to the copper grids and counterstained with 2% uranyl acetate solution in distilled water for 5 minutes. The samples were dried at room temperature for 15 minutes before analysis. TEM of two samples were performed using a Phillips CM200 Transmission Electron Microscope, operating at 200 kV, with a LaB6 (lanthanum hexaboride) filament.

For SEM, the coverslips with Ce6-loaded nanoemulsion previously diluted were dried at room temperature and placed in a vacuum desiccator for 7 days with silica gel. Subsequently, the analyzed samples were metalized with gold-palladium and fixed to the stubs using a conductive adhesive and the morphological analysis of the samples was performed using a Zeiss electron microscope (Jeol, model JSM-7500F) with a voltage of 5.00 kV.

## 2.4 Photo-physical analysis

### 2.4.1 Ultraviolet/Visible Spectroscopy (UV-Vis)

UV-Vis analysis was performed to identify changes in the absorption spectrum of the photosensitizer incorporated in the nanoemulsion (350 to 770nm) [24]. The solutions were prepared at a concentration of 200µM pure Ce6 and Ce6 with LL-37 in NE. A light dose of 60 J/cm<sup>2</sup> was divided into 6 irradiation cycles of 10 seconds each. Measurements were taken in Synergy H1M equipment (Synergy H1 Multi-Mode Reader, BioTek, Winooski, VT, USA). This evaluation allowed us to identify if any component of the nanoemulsion system or if LL-37 would interfere with the absorption wavelengths of the photosensitizer.

### 2.4.2 Reactive Oxygen Species (ROS)

Reactive oxygen species were detected by fluorescence using the Diacetate 3',7' - dichlorofluorescein - H2-DCF-DA probe (Thermo Fischer Scientific, MA, USA), following the manufacturer's protocol. Pure Ce6 (200µM) and Ce6 with LL-37 in NE were diluted in phosphate buffer (0.1M; pH 7.2), 3 µL of the mentioned probe was added in a 96-well black plate [24]. The solutions were irradiated following the previous description (**section 2.1**). Fluorescence readings were performed immediately after irradiation using Synergy H1M (Synergy H1 Multi-Mode Reader, BioTek, Winooski, VT, USA). The excitation and emission wavelengths were set at 490/515 nm and 505/525 nm, respectively.

## 2.5 *In vitro* Biological assay

### 2.5.1 Human Saliva

The present study was previously reviewed and approved by the Ethics Committee of the School Dentistry at São Paulo State University (UNESP), Brazil, CAAE: 23396619.5.0000.5416. Unstimulated saliva was collected and proceeded according to a previous study [11]. Human saliva composed the first layer of the multi-species biofilm described latterly.

### 2.5.2 Bacterial strain, growth conditions, and biofilm formation

*Streptococcus oralis* (So; American Type Culture Collection - ATCC 35037), *Fusobacterium nucleatum* (Fn; ATCC 25586), *Porphyromonas gingivalis* (Pg; ATCC 33277)

and *Aggregatibacter actinomycetemcomitans* (Aa; JP2) were cultured in Brucella agar Petri dishes (Brucella Agar, Neogen, Idaiatuba, SP, Brazil; Petri dish 90 x 10 cm, Kasvi, Sao Jose do Pinhais, PR, Brazil) with 5% defibrinated sheep blood (Microlab, Campinas, SP, Brazil) supplemented with yeast extract (6 g/L), hemin (10 mg/mL) and menadione (5 mg/mL) at 37°C. Atmosphere condition was considered for each bacterial strain; an anaerobic condition for *F. nucleatum* and *P. gingivalis* (85% N<sub>2</sub>, 10% H<sub>2</sub> e 5% CO<sub>2</sub>, White Martins, Rio de Janeiro, RJ, Brazil; anaerobic workstation, VA 500, Don Whitley Scientific, England), and incubation in a 5% CO<sub>2</sub> environment (Incubator, GE Healthcare Life Sciences, Chicago, IL, USA) for *S. oralis* and *A. actinomycetemcomitans*.

Bacterial suspensions were later adjusted according to the mid-exponential growth phase established previously by optical density (OD600 nm, BioSpectrometer® D30, Eppendorf, Hamburg, Germany) and colony-forming units (CFU mL<sup>-1</sup>) (*P. gingivalis*, 15 h, OD600 nm = 0.95 ± 0.01, CFU mL<sup>-1</sup> = 4.3 x 10<sup>9</sup>; *F. nucleatum*, 10 h, OD600 nm = 0.45 ± 0.01, CFU mL<sup>-1</sup> = 1 x 10<sup>8</sup>, *S. oralis*, 4 h, OD600 nm = 0.55 ± 0.01, CFU mL<sup>-1</sup> = 1.5 x 10<sup>9</sup>, *A. actinomycetemcomitans*, 8 h, OD600 nm = 0.18 ± 0.01, CFU mL<sup>-1</sup> = 5.66 x 10<sup>8</sup>) until reaching 10<sup>7</sup> CFU mL<sup>-1</sup> [11].

Fifty microliters of sterilized saliva were placed at the bottom of each well of a 96-well plate (Kasvi, São Jose do Pinhais, PR, Brazil) and kept in an orbital shaker (430 RD incubator, Nova Etica, Brazil), 75 rpm at 37 °C for 4 h in order to promote initial bacterial attachment [8]. A 200 µL aliquot of the standardized suspension (10<sup>7</sup> CFU mL<sup>-1</sup>) of *S. oralis* was added first to each well (pretreated with a saliva-derived film) and incubated in aerobic conditions, at 37 °C. After 24 h, nonadherent bacteria and culture media were removed and 200 µL of the same concentration of *F. nucleatum* was added in fresh medium to each well, then plates were incubated in anaerobic conditions. After 24 h, the suspension was removed and 100 µL of *P. gingivalis* and 100 µL of *A. actinomycetemcomitans* (both 10<sup>7</sup> CFU mL<sup>-1</sup>) were added to each well to continue biofilm formation (anaerobic condition). The culture media was replaced daily, for 3 more days, very slowly, to avoid biofilm disruption. For all groups, the wells were washed twice at the end of the incubation period and before further analyses.

### 2.5.3 Control and investigated groups

The groups were divided considering the light irradiation and the presence of Ce6, LL-37, H<sub>2</sub>O<sub>2</sub>, and nanoemulsion, in three independent plates. All variables were tested in isolation

to identify the effect of each of these further. So, the groups were divided as follows: 1) Negative control – **Ctrl**, no Ce6 (distilled water was applied to avoid biofilm dryness) and no light; 2) **Ce6**, chlorin-e6 formulated in nanoemulsion at 200  $\mu\text{M}$  with no light; 3) **LL-37**, antimicrobial peptide LL-37 at 8  $\mu\text{M}$  with no light; 4) **Nano**, the formulated nanoemulsion was tested unloaded with no light; 5) **H<sub>2</sub>O<sub>2</sub>**, 33.3mM of H<sub>2</sub>O<sub>2</sub> with no light; 6) **Blue**, blue light irradiation (60 J/cm<sup>2</sup>); 7) **Blue + LL-37**, antimicrobial peptide LL-37 at 8  $\mu\text{M}$  with blue light; 8) **Blue + Ce6 + LL-37**, chlorin-e6 formulated in nanoemulsion at 200  $\mu\text{M}$  and antimicrobial peptide LL-37 at 8  $\mu\text{M}$  with blue light; 9) **Blue + Ce6 + LL-37 + H<sub>2</sub>O<sub>2</sub>**, pre-treatment with 33.3mM of H<sub>2</sub>O<sub>2</sub>, then chlorin-e6 formulated in nanoemulsion at 200  $\mu\text{M}$  and antimicrobial peptide LL-37 at 8  $\mu\text{M}$  with blue light.

#### 2.5.4 Photodynamic Inactivation

After biofilm formation, the culture media was removed and 100  $\mu\text{L}$  of treatment solutions were added according to the description on **section 2.5.3**. All groups with Ce6 were incubated in the dark at room temperature for 5 min (pre-irradiation time). Subsequently, the 96-well plate with the groups 6 to 9 was irradiated with blue light (450 nm) under the light dose described previously (**section 2.1**). The plate with no light regimen was kept in the dark for the same time as the plate exposed to light to analyze any possible dark toxicity effect of Ce6 against the biofilm.

#### 2.5.5 DNA extraction and qPCR assay

After aPDT application, biofilms were carefully washed twice with phosphate buffer saline (PBS), removed (ultrasonic generator, Cristofoli, Parana, Brazil), and transferred to microtubes for subsequent DNA extraction. Before DNA extraction, samples were exposed to PMA (propidium monoazide) (Biotium, USA), a photoreactive DNA binding dye used to detect viable microorganisms, preventing from the amplification of dead cells in qPCR reactions. Then, DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The purified sample was stored at -80°C (Thermo Scientific 88400D, Massachusetts, EUA) until qPCR reaction.

The qPCR quantification reactions were performed on the CFX96 real-time system equipment (BioRad, Hercules, CA). The Taqman system with specific primers and probes was used [8]. For the Taqman reactions, 12.5  $\mu\text{L}$  of Mastermix (Eurogentec, Seraing, Belgium), 4.5

$\mu\text{L}$  of sterile  $\text{H}_2\text{O}$ , 1  $\mu\text{L}$  of each primer and probe and 5  $\mu\text{L}$  of each sample were used. Primers and probes were used at different concentrations depending on the bacteria. The amplification conditions were: 2 minutes at 50 °C, 10 minutes at 95 °C, followed by 45 cycles of 95 °C for 15 s and 60 s at 60 °C. This experiment was carried out in triplicate, in 3 independent replications (n=9).

## 2.6 Statistical Analyses

Three independent experiments were performed in triplicate at different times for qPCR analysis. Thus, nine wells were obtained for each group (n = 9). Data distribution was checked using descriptive statistical analysis and the Shapiro-Wilk test. Data were submitted to one-way ANOVA with Welch's correction, and Tukey's post-hoc test for multiple comparisons (p<0.05). For the characterization of NE, all tests were taken in triplicate, and descriptive analyses were performed. Statistical analyzes were performed using IBM® SPSS® Statistics Grad Pack 26.0 (New York, USA), with a significance level of 5%.

## 3 – Results

### Characterization of nanoemulsion

Twenty-four hours after the preparation of NE, organoleptic characteristics of loaded (Ce6-NE) and unloaded (NE) nanoemulsion were evaluated by observing signs of phase separation, flocculation, and sedimentation, in addition to physical aspects such as translucency, fluidity and odor. Alterations were not observed. The formulations presented an opaque and milk characteristic.

Average hydrodynamic diameter and polydispersity index were determined by DLS and zeta potential using electrophoretic mobility. **Table 1** shows mean values of average hydrodynamic diameter and PDI of Ce6-NE and NE, where it is observed that the diameter of NEs particles (blank) was 134.2 d.nm, and 146.9 d.nm for Ce6-NE. The slight increase in particle size may be due to the incorporation of Ce6. It is important to highlight the homogeneity of the formulation, before and after incorporation of Ce6, where 97.3% and 92% of particle sizes are distributed around the main peak range, respectively (**Figure 1**). For Ce6-NE, light-scattering analysis showed a mean PDI value of 0.308 after the incorporation of Ce6 to NE, indicating a good droplet size distribution and stability of NE system. This parameter directly reflects droplet size homogeneity in total NE (**Figure 1 A, C**).

Zeta potential (ZP) is an indication of repulsive forces between oil droplets in an

emulsion. High ZP values (positive or negative) indicate the difficulty of droplet coalescence and high stability of the emulsion. There is a small increase of zeta potential after incorporation, which can be considered as evidence of the increased stability (**Figure 1 B, D**). The formulations may be considered stable, with little tendency to flocculate [30].

**Table 1** - Particle size, polydispersity index and zeta potential value of NE and GE-NE

| Formulation | Particle Size (d.nm) | Polydispersity index (PDI) | Zeta Potential (mV) |
|-------------|----------------------|----------------------------|---------------------|
| NE          | 134.2                | 0.369                      | -3.16               |
| Ce6-NE      | 146.9                | 0.308                      | -4.39               |



**Figure 1:** Droplet size, polydispersity index and zeta potential of nanoemulsion (A e B) and Ce6-loaded nanoemulsion (C e D).

The particles of Ce6-NE can be seen in **Figure 2** for TEM (**A**) and SEM (**B**), note the rounded appearance of the particles and the size corroborates the quantification described in

DLS. Considering the TEM image, we speculate that Ce6 is concentrated in the core (oil phase) and LL-37 is dispersed around the core in the water phase.

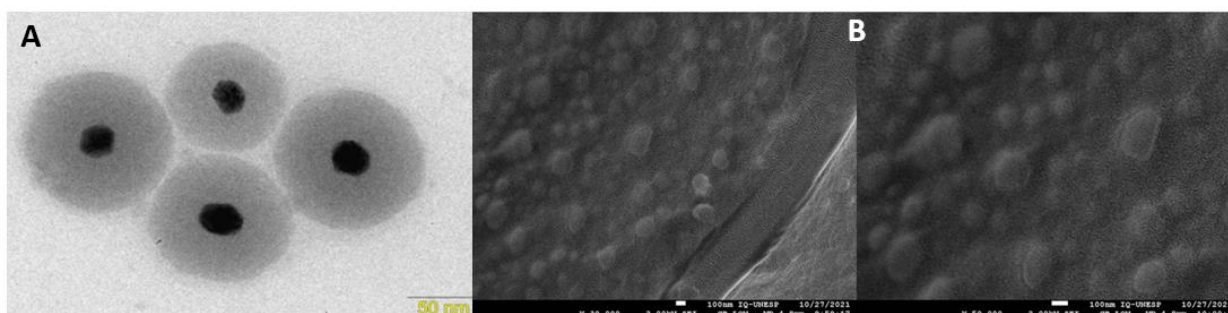


**Figure 2:** Images obtained by transmission electron microscopy (TEM - **A**) and scanning electron microscopy (SEM - **B**) represent the Ce6-loaded nanoemulsion.

### Photonic analysis

In order to check any alteration in the absorption spectrum or ROS production of Ce6 after its incorporation in NE, Ce6 and Ce6-NE were submitted to photonic analysis. **Figure 3** shows the absorption spectrum of Ce6 and NE loaded with Ce6 and LL-37, some small differences can be observed along the entire absorption spectrum, but differences in absorption peaks cannot be noticed though, note the Soret (~400nm) and q bands (~660nm) appears to be right at the same range after incorporation.

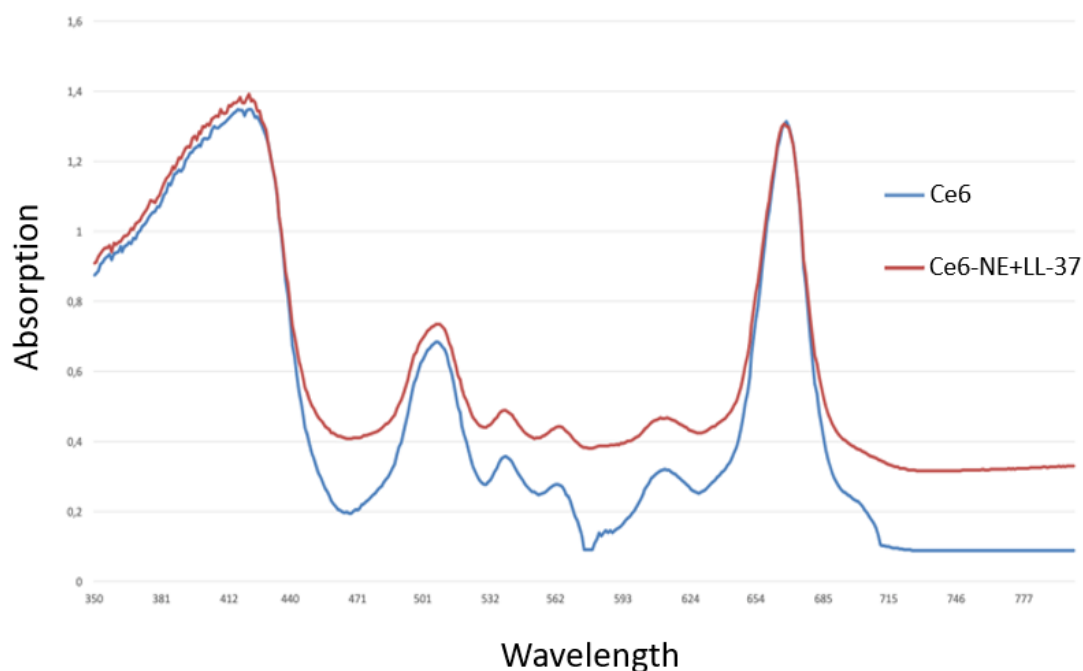


**Figure 3:** Absorption spectrum of the photosensitizer (Ce6) pure (in blue) and Ce6-NE in the presence of LL-37 (in red).

The production of reactive oxygen species was estimated by the fluorescence intensity after the incorporation of the detection probe (**section 2.4.2**) and irradiation with blue light (**section 2.1**). The application of blue light was responsible for a significant increase in fluorescence intensity (**B and C**), the LL-37 did not affect the ROS production. The highest fluorescence intensity was observed in the Ce6+LL-37 group incorporated in NE (**Figure 4**).

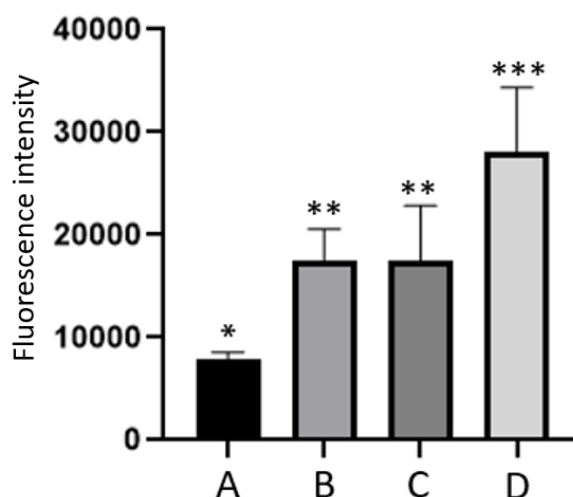


**Figure 4:** Detection of Reactive Oxygen Species (ROS). Group A = light application only, B = Ce6 + blue light, C = Ce6 + LL-37 + blue light, D = Nanoemulsion (Ce6 + LL-37) + blue light.

### Antimicrobial activity

The qPCR assay showed that all bacterial strains were similarly present in the mature multi-species biofilm on the surface of the wells. *S. oralis* was found in the highest concentration ( $9.08 \text{ Log}_{10} \text{ GeQ mL}^{-1}$ ) and *P. gingivalis* was the least present ( $5.04 \text{ Log}_{10} \text{ GeQ mL}^{-1}$ ) in the negative controls (**Figure 5**). The photosensitizer in the absence of light did not demonstrate any impact on bacteria viability, so no dark toxicity was identified for Ce6 in this experiment, just as any variable tested.

Briefly, the most significant bacterial elimination was observed in the groups where Ce6-NE was applied with LL-37 and  $\text{H}_2\text{O}_2$  irradiated by blue light, for *S. oralis* ( $1.8 \text{ Log}_{10} \text{ GeQ mL}^{-1}$  for,  $p < 0.0001$ ), for *F. nucleatum* ( $1.21 \text{ Log}_{10} \text{ GeQ mL}^{-1}$  for,  $p < 0.01$ ), for *A. actinomycetemcomitans* ( $0.93 \text{ Log}_{10} \text{ GeQ mL}^{-1}$  for,  $p < 0.05$ ), and for *P. gingivalis* ( $0.9 \text{ Log}_{10} \text{ GeQ mL}^{-1}$ ,  $p < 0.001$ ), compared to the control. All tested variables exhibit a significant impact on the bacteria viability of *S. oralis* compared to the control. For *P. gingivalis*, groups where the blue light was used (Blue + LL-37, Blue + Ce6 + LL-37, Blue + Ce6 + LL-37 +  $\text{H}_2\text{O}_2$ ) were

found as significant different when compared to the control.

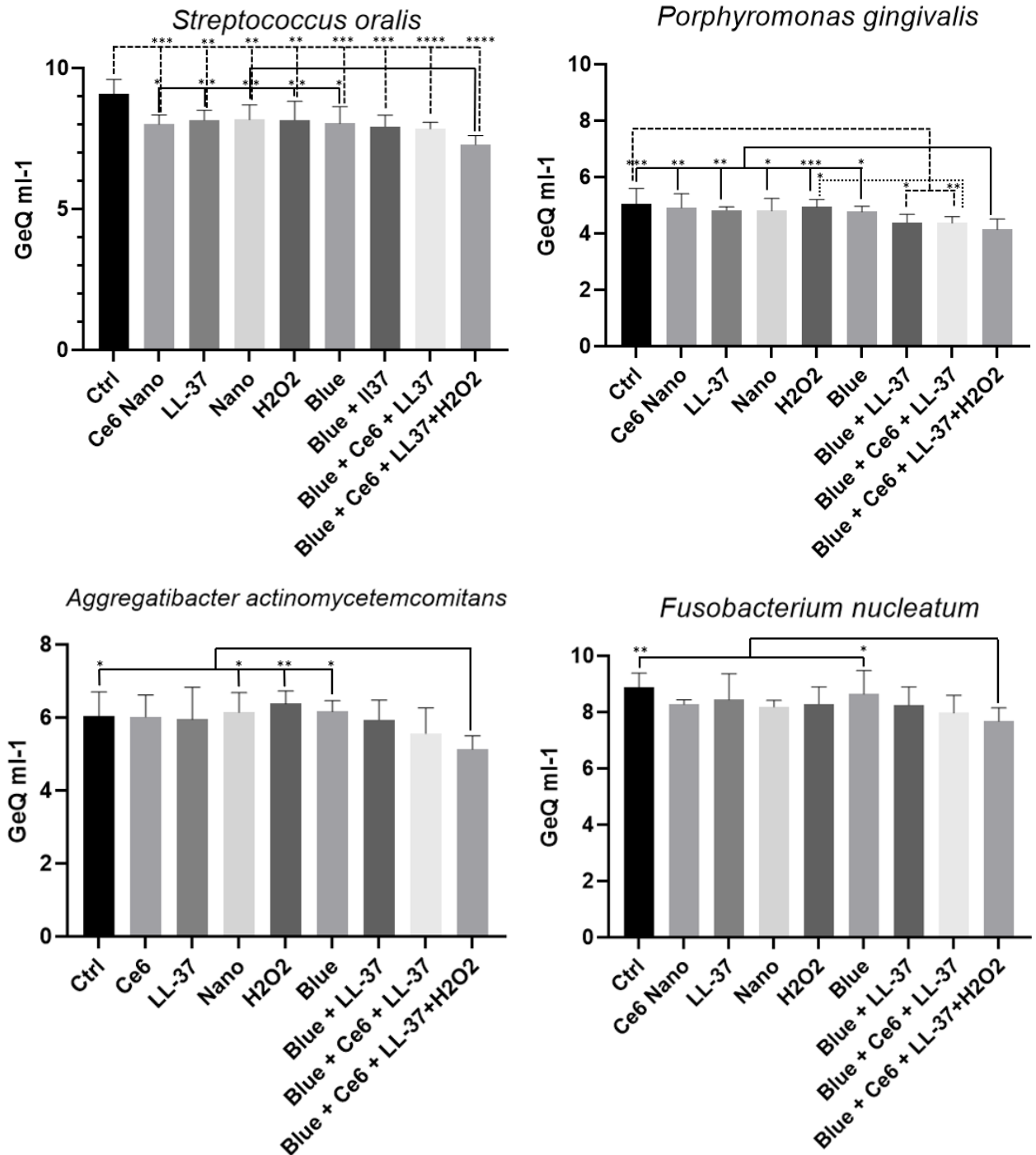


**Figure 5:** Effect of light irradiation and the presence of Ce6, LL-37, H2O2, and nanoemulsion against multi-species biofilm of *S. oralis*, *A. actinomycetemcomitans*, *F. nucleatum* and *P. gingivalis*. Quantitative data of bacteria viability after treatments is shown in (Log<sub>10</sub> GeQ mL<sup>-1</sup>). Linked lines mean differences between groups. Data represent the mean and  $\pm$  SD of three independent trials performed in triplicate at different times (\* -  $p < 0.05$ ; \*\* -  $p < 0.01$ ; \*\*\* -  $p < 0.001$ ; \*\*\*\* -  $p < 0.0001$ ).

#### 4 – Discussion

A multi-species biofilm was formed to mimic the challenger clinical condition of bacterial colonization associated with periodontal disease. The hypothesis that Ce6 combined with LL-37 and H<sub>2</sub>O<sub>2</sub> would be able to reduce bacterial viability against the mentioned biofilm model was confirmed. It is possible to notice a trend of bacterial reduction as the variables were added separately to the aPDT treatment until the moment when all were tested simultaneously, in group Blue+Ce6+LL-37+H<sub>2</sub>O<sub>2</sub>, where the highest antimicrobial effect was observed. The tested combination promoted a significant decrease in all the evaluated bacteria strains.

In this study, a nanoemulsion was developed and the main physiochemical parameters were investigated. The formed nanoformulation was kinetically stable, as no gravitational separation was observed; optically transparent with a relatively small droplet diameter (134.2 unloaded and 146.9 loaded), and weak light scattering [31]. The sonication method was used because it is effective, and allows better control of granulometry and a wide choice of formulation constituents [32, 33]. Lecithin is a non-toxic amphoteric surfactant that is well tolerated by the body and it can be fully metabolized [33]. In addition, they contain negatively charged polar lipids that are of great importance in the long-term stability of emulsions, as they confer a negative zeta potential at the interface, allowing electrostatic repulsion between the dispersed droplets.

Several drug delivery systems have been studied for improving aPDT, such as liposomes, microemulsions, and nanoparticles [34, 35]. The nature of neutral porphyrinoid photosensitizers generally used in aPDT is typically tending to hydrophobic ( $\log p = 6.12$ ), thus, nanoemulsions can encapsulate these PS, resolving the solubility problems in the aqueous medium [34,36]. Nanoemulsions have several advantages such as the solubilization of hydrophobic compounds, excellent stability, sustained release, reduction of toxicity, and promotion of drug activity [37,38]. Then, we speculate that the formed nanoemulsion is able to carry Ce6 in its oil phase, and also able to carry LL-37 in its water phase, constituting an excellent carrier system for the compounds tested in the present study.

The nanoemulsion formulated in this study showed average particle sizes of 134.2 d.nm unloaded and 146.9 d.nm loaded, corroborating other studies that consider that the size of the NEs must be between 100 and 300 nm, and particles are expected to be found in a small range of size distribution to be considered stable against the processes of flocculation, creaming, sedimentation and coalescence [39,40]. The parameters of the nanoemulsion formulated in the

present study are similar to other NE parameters already mentioned before [41,42]. When comparing the unloaded and loaded formulations, an increase in particle size was observed after incorporation, indicating carryover to the lipid system. Images of TEM and SEM corroborated the results from NE characterization.

The photosensitizer was tested for its photophysical properties, considering possible ROS production and absorption spectrum changes after incorporation into the nanoemulsion system and aggregation to the antimicrobial peptide. The results proved that the NE system did not impact the standard UV-VIS spectra of Ce6, similarly to the results obtained by Freitas et al [24], where Ce6, methylene blue, and curcumin were enhanced by the peptide aurein 1.2, and no significant variations were observed in the absorbance spectrum of the three photosensitizers tested. For ROS production, there was no change when LL-37 was added to the system, but when LL-37 was incorporated into the NE system, there was a significant increase in ROS production. We theorize that NE system gives extra protection against photobleaching to the Ce6, then more intact PS is preserved until the blue light is irradiated. The photobleaching assay should be addressed later to better understand this phenomenon. The blue light tested proved to be effective for PS activation and ROS production in the present study

There is a raising interest in the conjugation of photosensitizers to antimicrobial peptides, different PS and AMP have already been tested [25, 43, 44]. Studies hypothesize that AMP works as promising bacteria-targeted delivery of photosensitizers, improving PS internalization into bacteria, and allowing damage closer to bacterial DNA [24, 45]. A recent systemic review and meta-analysis showed better outcomes of aPDT associated with peptides than aPDT alone for controlling the microbial load (OR = 0.14/p = 0.0235/I-squared = 0%) [46]. Our results corroborate the current literature on the topic of AMP combined with aPDT to enhance microbial reduction, we proved that Ce6 and LL-37 have a synergic effect against Gram-positive and Gram-negative bacteria, and promote extra microbial reduction compared to our previous results [8].

The combination of Ce6, LL-37 and H<sub>2</sub>O<sub>2</sub> effectively reduced the viability of all bacteria tested. As far as our knowledge goes, this is the first time that Ce6 is tested in combination with LL-37 and H<sub>2</sub>O<sub>2</sub> against a multi-species biofilm. The antimicrobial effect of Ce6 and AMP against single species biofilm of *E. faecalis* [25], *S. aureus* [25], *E. faecium* [25], *P. aeruginosa* [47], *E. coli* [48] has already been described. Nie and collaborators reported the synergetic antimicrobial effect of Ce6, under blue light; and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) against multi-species biofilms [12]. Our results corroborate recent studies on the topic and encourage the

development of animal studies to investigate further this combined formulation for aPDT.

The biofilm model developed in the present study needs to be highlighted, each bacterium was selected to mimic the biofilm associated with periodontal disease. *A. actinomycetemcomitans* (Aa), *F. nucleatum* (Fn), and *P. gingivalis* (Pg) have an important role in the development of the so-called periodontal pathogenic biofilm, Pg and Aa are directly involved in tissue damage and host's inflammatory response, and Fn in biofilm maturation by allowing colonization of other bacteria [49]. The first is responsible for the initial colonization of the subgingival biofilm, bacterial growth, and exacerbated inflammatory response by the host, and it is frequently associated with rapid disease development [50]. *F. nucleatum* is noted by its ability to connect to numerous microorganisms in the biofilm (providing specific binding sites for subsequent coaggregation), and that is the reason why this specie is difficult to eradicate and important in the transition to a complex and highly pathogenic biofilm [49]. The latter is the one identified as the major producer of virulence factors contributing to disease progression and tissue damage [51]. *S. orallis* is usually present in the incipient aerobic plaque, and it was added to the biofilm model as a pioneer microorganism to establish initial connections to salivary film. However, we need to keep in mind that the biofilm developed is still far from the actual situation in the mouth. The biofilm used in this study cannot completely mimic the original subgingival biofilm around the tooth and implants.

## 5 – Conclusion

The Ce6 was effectively incorporated in a nanoemulsion with adequate physiochemical parameters and no implication in the biophotonic characteristic. The antimicrobial effect of chlorin-e6 with LL-37 and H<sub>2</sub>O<sub>2</sub> in NE against multi-species biofilm of periodontal pathogens was confirmed. When drawing conclusions from this research, it is important to note that the present study represents the most recent step in a series of studies investigating Ce6 in aPDT [7,8], in our research group experience, the current aPDT regimen was the best already tested by us against periodontal biofilm, and the one selected for following investigations.

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### Conflicts of Interest

The authors declare no conflicts of interest.

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### 3.3 Artigo 3\*

#### **Multiple PDT sessions with chlorin-e6 conjugated to the antimicrobial peptide LL-37 loaded nanoemulsion for experimental periodontitis treatment in rats**

**Running title:** Chlorin-mediated photodynamic therapy for periodontitis in rats

#### **Author role**

All authors contributed in all aspects of the study.

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#### **Declaration of Competing Interest**

None of the authors has any conflict of interest to be disclosed.

#### **Conflict of interest statement**

This manuscript has been read and approved in final form by all authors and the authors declare no conflicts of interest.

### RESEARCH ARTICLE

#### **Multiple PDT sessions with chlorin-e6 conjugated to the antimicrobial peptide LL-37 loaded nanoemulsion for experimental periodontitis treatment in rats**

### **1. Introduction**

Periodontitis is an oral infection triggered by pathogenic bacterial species and exacerbated host inflammatory response<sup>1</sup>. Controlling host response and bacterial biofilm is the way for disease resolution, then patient may start maintenance therapy. Scaling and root planning (SRP) is well-known as the professional mechanical removal of the pathogenic biofilm, although it has been around for a long time and is considered the standard treatment to restore periodontal health, when complicating factors (difficult access to instrumentation, incompetent host response, systemically compromised patient, poorly informed or unmotivated patient regarding oral hygiene,) are present, this traditional therapy might not be enough to reach disease resolution<sup>2</sup>.

Several randomized controlled clinical trials have shown satisfactory results in the use of photodynamic therapy (PDT) as an adjunct strategy in the treatment of periodontitis<sup>3-5</sup> and peri-implantitis<sup>6-8</sup>, since SRP is sufficient for the majority of the cases, PDT is suggested as an adjuvant for patients with type-II diabetes mellitus, residual pockets, and on maintenance

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\* Artigo formatado segundo as normas do periódico Journal of Clinical Periodontology para o qual será submetido.

therapy. However, some studies showed that the therapy may not be as effective as many researchers believe<sup>9-12</sup>. Most of the data noticed as positive results are related to microbiological and immunological parameters, when it comes to clinical parameter, especially reduction of probing depth (PD) and gain of clinical attachment level (CAL), the results are not promising enough to convince clinicians to the use PDT routinely. These controversial results are due to the variation of different treatment protocols used in PDT.

In our previous studies, our research team has demonstrated the effectiveness of the photodynamic therapy with chlorin-e6 (Ce6) and blue light against different bacteria related to periodontal disease<sup>13-14</sup>. Later, this photosensitizer (PS) was carried in a biphasic nanosystem composed of oil-in-water and an appropriate stabilizing surfactant, after characterization of this nanoemulsion (NE), Ce6 + NE demonstrated a great antimicrobial effect against biofilms related to periodontitis<sup>15</sup>. In the same study, our group confirmed the synergic effect of PDT with Ce6 and an antimicrobial peptide, LL-37 was added to the nanosystem described previously and a higher antimicrobial activity was noted when this combination was tested<sup>15</sup>.

Several animal studies have already demonstrated that PDT enhances the wound healing and reduction in inflammatory cells in periodontal tissue<sup>16-19</sup>. Repeated sessions of PDT as an adjunct or alternative therapy were even more effective at reducing alveolar bone loss, regulating bone metabolism, and reducing *Prevotella nigrescens* and *Aggregatibacter actinomycetemcomitans*, in rats treated with chemotherapy and induced periodontitis than SRP alone<sup>20</sup>. A systematic review and meta-analysis of animal studies looking for the influence of antimicrobial photodynamic therapy as an adjunctive to scaling and root planning on alveolar bone loss concluded that PDT favored the reduction of alveolar bone loss in experimental periodontitis (EP) in rats, and this result was more evident when systemic impairment is present<sup>21</sup>. However, the combination of PDT and LL-37 has never been tested in an animal study and our research team background suggest this strategy as a promising alternative to intensify disease resolution. Therefore, this study aimed to assess the effects of multiple sessions of PDT with chlorin-e6 conjugated to the antimicrobial peptide LL-37 loaded nanoemulsion, as adjunctive therapy in experimental periodontitis in rats.

## 2. Material and Methods

This study was carried out with 81 healthy three-month-old male adult rats (*Rattus norvegicus*, Albinus, Wistar) weighing from 260 to 360 g. Animals were kept in plastic cages with wood shavings and access to food and water *ad libitum*, under 12 hours/12 hours light/dark

cycles,  $22 \pm 2$  °C ambient temperature. Prior to the experimental steps, the rats were allowed to acclimatize to the laboratory environment for a period of 1 week. Procedures for experimental manipulation were conducted according to the guidelines established by the "Guide for the Care and Use of Laboratory Animals" and the experimental protocol was approved by the Ethics Committee on Animal Use (CEUA N° 14/2021) of the School of Dentistry, Araraquara, São Paulo State University. For all experimental procedures, the animals received general anesthesia with the combination of ketamine hydrochloride (70 mg/kg; Dopalen, SP, Brazil) with xylazine chlorhydrate (6 mg/kg; Xilazin, SP, Brazil) via intramuscular injection. Experimental periodontitis was induced through the presence of a cotton thread (n° 24, Linha Mamute, SP, Brazil) around the right and left second mandibular molars for a seven-day period<sup>22</sup>.

The animals were randomly assigned by a computer-generated table into three groups: SRP (scaling and root planning) (n=27); SRP + 1PDT (n=27), SRP followed by a single PDT session immediately after SRP; SRP + 4PDT (n=27), SRP followed by four PDT sessions at 0, 24, 48 and 72h after SRP (**Figure 1**). SRP was performed using mini-five hand curettes (#1-2, Hu-Friedy Co. Inc., Chicago, IL, USA) after ligature remotion. After treatment, nine animals of each group were subjected to euthanasia at 7, 14 and 28 days. The left and right hemimandibles were removed and fixed in 4% formaldehyde for 48 hours and latter submitted to histological (left hemimandible) and micro-CT ( $\mu$ CT – right hemimandible) analysis, a gingival collar bordering of the right mandibular second molar was also collected (**Figure 2**). Sample collection was carried out by a blinded investigator. All samples were randomly assigned by a computer-generated of codes during their histological processing until analysis of the results.

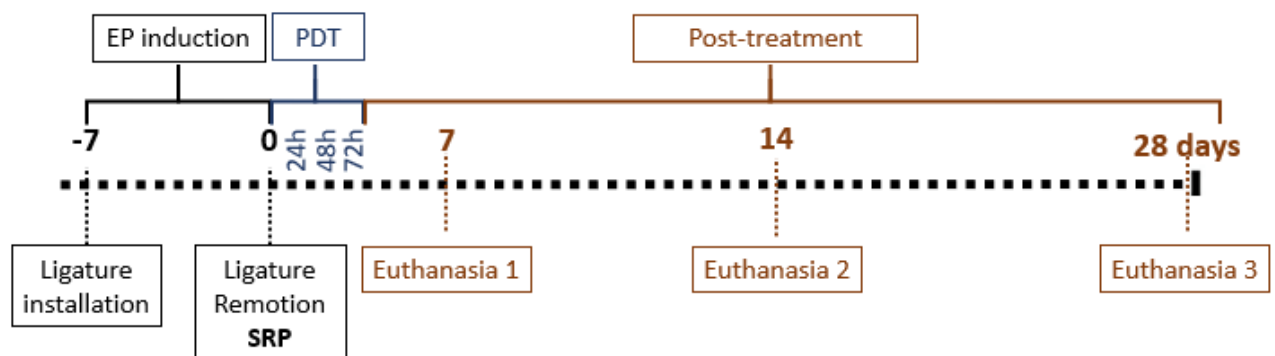


**Figure 1** – Timeline of the study

## 2.1 Photodynamic Therapy

Chlorin-e6 was semi-synthesized in the Bioorganic Chemistry Laboratory at Federal University of São Carlos - UFSCar (São Carlos-SP, Brazil), as previously described<sup>23</sup>. Later, 200  $\mu\text{M}$  (119,34 mg/L) of Ce6 was added to the nanosystem after the first sonication to reach the working concentration determined by previous studies<sup>13,14</sup>. The antimicrobial peptide (LL-37) was incorporated into a nanoemulsion system conjugated to Ce6, concentration was set at 8  $\mu\text{M}$  (35.92  $\mu\text{g/ml}$ ).

A dental curing-light equipment (VALO™ Cordless-LED Curing Light, Ultradent, South Jordan, UT, USA) was used as the light source for PDT application. The blue light is generated at wavelengths between 385-515 nm, producing high-intensity light with two peaks at  $\sim 410$  (right at the Soret absorption band of Ce6) and  $\sim 470$  nm. The light intensity of the equipment was set at 1000  $\text{mW/cm}^2$ , and 60 seconds was required to reach a light dose of 60  $\text{J/cm}^2$ , in 3 intermittent cycles of 20 seconds of application<sup>14</sup>.

All components of the photodynamic therapy protocol used in the present study were tested alone or in combination with the others, different concentrations were also tested, and the nanocarrier was characterized previously<sup>13-15</sup>. For more details about the photodynamic therapy protocol used in the present study, please refer to de Carvalho et al, 2023<sup>15</sup>.

Chlorin-e6 + LL-37 loaded nanoemulsion (0,05mL) was slowly poured into the periodontal pocket around the left and right mandibular second molar, using a syringe (1 mL) and an insulin needle. After 1 min of PS application (pre-irradiation time), light was applied in both sides of mandibular according to the **Figure 2**.

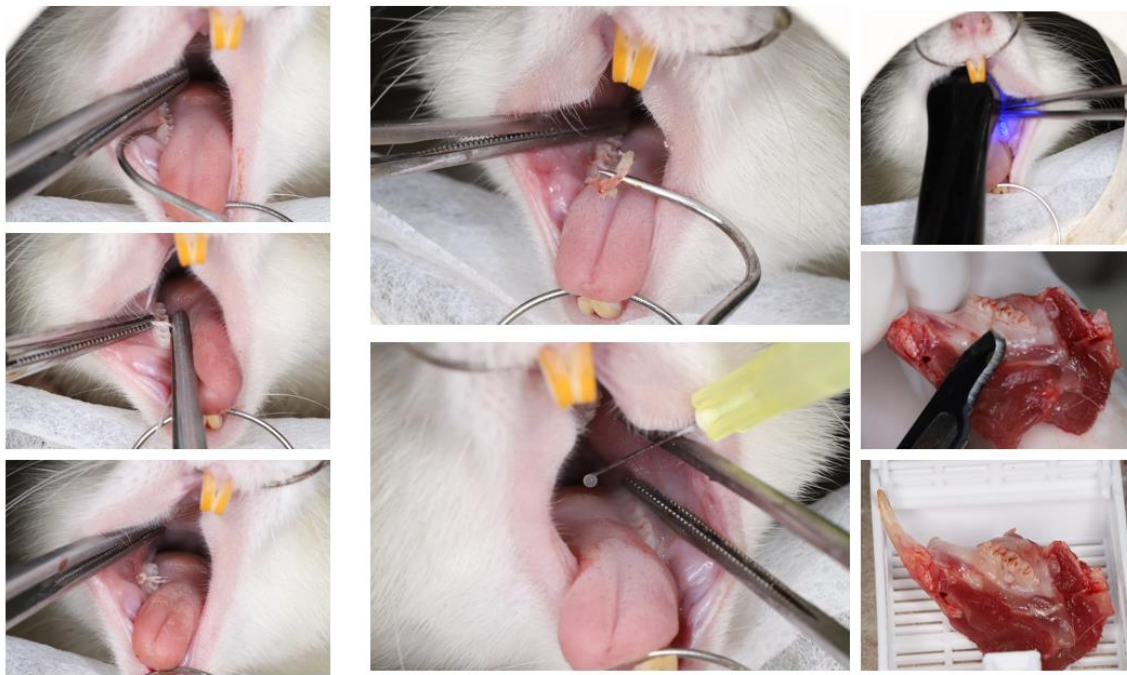


**Figure 2** – Animal study flowchart. Experimental periodontitis induction (left), Treatment (middle and upper right) and sample collection (right).

## 2.2 Osteoclast quantification

After euthanasia, the hemimandibles were removed and fixed for 48h in 4% formaldehyde buffered with 0.1 M sodium phosphate at pH 7.2. After fixation, the samples were decalcified in 7% EDTA at pH 7.2 and processed for paraffin embedding. Buccolingual non serial sections were stained with H&E or subjected to the TRAP reaction, used as an osteoclast marker.

Deparaffinized sections were immersed in an incubation medium freshly prepared containing 70 mg of Fast Red Salt TR (Sigma Chemical Company, St Louis, MO, USA) dissolved in 50 mL of 0.2 M sodium acetate buffer (pH 5.0). Subsequently, 20 mg of naphthol AS-BI (Sigma Chemical Company, St Louis, MO, USA) dissolved in 250  $\mu$ L N-N-dimethylformamide was added to the solution of sodium acetate buffer. Afterwards, 50 mM sodium tartrate dihydrate was added and the medium was filtered. After incubation at 37°C, the sections were washed in distilled water, counterstained with hematoxylin and mounted with glycerin.

In each specimen, two non-serial sections subjected to the TRAP reaction were captured using a camera (DP71, Olympus) attached to a light microscope (BX51, Olympus) at x65 magnification. Using an image analysis software (Image-Pro Express 6.0, Olympus), the linear

surface of alveolar process was measured (**Figure 3a**). In this surface previously measured, the number of multinucleated TRAP-positive osteoclasts (**Figure 3b**) adjacent to the alveolar bone was computed under a light microscope (Carl Zeiss, Inc., Jenamed 2, Germany) at x400. In each specimen, the total number of osteoclasts was divided by the total length of the bone surface.

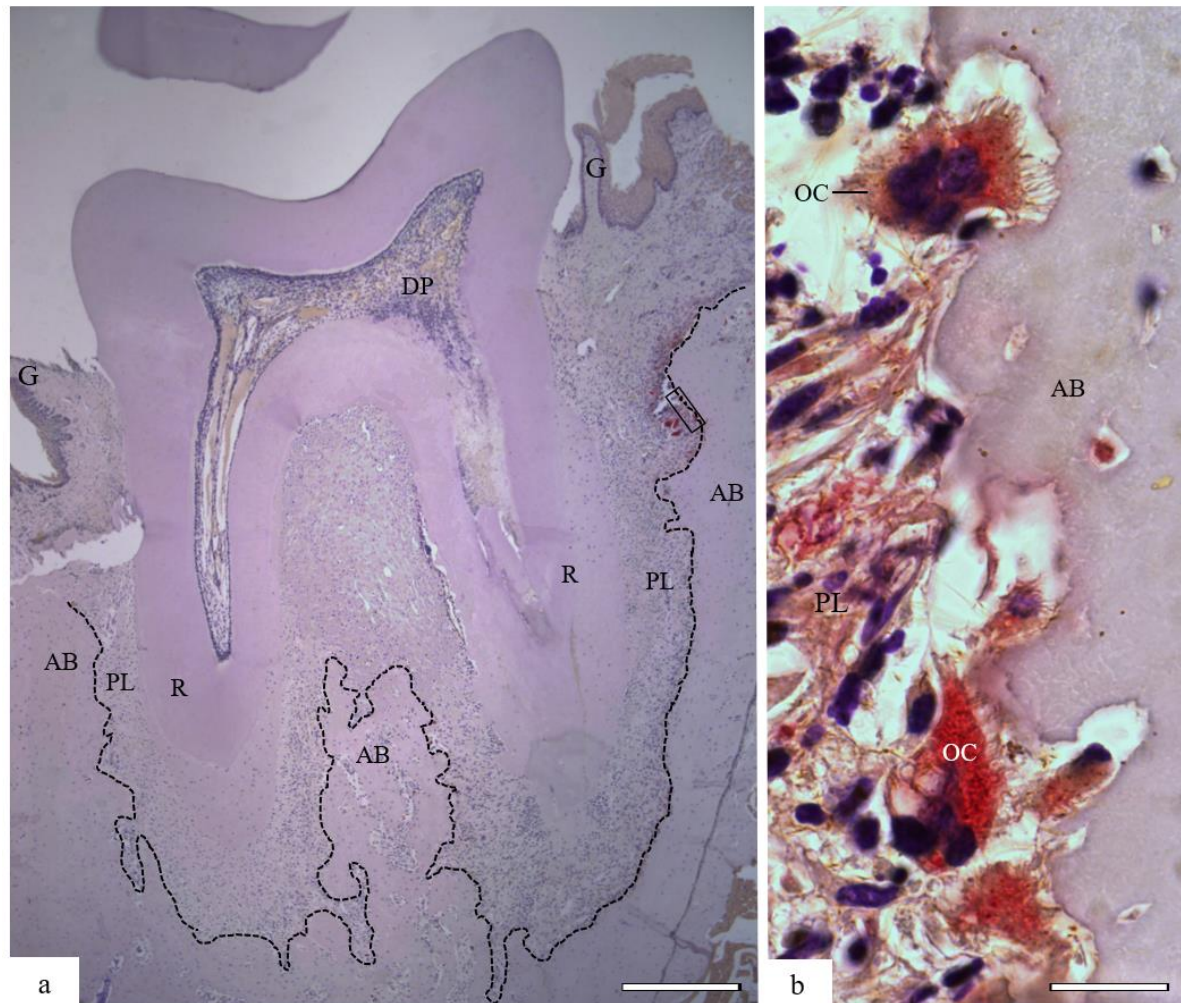


**Figure 3a and 3b** - Light micrographs of a section of the lower molar tooth submitted to the TRAP reaction and counterstained with hematoxylin. (a) The alveolar bone (AB) surrounding the molar roots (R) exhibits irregular surface, demarcated by hatched line. Osteoclasts (OC) exhibiting strong tartrate-resistant phosphatase acid (TRAP) activity in their cytoplasm (red colour) are observed in the alveolar bone surface, as shown in (b). (b) High magnification of outlined area in (a) showing TRAP-positive multinucleated osteoclasts (OC) in close juxtapposition to the bone surface. DP, dental pulp; PL, periodontal ligament; G, gingiva. Scale bars: 570  $\mu\text{m}$  (a) and 18  $\mu\text{m}$  (b).

### 2.3 Immunological analysis

Total protein was extracted from gingival samples using RIPA Lysis Buffer reagent (Merck, Millipore, Massachusetts, EUA) with protease inhibitors (Protease Inhibitor Cocktail Set I, #cod 539131-1VL, Calbiochem®, Massachusetts, EUA), then quantified by the BCA Protein Assay method (Protein Assay, #cod 23225, Pierce™, ThermoFisher, Massachusetts, EUA) according to manufacturer's instructions.

TNF- $\alpha$ , IL-1 $\beta$ , and IL-10 were evaluated by a multiplex assay using a Milliplex Map Mouse Cytokine/Chemokine Magnetic Bead Panel (Merck, Millipore, Massachusetts, EUA) according to the previous study<sup>24</sup>. The assay was performed following the instructions of the manufacturer.

### 2.4 Micro tomography analysis

The samples were scanned using microtomography scanner (Micro-CT Skyscan 1172; Bruker, Kontich, Belgium) from the Biological Microscopy and Imaging Laboratory (LAMIB) of the Federal University of Paraíba (UFPB). The following parameters were used to acquire the images: 80kv, 124mA, 0.5mm aluminum filter, large matrix (2000x2000), pixel size of 27.7 $\mu$ m, rotation steepness of 0.4, averaging 3, rotation of 180 degrees and random movement disabled. Approximately 1,100 images per sample were reconstructed using Nrecon software (Bruker, Kontich, Belgium). The parameters used for reconstruction were: beam harding 5%, ring artefact of 5, smoothing 02 with standardized minimum and maximum output values from 0.008524 to 0.102154, respectively. The two-dimensional images were analyzed by the Data Viewer software (Bruker, Kontich, Belgium) in three sections (axial, coronal and sagittal).

Linear alveolar bone loss assessment was performed using the CT Analyzer software (CTan, Bruker, Kontich, Belgium), cemento-enamel junctions (CEJ) of the mandibular right second molar was set parallel to each other in the coronal view. In the axial plane, the middle of the crown was identified and, in the sagittal plane, linear measurements (mm) were recorded from the CEJ to the alveolar crest (**Figure 4**). Altogether, five measurements were taken as described previously<sup>25,25</sup>: on the mesial, distal, buccal, lingual face and in the bifurcation region.

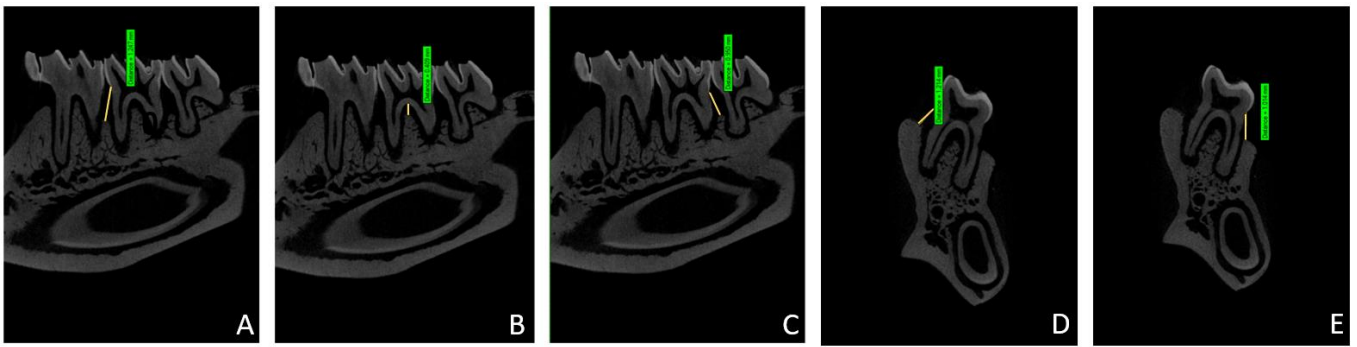


**Figure 4** – In the coronal plane, analysis of mesial (A), bifurcation (B) and distal (C) measurements (mm). In the sagittal plane, analysis of buccal (D) and lingual (E) measurements (mm). Orientation of measurements in the CT-Analyzer software.

Assessment of volumetric bone loss in the second molar region was performed in the axial axis, where the crowns of the three molars were entirely visible. The section in which the three roots of the second molar were completely separated was used as the starting point (top) for this analysis, and the final section (bottom) was set when it was no longer possible to visualize all the roots<sup>27</sup>. The volume of interest (VOI) was obtained by manually contouring the entire inter-radicular bone area around the roots without including the periodontal ligament space, repeatedly in an approximate number of 110 sections, then bone and dental structures were separated through the mineral density using gray scale (25– 255; grayscale threshold 0– 255) (**Figure 5**). The parameters determined in this volumetric analysis were the percentage of bone volume divided by tissue volume (BV/TV, %), porosity (%), and trabecular (TB) thickness (mm<sup>3</sup>). All micro-CT analyzes were performed by a blind and calibrated examiner.

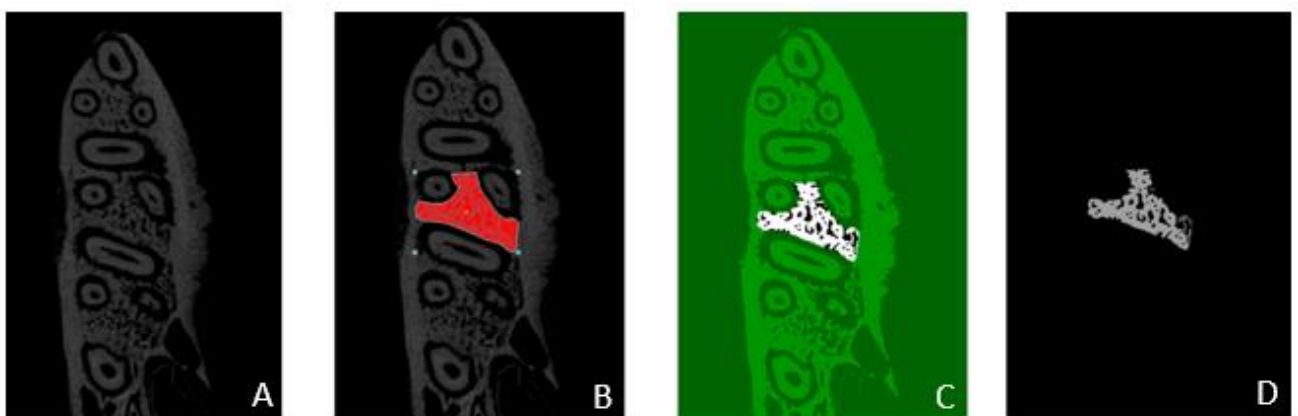


**Figure 5** - Volumetric bone level assessment in the CT-Analyser software. Axial axis of  $\mu$ CT image showing the separated roots of all molar teeth (A). Red area showing the volume of interest (VOI - B). VOI separated from the whole sample (C and D).

## 2.5 Statistical analysis

The data obtained were submitted to descriptive and inferential statistics in Prism 5.0 software (GraphPad, La Jolla, California). The animal was considered as the statistical unit. Nonparametric tests were performed in the absence of data normality. One-way ANOVA followed by Tukey's multiple comparison test was used to compare the mean values between groups for BV/TV, porosity, and TB thickness, and also the linear measurements in the faces buccal, mesial and distal. Kruskal–Wallis test followed by the Dunn's multiple comparison test was used to compare the median values between groups for the linear measurements in the lingual face and in the bifurcation region. Cytokine assay data was the average obtained from triplicate experiments. Such statistical tests were performed using a margin of error of 5%. The sample size calculation ( $n = 9$ ) showed a study power of 85% ( $p < 0.05$ ) considering the data from a previous study<sup>28</sup>, using the software Bioestat (version 5.3, Instituto Mamirauá, Manaus, Brazil).

## 3. Results

The experimental periodontitis was effectively induced in all animals, severe bone resorption with root exposure was clinically evident, and later confirmed in histological and micro-CT sections. The animals euthanized after 7 days showed the lowest ratio between bone volume and tissue volume (around 46.5%) (**Figure 6**). For the animals euthanized after 14 and 28 days, all groups demonstrated more than 65% of proportional bone volume presence after 14 days, this result means that all treatment were sufficient to restore periodontal health, but at least two weeks were required to restore tissue damage. The statistical analysis revealed no differences between the control group (SRP) and the groups with single and multiple applications of PDT, an increase of only 3-5% of bone volume was seen in these groups when compared to SRP group.

The results of porosity and TB thickness corroborates the bone volume assessment, a reduction in porosity is observed after 14 days just as the TB thickness increases in the same proportion, what indicates bone neoformation and remineralization. No statistical difference was seen between groups. Statistically significant difference was observed when compare the timeline of 7 days of euthanasia with the 14 and 28 days for all groups in all volumetric parameters (BV/TV, Porosity, and TB thickness).

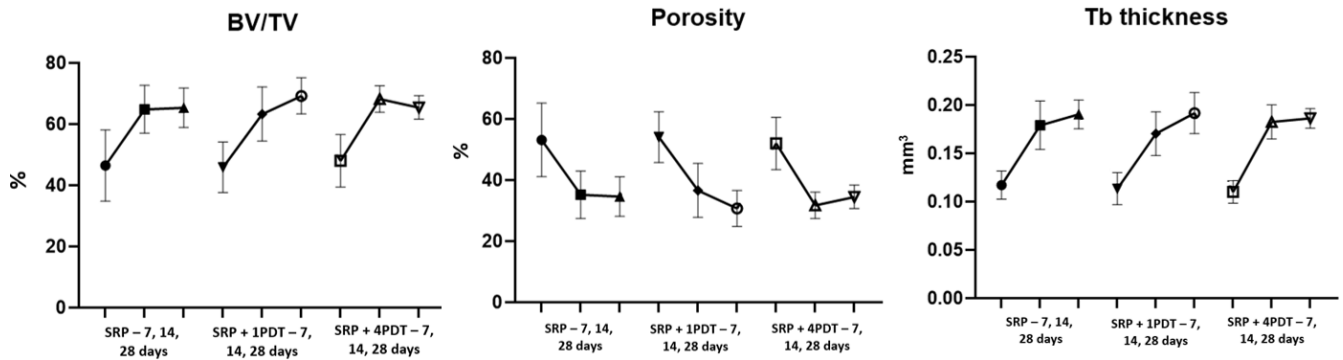


**Figure 6** – Volumetric assessment in radiographic evaluation. Graph represents the mean of the ratio between bone and tissue volume (BV/TV, %), porosity (%) and trabecular thickness (mm<sup>3</sup>) for the 3 groups SRP, SRP +1PDT, SRP +4PDT, and 3 timepoint euthanasia 7, 14, 28 days.

Concerning 2D radiographic analysis, reduction in the distance from cemento-enamel junction (CEJ) to the alveolar crest indicates bone formation, some reduction was observed for all groups after 14 and 28 days, especially for bifurcation and interproximal region (mesial and distal), but no statically difference was observed when compare groups and timepoints (**Fig 6**).

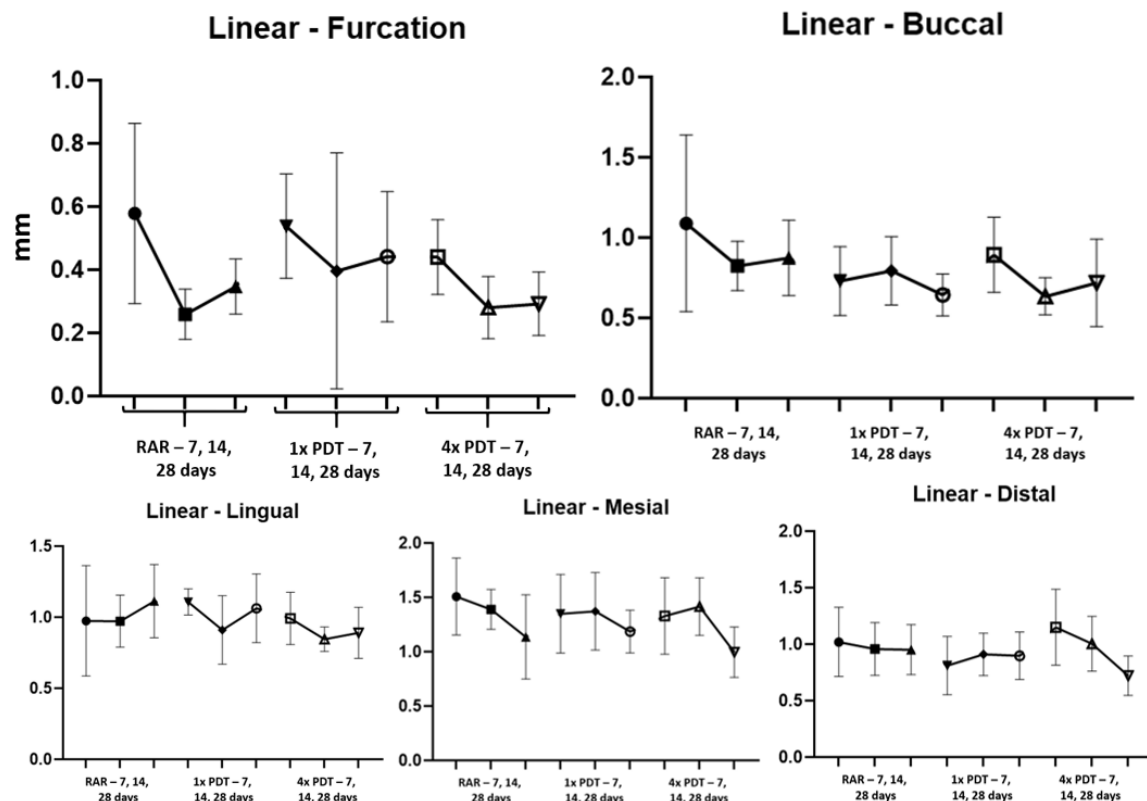


**Figure 7** – 2D radiographic evaluation. Linear measurements (mean- buccal, mesial and distal; and medians- furcation and lingual; mm) for the 3 groups SRP, SRP +1PDT, SRP +4PDT, and

3 timepoint euthanasia 7, 14, 28 days.

The levels of the proinflammatory cytokine IL-1 $\beta$  showed a slightly decreased in the groups treated with additional single application of PDT (4-8%), the animals treated with SRP and multiple PDT application showed a reduction of 11-18% in the levels of IL-1 $\beta$  after 28 days of treatment, but this reduction was not considered statistically significant. Regarding IL-10, 27% reduction was observed in the groups treated with PDT adjuvant to SRP after 28 days, this reduction was not considered statistically significant either. There was no statistically significant difference between the groups and timepoints for cytokine levels. For TNF- $\alpha$ , there was not enough count in the samples in the multiplex assay.

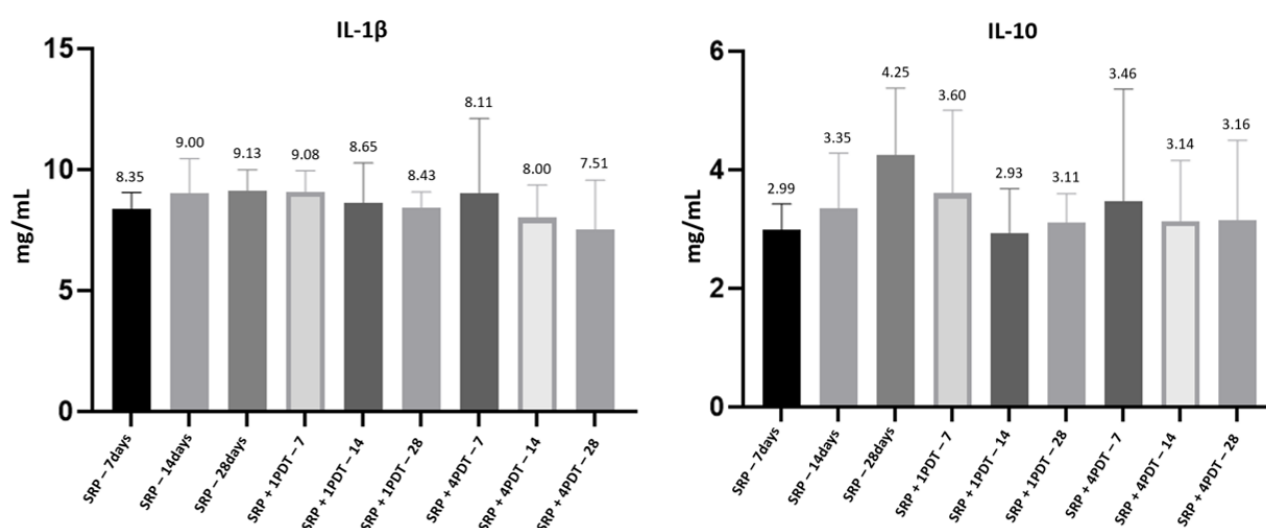


**Figure 8** – Immunological analysis of the levels of cytokine IL-1 $\beta$  and IL-10. The values represent the mean of triplicate experiments for the 3 groups SRP, SRP +1PDT, SRP +4PDT, and 3 timepoint euthanasia 7, 14, 28 days.

The quantitative analysis of osteoclast revealed that the number of TRAP-positive cells per mm of alveolar bone surface decreased in the groups treated with a single PDT application (after 14 days) and the groups treated with multiple applications of PDT (after 14 and 28 days), compared with the group treated with the conventional treatment (SRP), after 28 days. No statistically difference was found for the other groups (**Figure 9**).

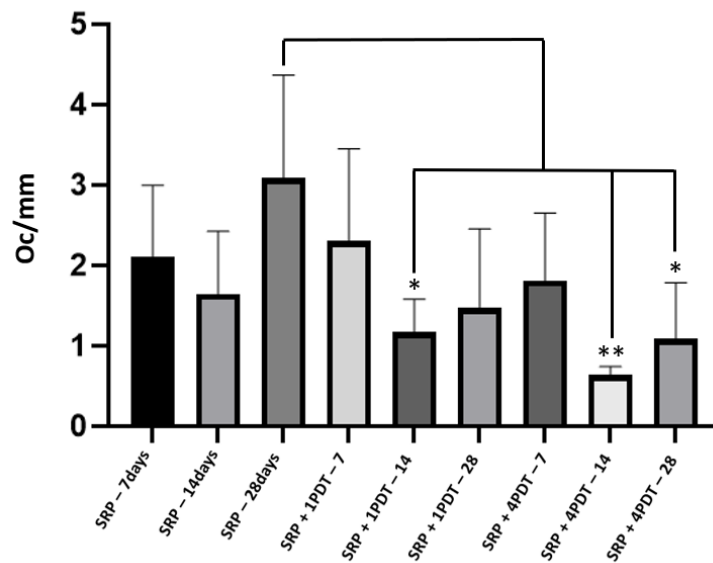


**Figure 9** - Number of TRAP-positive osteoclasts per mm of alveolar bone linear surface in the animals. The values represent the mean for the 3 groups SRP, SRP +1PDT, SRP +4PDT at 7, 14, 28 days. Different means. Statistically significant differences between the means are indicated by \* (>0.01) and \*\* (>0.001)

## Discussion

The results of this study demonstrated that single or multiple sessions of PDT with chlorin-e6 conjugated to the antimicrobial peptide LL-37 loaded nanoemulsion as adjunct to SRP in the treatment of periodontitis provided limited benefits compared to SRP alone in rats, regarding inflammatory response and periodontal repair process. All treatments resulted in significant periodontal improvements and there was no significant difference between the groups in both local inflammatory response and healing process. Minimal adjunctive effects could be found for the combined therapy in terms of cytokine levels (IL-1 $\beta$  and IL-10), with no statistically significance. However, the number of TRAP-positive osteoclasts per mm of alveolar bone linear surface for the group treated with PDT sessions was significantly lower than those treated with SRP only.

Our research group has been trying to optimize the composition of chlorin-e6, concentration of PS, parameter of light, nanocarrier and synergic effect with hydrogen peroxide and antimicrobial peptide (LL-37) to verify whether they cause an impact on its efficiency, thus increasing the evidence for further clinical use. Ce6 demonstrated great bacterial reduction in past studies<sup>13-15</sup>. In line with literature and our research experience, it was decided to carry out this animal study, anterior investigations supported the treatment protocol tested in the present

study<sup>13-15,23</sup>. However, part of the results disagrees from those found in other animal studies testing PDT for periodontal disease<sup>16,18,20,29</sup>. It is important to highlight that most of the animal studies evaluating the influence of photodynamic therapy as an adjunctive to scaling and root planning on alveolar bone loss tests toluidine blue or methylene blue as photosensitizers, at the concentration of 0.1 mg/mL. A systematic review and meta-analysis of animal studies found that PDT favored the reduction of alveolar bone loss in experimental periodontitis in rats, and this result was more evident in systemically compromised rats<sup>21</sup>, but in the present study benefits were found only in the quantification of osteoclasts for groups treated with PDT. Seven out of the nine included studies in this mentioned systematic review and meta-analysis came from the same research group, all of studies concentrated their efforts in histomorphometrical and immunohistochemical analysis. Our study is the first in this topic to evaluate bone repair through micro-CT. Assessment using micro-CT provides us with more reliable volumetric data on bone repair.

The pre-irradiation and irradiation time of 1 min seemed to be clinically possible and comfortable, both for the operator and patient<sup>30-32</sup>. The incubation time of the drug was found as the main parameter for the effectiveness of PDT, period of 20 and 60 minutes were tested in vitro studies<sup>33</sup>, and this parameter may be the first that justifies the slight benefit of PDT in the experimental groups for the present study. Higher incubation time would allow greater interaction between the photosensitizer and bacteria, the efficacy of a PS agent is determined by the degree of its interaction with the target<sup>34</sup>, but long incubation time means low acceptance by dentists and patients.

The dental curing-light equipment daily used at the dental office seems promising for PDT with active PS under blue light, it is important to note that this equipment is already available for any dental surgeon what improves clinical adherence and minimize costs augmentation. Additionally, effective photoantimicrobial action is achieved with an efficient light source that has the correct wavelength range and sufficient power output<sup>35</sup>. The light delivery equipment used in this study is quite specific for the absorption wavelength peak of Ce6 (around 410nm). Since its output power is fairly high, the time to reach the desired dose is very low. Although, the transgingival irradiation method is commonly used in other similar studies<sup>21</sup>, there is no evidence on how much light reaches inside the periodontal pocket, and it might be considered a limitation of this study. PDT treatment was performed with the light output positioned perpendicular to the long axis of the tooth and in contact with the gingiva.

Increasing the number of PDT session seems to be a factor that can improve the phototoxic effects of PS<sup>31</sup>. It's still questionable if repeated applications of PDT result in superior outcomes compared to single applications or not, a systematic review and network meta-analysis suggest that a repeated application of PDT might not result in superior outcomes compared with its single application<sup>36</sup>, but in other study, multiple sessions of PDT as an adjunct to surgical periodontal treatment significantly improved clinical parameters at 90 postoperative days<sup>37</sup>. In the present study, all treatments were efficient to restore periodontal health, no difference was found between single and multiple sessions of PDT for quantification of OC, cytokines levels and bone repair. These findings do not align with the results of a pairwise animal study, whereas significant less alveolar bone loss was found only in the group where conventional periodontal treatment and four applications of PDT was tested<sup>20</sup>.

In the experimental periodontitis model used in this study, ligature installation leads to plaque accumulation and trauma to the surrounding tissues, acting as a key factor for the development of a dysbiotic microbiota and consequently bone resorption<sup>38</sup>. The dysbiotic microbiota induces periodontal tissue destruction by means of a dysregulated inflammatory immune response of the host<sup>39</sup>. In this experimental model, bone loss occurs predictably over a period of 7 days<sup>40</sup>. The periodontitis induced by ligature in rat and mice has been frequently explored since naturally existent bacteria is responsible for biofilm formation and their metabolism products stimulates infiltration of inflammatory cells, disruption of epithelial integrity, connective tissue attachment loss, and alveolar bone resorption<sup>41,42</sup>.

Periodontal treatment results in reduction of local inflammation, including a decrease in serum and gingival crevicular fluid IL-1 $\beta$  levels in periodontitis patients<sup>43,44</sup>. It has been reported that IL-10 mediates the control of periodontal disease progression<sup>45</sup>. In the early phases of inflammation, IL-10 has pro-inflammatory properties, later, the function of IL-10 converges into a congruent attempt to limit the tissue damage<sup>46</sup>. In the present study, no difference was observed in multiple comparison of the groups considering the levels of IL-1 $\beta$  and IL-10.

Chlorin-e6 has proved to be effective against periodontal biofilm in past studies<sup>13-15</sup>. Nevertheless, it is assumed that in vitro models cannot completely reproduce the periodontal infection. In the animal model, some inflammation resolution associated with Ce6 and LL-37 during PDT was expected to be observed, plenty reasons may explain this fact and some of them are pointed bellow: blue light may not have reached the periodontal pocket at a sufficient level, higher incubation time might be needed, biofilm present in the host are different from the

bacteria evaluated in previous studies, photosensitizer formulation should be more viscous to be applied and stayed non-washed by gingival crevicular fluid. In the light of the previously discussed results and limitations of this study, there is a need of more investigations around Ce6 formulation and light delivery previous to preclinical studies.

## Conclusion

Multiple PDT sessions with chlorin-e6 and LL-37 nanoemulsion as an adjunct to scaling and root planning reduced the presence of osteoclast in local site but did not contribute towards bone regeneration and IL-1 $\beta$  and IL-10 levels.

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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*. O efeito antimicrobiano mais expressivo foi observado quando a terapia fotodinâmica ocorreu na presença de Ce6 + LL-37 em nanoemulsão. Em estudo *in vivo*, sessões únicas ou múltiplas de terapia fotodinâmica com clorina-e6 conjugada com peptídeo antimicrobiano LL-37 em nanoemulsão como adjuvante da raspagem e alisamento radicular no tratamento da periodontite não proporcionaram benefícios em comparação à terapia convencional isoladamente.

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## APÊNDICE A – METODOLOGIA

### Fotossensibilizador, Peptídeo Antimicrobiano, Nanoemulsão e Fonte de Luz

A clorina-e6 (Ce6) foi semi-sintetizada pelo Prof. Dr. Kleber Thiago de Oliveira (Departamento de Química, Laboratório de Química Bioorgânica UFSCar), a partir da extração da clorofila presente na *Spirulina maxima*, alga utilizada em suplementação alimentar (rica em vitaminas e proteínas), seguindo protocolo descrito<sup>1</sup>. Solução de estoque foi preparada dissolvendo 11,93 mg de Ce6 em 1 mL DMSO (Dimethyl sulfoxide, Sigma-Aldrich, St. Louis, MO, USA) para atingir a concentração de 20 mM. Esta solução foi mantida em freezer -20 °C até o uso. As concentrações de 100 µM (59,7 mg/L) e 200 µM (119,34 mg/L) foram adotadas para o estudo microbiológico descrito na sequência.

O peptídeo antimicrobiano (PAM) LL-37 (1 mg) foi adquirido na SmartBioscience (Saint Egrève, France). Considerando um estudo prévio<sup>2</sup> que determinou a média da concentração inibitória mínima do LL-37 para microrganismos periodontopatogênicos em 56,51 µg/ml e que, em nosso estudo, o LL-37 foi incorporado em sistema de nanoemulsão e seu efeito antimicrobiano somado ao do aPDT, espera-se que uma menor concentração seja necessária para obter o resultado desejado, logo utilizamos 8 µM (35,92 µg/ml).

A associação do fotossensibilizador e peptídeo antimicrobiano ao sistema em nanoemulsão foi realizado conforme descrito em estudo prévio<sup>3</sup>. O sistema lipídico nanoestruturado (nanoemulsão) foi preparado com 10 % de colesterol (fase oleosa), 10 % de Brij 35<sup>®</sup> e fosfatidilcolina (Epikuron<sup>®</sup> 200 – Surfactante 2:1) e 80 % de solução salina tamponada de fosfato (PBS – fase aquosa)<sup>3</sup>. A mistura foi preparada sob baixas temperatura em ultrassom (Q500 – Qsonica, Newtown, CT, USA), com potência de 500 W em modo descontinuo por 10 min (30 seg de atividade a cada 1 min).

O peróxido de hidrogênio (Sigma-Aldrich, Darmstadt, Alemanha) foi testado sozinho ou em combinação com Ce6 e LL-37. Quando a aPDT foi realizada com H<sub>2</sub>O<sub>2</sub>, 33,3mM de H<sub>2</sub>O<sub>2</sub> foi colocado em contato com o biofilme primeiro e Ce6 + LL-37 foi adicionado posteriormente (por 5 min, tempo pré-irradiação), não houve washout entre eles. A concentração de H<sub>2</sub>O<sub>2</sub> foi definida de acordo com um estudo anterior<sup>4</sup>.

De maneira pioneira, nosso grupo testou a utilização de um fotopolimerizador como fonte de luz na aPDT. Fotopolimerizadores são equipamentos de luz azul utilizados rotineiramente na prática odontológica para tratamentos de restauração, clareamento e cimentação de peças protéticas, logo, consagrado e seguro sobre tecidos orais. Com objetivo de reduzir custos adicionais e facilitar o emprego da terapia alternativa proposta, o

fotopolimerizador Valo<sup>®</sup> (Ultradent, South Jordan, USA) foi utilizado na ativação do fotossensibilizador, uma vez que utiliza díodo emissor de luz (LED) personalizado em comprimentos de onda entre 385-515 nm (amplo espectro na região azul), produzindo luz de alta intensidade com dois picos em  $\sim 410$  e  $\sim 470$  nm. A intensidade de luz do equipamento foi definida em  $1000 \text{ mW/cm}^2$ , e um tempo de 60 segundos foi necessário para atingir a dose de  $60 \text{ J/cm}^2$ , em ciclos intermitentes de 20 segundos de aplicação. Esse parâmetro foi definido com base em estudo prévio já publicado<sup>5</sup>.

### **Índice de Polidispersão (PDI) e Potencial Zeta**

A determinação do diâmetro da partícula e do índice de polidispersão (PDI) da nanoemulsão pura ou associada à Ce6 foi realizada por espalhamento de luz dinâmico (DLS) a  $20^\circ\text{C}$ . A nanoemulsão pura ou associada ao fotossensibilizador (FS) também foi caracterizada quanto o potencial zeta usando a mobilidade eletroforética. As nanopartículas foram diluídas em água ultrapura tipo 1 ( $10 \mu\text{L}$  da nanoemulsão +  $990 \mu\text{L}$  de água) e avaliadas em equipamento Zetasizer Nano NS (Malvern Instruments, Worcestershire, Reino Unido). Os resultados de tamanho, diversidade relativa e potencial zeta das nanopartículas foram obtidos com o auxílio do software Zetasizer<sup>6</sup>.

### **Espectroscopia Ultravioleta/visível (UV-Vis)**

A análise de UV-Vis foi realizada para identificar mudanças no espectro de absorção do fotossensibilizador incorporado em nanoemulsão<sup>7</sup>. As soluções foram preparadas na concentração de  $100 \mu\text{M}$  Ce6 livre e em nanoemulsão com LL-37. Uma dose de luz de  $60 \text{ J/cm}^2$  foi empregada dividida em 6 ciclos de irradiação de 10 segundos cada. As medições foram tomadas em equipamento Synergy H1M (Synergy H1 Multi-Mode Reader, BioTek, Winooski, VT, USA). Essa avaliação nos permitiu identificar se algum componente do sistema de nanoemulsão ou se o LL-37 interfeririam nos comprimentos de onda de absorção do fotossensibilizador.

### **Espécies Reativas de Oxigênio (ROS)**

Espécies reativas de oxigênio foram detectadas por fluorescência utilizando a sonda Diacetato 3',7' – diclorofluoresceína – H<sub>2</sub>-DCF-DA (Thermo Fischer Scientific, MA, EUA), seguindo protocolo do fabricante. Soluções de Ce6 ( $100 \mu\text{M}$ ) em nanoemulsão e sem nanoemulsão, com e sem LL-37, foram preparadas em tampão fosfato ( $0,1\text{M}$ ; pH 7,2), adicionadas a  $3 \mu\text{M}$  da sonda citada em placas de 96 poços de fundo preto. As soluções foram irradiadas seguindo protocolo de aPDT descrito nas seções anteriores. As leituras de

fluorescência foram realizadas imediatamente após irradiação utilizando Synergy H1M (Synergy H1 Multi-Mode Reader, BioTek, Winooski, VT, USA)<sup>7</sup>. Os comprimentos de onda de excitação/emissão para 490/515 nm e 505/525 nm.

### **Microscopia Eletrônica de Transmissão (MET) e Microscopia Eletrônica de Varredura (MEV)**

A nanoemulsão carregada com Ce6 foi diluída (1:20) em água deionizada, 3 µL foram adicionados às grades de cobre e corados com solução de acetato de uranila 2% em água destilada por 5 minutos. As amostras foram secas à temperatura ambiente por 15 minutos antes da análise. A MET de duas amostras foi realizada em microscópio eletrônico de transmissão Phillips CM200, operando a 200 kV, com filamento LaB6 (hexaboreto de lantânio).

Para MEV, as lamínulas com nanoemulsão contendo Ce6 previamente diluídas foram secas em temperatura ambiente e colocadas em dessecador a vácuo por 7 dias com sílica gel. Posteriormente, as amostras analisadas foram metalizadas com ouro-paládio e fixadas aos suportes com adesivo condutivo e a análise morfológica das amostras foi realizada em microscópio eletrônico Zeiss (Jeol, modelo JSM-7500F) com tensão de 5,00 kV.

### **Cepa bacteriana e condições de crescimento**

As cepas bacterianas utilizadas neste estudo foram *Streptococcus oralis* (American Type Culture Collection - ATCC<sup>®</sup> 35037<sup>TM</sup>), *Fusobacterium nucleatum* (ATCC<sup>®</sup> 25586<sup>TM</sup>), *Porphyromonas gingivalis* (ATCC<sup>®</sup> 33277<sup>TM</sup>) e *Aggregatibacter actinomycetemcomitans* (JP2, gentilmente fornecida pelo Laboratório do Prof. Mario Júlio Ávila-Campos (*in memoriam*) da Universidade de São Paulo - USP, São Paulo, Brasil). Todas as cepas bacterianas foram cultivadas em ágar brucela (Agar Brucella, Neogen, Idaiatuba, SP, Brasil; placa de Petri 90x10cm, Kasvi, São José do Pinhais, PR, Brasil) suplementado com sangue de carneiro desfibrinado a 5 % (Microlab, Campinas, SP, Brasil), extrato de levedura (6 g / L), hemina (10 mg/mL) e menodiona (5 mg/mL), a 37 °C em condições atmosféricas adequadas para formação de biofilme periodontopatogênico com cepas bacterianas anaeróbias, e mantidas em câmara de anaerobiose (VA 500, Don Whitley Scientific, Inglaterra) com mistura de gases 85 % N<sub>2</sub>, 10 % H<sub>2</sub> e 5 % CO<sub>2</sub>, (White Martins, Rio de Janeiro, RJ, Brasil).

Após 48 h, as colônias bacterianas foram transferidas para 10 mL de meio líquido (BHI - Difco Laboratories Inc, MI, EUA) suplementado com extrato de levedura (6 g/L), hemina (10 mg/mL) e menadiona (5 mg/mL), e incubadas nas condições descritas anteriormente por 24 h. Finalmente, as células bacterianas foram ajustadas por densidade ótica em meio fresco, de

acordo com a fase exponencial de crescimento previamente estabelecida (*P. gingivalis*, 15 h, OD<sub>600 nm</sub> = 0.95 ± 0.01; *F. nucleatum*, 10 h, OD<sub>600 nm</sub> = 0.45 ± 0.01; *S. oralis*, 4 h, OD<sub>600 nm</sub> = 0.55 ± 0.01; *A. actinomycetemcomitans*, 8 h, OD<sub>600 nm</sub> = 0.18 ± 0.01) usando espectrofotômetro (BioSpectrometer® D30, Eppendorf, Hamburgo, Alemanha)<sup>8</sup>.

### **Processamento da saliva humana**

Antes da coleta da saliva humana, o protocolo de pesquisa foi submetido a avaliação pelo Comitê de Ética em Pesquisa da Faculdade de Odontologia de Araraquara, Universidade do Estado de São Paulo (CAAE 23396619.5.0000.5416). A saliva foi obtida, sem estímulo, de homens jovens saudáveis, sem cárie ativa ou doença periodontal, sem aparelho ortodôntico, sem doença sistêmica, em jejum e sem escovação dentária nas 12 h que antecedem a coleta, e sem relato de uso de antibióticos por pelo menos seis meses antes da coleta. A saliva foi processada seguindo protocolo de Tavares et al<sup>9</sup>.

### **Formação do biofilme**

A formação de película salivar ocorreu no fundo da superfície de placas de 96 poços convencionais (Kasvi, São José do Pinhais, PR, Brasil). Cinquenta microlitros de saliva foram adicionados aos poços e a placa foi mantida em agitador orbital (Incubadora 430 RD, Nova Etica, Brasil), 75 rpm a 37 °C por 4 h<sup>9</sup>. Na sequência, alíquotas padronizadas de 200 µL da suspensão de cada espécie bacteriana foram distribuídas em cada poço, seguindo a ordem programada (24 h - *S. oralis*; 48 h - *F. nucleatum*; 72 h - *P. gingivalis* e *A. actinomycetemcomitans*). Depois de adicionadas todas as cepas (72 h), as células não aderentes foram removidas por meio de aspiração de meio consumido e 200 µL de meio fresco foi adicionado para continuar a formação do biofilme. O meio de cultura foi substituído diariamente, por 4 dias, e os biofilmes foram mantidos a 37 °C em condições anaeróbias<sup>10</sup>.

### **Tratamento do Biofilme**

Os grupos foram divididos considerando a irradiação de luz e a presença de Ce6, LL-37, H<sub>2</sub>O<sub>2</sub> e NE, em três placas independentes. Todas as variáveis foram testadas isoladamente para identificar melhor o efeito de cada uma delas. Assim, os grupos foram divididos da seguinte forma: 1) Controle negativo – Ctrl, sem Ce6 (foi aplicada água destilada para evitar o ressecamento do biofilme) e sem luz; 2) Ce6, clorina-e6 incorporada em NE a 200 µM sem luz; 3) LL-37, peptídeo antimicrobiano LL-37 a 8 µM sem luz; 4) Nano, o NE formulado foi testado sem carga e sem luz; 5) H<sub>2</sub>O<sub>2</sub>, 33,3mM de H<sub>2</sub>O<sub>2</sub> sem luz; 6) Azul, irradiação de luz azul (60

J/cm<sup>2</sup>); 7) Azul + LL-37, peptídeo antimicrobiano LL-37 a 8 µM com luz azul; 8) Azul + Ce6 + LL-37, clorina-e6 formulada em NE a 200 µM e peptídeo antimicrobiano LL-37 a 8 µM com luz azul; 9) Azul + Ce6 + LL-37 + H<sub>2</sub>O<sub>2</sub>, pré-tratamento com 33,3 mM de H<sub>2</sub>O<sub>2</sub>, depois clorina-e6 formulada em NE a 200 µM e peptídeo antimicrobiano LL-37 a 8 µM com luz azul. A placa sem irradiação de luz foi mantida no escuro pelo mesmo tempo que os grupos irradiados, com o objetivo de detectar possível toxicidade no escuro.

### **Extração do DNA**

Após tratamento, o biofilme foi lavado e submetido a extração de DNA usando PMA (propidium monoazida) (Biotium, EUA), um corante de ligação de DNA fotoreativo utilizado para detectar microrganismos viáveis por qPCR. Após a fotólise, o PMA modifica seletivamente (uma vez que é impermeável à membrana celular intacta) o DNA das bactérias mortas, sem causar danos às bactérias viáveis. O uso de PMA permitiu a amplificação apenas de DNA de bactérias viáveis. A extração do DNA foi realizada usando o QIAamp DNA Mini Kit, seguindo as instruções do fabricante.

### **Análise por PCR em tempo real**

As reações de quantificação por qPCR foram realizadas no equipamento CFX96 real-time system equipment (BioRad, Hercules, CA). O sistema Taqman com primers e sondas específicos foi utilizado (**Quadro 1**). Para as reações de Taqman foram usados 12,5 µL de Mastermix (Eurogentec, Seraing, Bélgica), 4,5 µL de H<sub>2</sub>O estéril, 1 µL de cada primer e sonda e 5 µL de cada amostra. Primers e sondas foram utilizados em diferentes concentrações, dependendo das bactérias. As condições de amplificação foram: 2 minutos a 50 °C, 10 minutos a 95 °C, seguidos de 45 ciclos de 95 °C por 15s e 60s a 60 °C. A quantificação foi baseada em curva padrão de plasmídeos<sup>12</sup>. Esse experimento foi conduzido em triplicata, em 3 repetições independentes (n=9).

**Quadro 1** – Primers e Sondas usados para detecção e quantificação de PMA-qPCR

| Cepa bacteriana                |         | Primer/Sondas (5'-3')                   | Comprimento do fragmento |
|--------------------------------|---------|-----------------------------------------|--------------------------|
| <i>A.actinomycetemcomitans</i> | Forward | GAA CCT TAC CTA CTC TTG ACA TCC GAA     | 80 bp                    |
|                                | Reverse | TGC AGC ACC TGT CTC AAA GC              |                          |
|                                | Probe   | AGA ACT CAG AGA TGG GTT TGT GCC TTA GGG |                          |
| <i>F. nucleatum</i>            | Forward | GGA TTT ATT GGG CGT AAA GC              | 162 bp                   |
|                                | Reverse | GGC ATT CCT ACA AAT ATC TAC GAA         |                          |
|                                | Probe   | CTC TAC ACT TGT AGT TCC G               |                          |
| <i>P. gingivalis</i>           | Forward | GCG CTC AAC GTT CAG CC                  | 68 bp                    |
|                                | Reverse | CAC GAA TTC CGC CTG C                   |                          |
|                                | Probe   | CAC TGA ACT CAA GCC CGG CAG TTT CAA     |                          |
|                                | Reverse | ACC GTA ACG TGG GAA AAC TG              |                          |
|                                | Probe   | GTA GCG TCA GAG TGG TTG AC              |                          |
| <i>S. oralis</i>               | Forward | ACC AGC AGA TAC GAA AGA AGC AT          | 229 bp                   |
|                                | Reverse | AGG TTC GGG CAA GCG ATC TTT CT          |                          |
|                                | Probe   | AAG GCT GCT GTT GCT GAA GAA GT          |                          |

Fonte: Herrero et al., 2018<sup>13</sup>.

### **Análise confocal de microscopia de varredura a laser (CLSM)**

Para este experimento os biofilmes foram cultivados em placas de 48 poços e tratados conforme descrito nas seções anteriores. A viabilidade bacteriana foi avaliada após coloração com o kit Live/Dead BacLight™ Bacterial Viability (Invitrogen, Carlsbad, CA, EUA) seguindo as instruções do fabricante. Após a coloração, os biofilmes foram lavados e analisados com microscópio confocal de varredura a laser LSM 800 (Zeiss, Jena, Alemanha). As imagens foram obtidas através de objetiva 10× (EC Plan-Neofluar NA 0.3 air). Comprimentos de onda de excitação de 488 nm (ar) e 561 nm (laser HeNe) foram empregados para visualizar a distribuição de bactérias vivas e mortas.

### **Animais e indução de doença periodontal**

O protocolo experimental proposto foi aprovado pelo Comitê de Ética no Uso de Animais (CEUA 14/2021) da Faculdade de Odontologia, Araraquara, UNESP (**Anexo B**). Este estudo foi realizado com 81 ratos machos adultos (3 meses de idade) (*Rattus norvegicus albinus*, Holtzman), com peso médio de 300 ± 50g, procedentes do Biotério Central da FOAr (Campus Araraquara). Os animais foram mantidos no biotério 2 da Faculdade de Odontologia de Araraquara sob temperatura de 21±1 °C, umidade de 65% - 75% e ciclos de luz controlado (12h claro - 12h escuro), de acordo com as recomendações do Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com padrões éticos que cumprem as recomendações da ARRIVE (*Animal Research: Reporting of In Vivo Experiments*). Os ratos foram divididos em gaiolas plásticas e água e ração padrão foram disponibilizadas *ad libitum*. Inicialmente, os

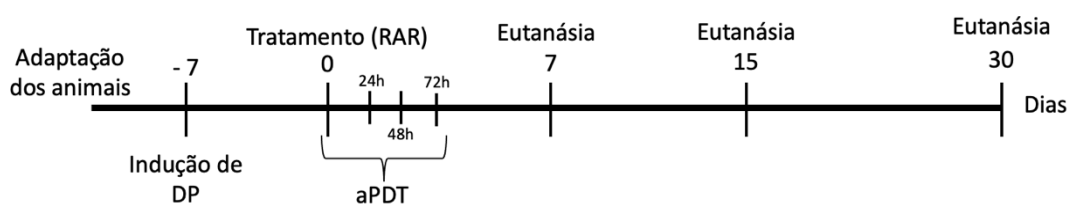
animais permaneceram em adaptação ao ambiente do biotério por período de 1 semana. Todos os procedimentos para o uso experimental dos animais foram realizados sob anestesia com cloridrato de ketamina (80 mg / kg; Vetaset - Fort Dodge, IA, EUA) e com cloridrato de xilazina (8mg/kg; Coopazina, Coopers, SP, Brasil) via injeção intramuscular. A periodontite experimental foi induzida pela presença de fio de algodão (Corrente Algodão no 24, Coats Corrente, SP, Brasil) ao redor dos primeiros molares inferiores direito e esquerdo por um período de sete dias<sup>14</sup>. A ligadura foi retirada sete dias após sua instalação, e os animais foram aleatoriamente distribuídos em grupos.

### Grupos experimentais

Os animais foram distribuídos nos seguintes grupos: RAR (n = 27), raspagem e alisamento radicular (RAR); RAR + 1aPDT (n = 27), RAR seguido de uma única sessão de aPDT; RAR + 4aPDT (n = 27), RAR seguido de quatro sessões de aPDT em 0, 24, 48 e 72 h após RAR; (**Figura 1**). A RAR foi realizada utilizando mini curetas de mão (# 1-2, Hu-Friedy Co. Inc., Chicago, IL, EUA) por meio de 5 movimentos de tração distal-mesial nas faces vestibular e lingual e 5 movimentos de tração cervical-oclusal em as regiões interproximal e de furca seguiram o protocolo relatado por Garcia et al.<sup>14</sup>. Os animais receberam o mesmo tratamento em ambos os lados

Nos grupos que receberam aPDT, a solução com clorina-e6 (200  $\mu$ M) e LL-37 (8 $\mu$ M) foi lentamente adicionada na bolsa periodontal ao redor do primeiro molar inferior esquerdo após RAR, usando uma seringa (0,5 mL) e uma agulha de insulina<sup>14</sup>. Após 1 minuto da aplicação do FS, a luz foi aplicada nas faces vestibular e lingual dos elementos onde a doença periodontal foi induzida.

**Figura 1** - Linha do tempo do estudo



Fonte: Elaboração própria.

### Coleta e processamento das amostras

Nove animais de cada grupo foram submetidos à eutanásia em cada momento descrito (**Figura 1**) e as hemimandíbulas esquerdas foram retiradas e fixadas em formol a 4 % por 48 h para análise histológica e imunohistoquímica. Cinco cortes equidistantes foram corados com hematoxilina e eosina (HE) para análise histológica e histomorfométrica. Para a técnica de imunoperoxidase indireta, cinco cortes foram submetidos aos seguintes anticorpos primários: anticorpo anti-fosfatase ácida tartarato-resistente (TRAP) (Santa Cruz Biotechnology, Santa Cruz, CA, EUA), anti RANKL (Ligante do Receptor Ativador do Fator Nuclear kappa B – RANKL) (Santa Cruz Biotechnology); fator nuclear kappa B (NF- $\kappa$ B), (Santa Cruz Biotechnology). O processamento imunohistoquímico seguiu o protocolo relatado por Garcia et al.<sup>15</sup>.

Uma amostra do colar gengival do primeiro molar inferior direito (vestibular) de todos os animais foi coletada antes da eutanásia dos animais (7, 15 e 30 dias), congelada em nitrogênio líquido e armazenada a -80 ° C22. As proteínas totais foram extraídas de amostras de tecido gengival usando tampão de extração (Tissue Protein Extraction Reagent [T-PER], Pierce Biotechnology; Thermo Fischer Scientific, Rockford, IL, EUA) contendo inibidores de protease (Protease Inhibitor Cocktail Set I – Calbiochem, Sigma-Aldrich, St. Louis, MO, USA), seguindo as instruções do fabricante (Pierce Biotechnology, Rockford, IL, USA). As amostras de tecido foram maceradas em tampão (50  $\mu$ L/mg de tecido) e centrifugadas por 5 min a 16.000g a 4 °C, e o sobrenadante descartado. A **Figura 2** mostra as etapas do experimento em animal.

**Figura 2** - Etapas do experimento em animal



Fonte: Arquivo pessoal do autor.

## **Análise imunológica**

A proteína total foi extraída das amostras de gengivais utilizando o reagente RIPA Lysis Buffer (Merck, Millipore, Massachusetts, EUA) com inibidores de protease (Protease Inhibitor Cocktail Set I, #cod 539131-1VL, Calbiochem®, Massachusetts, EUA), sendo então quantificada pelo método de ensaio de proteína BCA (Protein Assay, #cod 23225, Pierce™, ThermoFisher, Massachusetts, EUA) de acordo com as instruções do fabricante.

TNF- $\alpha$ , IL-1 $\beta$  e IL-10 foram avaliados por um ensaio multiplex usando o kit Milliplex Map Mouse Cytokine/Chemokine Magnetic Bead Panel (Merck, Millipore, Massachusetts, EUA) de acordo com o estudo anterior<sup>16</sup>. O ensaio foi realizado seguindo as instruções do fabricante.

## **Análise microtomográfica**

As amostras foram escaneadas em scanner microtomográfico (Micro-CT Skyscan 1172; Bruker, Kontich, Bélgica) do Laboratório de Microscopia e Imagem Biológica (LAMIB) da Universidade Federal da Paraíba (UFPB). Os seguintes parâmetros foram utilizados para aquisição das imagens: 80kv, 124mA, filtro de alumínio de 0,5mm, matriz grande (2000x2000), tamanho de pixel de 27,7 $\mu$ m, inclinação de rotação de 0,4, média 3, rotação de 180 graus e movimento aleatório desativado. Aproximadamente 1.100 imagens por amostra foram reconstruídas usando o software Nrecon (Bruker, Kontich, Bélgica). Os parâmetros utilizados para reconstrução foram: beam harding 5%, ring artefato de 5, smoothing 02 com valores de saída mínimo e máximo padronizados de 0,008524 a 0,102154, respectivamente. As imagens bidimensionais foram analisadas pelo software Data Viewer (Bruker, Kontich, Bélgica) em três cortes (axial, coronal e sagital).

A avaliação da perda óssea alveolar linear foi realizada usando o software CT Analyser (CTan, Bruker, Kontich, Bélgica), as junções amelocementárias (JAC) do segundo molar inferior direito foram colocadas paralelas entre si na vista coronal. No plano axial foi identificado o meio da coroa e no plano sagital foram registradas medidas lineares (mm) da JAC até a crista alveolar. Ao todo, foram realizadas cinco medidas conforme descrito anteriormente: na face mesial, distal, bucal, lingual e na região de bifurcação<sup>17,18</sup>.

A avaliação da perda óssea volumétrica na região dos segundos molares foi realizada no eixo axial, onde as coroas dos três molares estavam totalmente visíveis. O corte em que as três raízes do segundo molar estavam completamente separadas foi utilizado como ponto inicial

(topo) para esta análise, e o corte final (inferior) foi definido quando não era mais possível visualizar todas as raízes<sup>19</sup>.

O volume de interesse (VOI) foi obtido contornando manualmente toda a área óssea inter-radicular ao redor das raízes sem incluir o espaço do ligamento periodontal, repetidamente em um número aproximado de 110 cortes, em seguida as estruturas ósseas e dentárias foram separadas através da densidade mineral usando escala de cinza (25–255; limite da escala de cinza 0–255). Os parâmetros determinados nesta análise volumétrica foram a porcentagem do volume ósseo dividido pelo volume do tecido (BV/TV, %), porosidade (%) e espessura trabecular (TB) (mm<sup>3</sup>). Todas as análises de micro-TC foram realizadas por um examinador cego e calibrado.

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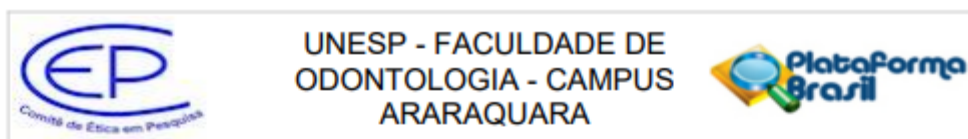
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\* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: <http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf>.

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## ANEXO A - CERTIFICADO DO COMITÊ DE ÉTICA



Continuação do Parecer: 5.825.313

Sugere-se aprovação do projeto de pesquisa.

**Considerações Finais a critério do CEP:**

Protocolo APROVADO Ad Referendum em 19 de dezembro de 2022.

O pesquisador deverá apresentar um relatório parcial no meio do período da pesquisa e um relatório final.

Deve ser apresentado somente o relatório final em pesquisas com duração de até 1 ano.

**Este parecer foi elaborado baseado nos documentos abaixo relacionados:**

| Tipo Documento                                            | Arquivo                                               | Postagem            | Autor                               | Situação |
|-----------------------------------------------------------|-------------------------------------------------------|---------------------|-------------------------------------|----------|
| Informações Básicas do Projeto                            | PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1865217.pdf         | 08/12/2022 20:37:40 |                                     | Aceito   |
| Projeto Detalhado / Brochura Investigador                 | Projeto_cep_saliva.pdf                                | 08/12/2022 20:37:34 | Denise Madalena Palomari Spolidorio | Aceito   |
| Cronograma                                                | Cronograma_saliva.pdf                                 | 08/12/2022 20:37:19 | Denise Madalena Palomari Spolidorio | Aceito   |
| Outros                                                    | Carta_Resposta_CAAE_64292522_4_000_5416_Nova.docx     | 08/12/2022 09:35:53 | Denise Madalena Palomari Spolidorio | Aceito   |
| Outros                                                    | Carta_Resposta_CAAE_64292522_4_000_5416.docx          | 21/11/2022 12:11:14 | Denise Madalena Palomari Spolidorio | Aceito   |
| TCLE / Termos de Assentimento / Justificativa de Ausência | Termo_Consentimento_Livre_Esclarecido_saliva.pdf      | 17/11/2022 10:59:04 | Denise Madalena Palomari Spolidorio | Aceito   |
| Outros                                                    | 1_Gabriel_saliva.pdf                                  | 18/10/2022 10:50:36 | GABRIEL GARCIA DE CARVALHO          | Aceito   |
| Outros                                                    | 2_Gabriel_saliva.pdf                                  | 18/10/2022 10:50:09 | GABRIEL GARCIA DE CARVALHO          | Aceito   |
| Outros                                                    | Cartacomitefinal.pdf                                  | 14/10/2022 08:59:05 | GABRIEL GARCIA DE CARVALHO          | Aceito   |
| Folha de Rosto                                            | Folha_de_rosto_2320221014_08570950.pdf                | 14/10/2022 08:57:39 | GABRIEL GARCIA DE CARVALHO          | Aceito   |
| Outros                                                    | Termo_compromisso_pesquisador_responsavel_Gabriel.pdf | 12/10/2022 20:05:16 | GABRIEL GARCIA DE CARVALHO          | Aceito   |

**Situação do Parecer:**

Aprovado

**Necessita Apreciação da CONEP:**

Endereço: HUMAITA 1680, SALA 512

Bairro: CENTRO

CEP: 14.801-903

UF: SP

Município: ARARAQUARA

Telefone: (16)3301-6459

E-mail: cep.foar@unesp.br

## ANEXO B - CERTIFICADO DO COMITÊ DE ÉTICA NO USO DE ANIMAIS



UNIVERSIDADE ESTADUAL PAULISTA  
"JÚLIO DE MESQUITA FILHO"  
Campus de Araraquara



FACULDADE DE ODONTOLOGIA

### CERTIFICADO

Certificamos que a proposta intitulada *"APLICAÇÕES MÚLTIPLAS DE APDT UTILIZANDO CLORINA-E6 EM RATOS COM DOENÇA PERIODONTAL INDUZIDA: ANÁLISE HISTOMORFOMÉTRICA, IMUNOHISTOQUÍMICA, IMUNOLÓGICAS E MICROBIOLÓGICA"*, registrada com o nº 14/2021, sob a responsabilidade do Prof<sup>a</sup> Dr<sup>a</sup> Denise Madalena Palomari Spolidorio – que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA FACULDADE DE ODONTOLOGIA DE ARARAQUARA em reunião de 31/08/2021.

|                         |                                               |
|-------------------------|-----------------------------------------------|
| Finalidade              | ( ) Ensino (X) Pesquisa Científica            |
| Vigência da autorização | Dezembro/2022                                 |
| Espécie/linhagem/raça   | Ratos Holtzman                                |
| Nº de animais           | 81                                            |
| Peso/Idade              | 250 a 350 g / 90 dias                         |
| Sexo                    | Macho                                         |
| Origem                  | Biotério Central da FOAr (Campus-Araraquara). |

*Marlise Inêz Klein Furlan*  
Dr<sup>a</sup>. MARLISE INÊZ KLEIN FURLAN  
Vice-Coordenadora da CEUA/FOAr

Comissão de Ética no Uso de Animais - CEUA  
Rua Humaitá nº 1.680 – Centro – CEP 14801-903 – Caixa Postal nº 331 - ARARAQUARA – SP  
5º andar – fone (16) 3301-6459 / fax (16) 3301-6433 / e-mail: ceua.foar@unesp.br - home page: <http://www.foar.unesp.br>

Não autorizo a publicação deste trabalho pelo prazo de 2 anos após a data de defesa.  
(Direitos de publicação reservado ao autor)

Araraquara, 27 de março de 2024

Gabriel Garcia de Carvalho