UNESP – Universidade Estadual Paulista
"Júlio de Mesquita Filho"
Faculdade de Odontologia de Araraquara

Sâmara Cruz Tfaile Corbi

Terapia fotodinâmica com ftalocianina de zinco tetracarboxi-N-metilglucamina na doença periodontal induzida em ratos

Araraquara
2017

UNESP – Universidade Estadual Paulista
"Júlio de Mesquita Filho"
Faculdade de Odontologia de Araraquara

Sâmara Cruz Tfaile Corbi

Terapia fotodinâmica com ftalocianina de zinco tetracarboxi-N-metilglucamina na doença periodontal induzida em ratos

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. Rosemary Adriana Chiérici Marcantonio

Araraquara
2017

Corbi, Sâmara Cruz Tfaile
Terapia fotodinâmica com ftalocianina de zinco tetracarboxi-N-metilglucamina na doença periodontal induzida em ratos / Sâmara Cruz Tfaile Corbi.-- Araraquara: [s.n.], 2017
84 f. ; 30 cm.

Tese (Doutorado em Odontologia) – Universidade Estadual Paulista, Faculdade de Odontologia
Orientadora: Profa. Dra. Rosemary Adriana Chiérici Marcantonio

1. Doenças periodontais 2. Fotoquimioterapia 3. Microtomografia por raio-X I. Título

Ficha catalográfica elaborada pela Bibliotecária Ana Cristina Jorge, CRB-8/5036
Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara
Serviço Técnico de Biblioteca e Documentação

Sâmara Cruz Tfaile Corbi

Terapia fotodinâmica com ftalocianina de zinco tetracarboxi-N-metilglucamina na doença periodontal induzida em ratos

Comissão Julgadora

Tese para obtenção do grau de Doutor em Periodontia

Presidente e Orientador: Profa. Dra. Rosemary Adriana Chiérici Marcantonio

2º Examinador: Prof. Dr. Joni Augusto Cirelli

3º Examinador: Profa. Dra. Raquel Mantuaneli Scarel-Caminaga

4º Examinador: Prof. Dr. Michel Reis Messora

5º Examinador: Profa. Dra. Chaine Pavone

Araraquara, 07 de Dezembro de 2017

DADOS CURRICULARES

Sâmara Cruz Tfaile Corbi

NASCIMENTO	10 de Outubro de 1986, Araraquara/SP
FILIAÇÃO	Sálua Cruz Tfaile Jeferson Luis Corbi
2007/2011	Curso de Graduação em Odontologia pela Faculdade de Odontologia de Araraquara – UNESP
2012/2014	Curso de Pós-Graduação em Odontologia, área de concentração em Periodontia, nível de Mestrado, na Faculdade de Odontologia de Araraquara – UNESP
2014/2017	Curso de Pós-Graduação em Odontologia, área de concentração em Periodontia, nível de Doutorado, na Faculdade de Odontologia de Araraquara – UNESP

Dedico este trabalho aos meus pais Sálua e Jeferson e

à minha irmã Sâmia, pela inspiração de todo dia e a vontade de vencer na vida.

Agradeço primeiramente a **Deus**, por colocar pessoas certas e especiais na minha vida. Pela sabedoria, força e amparo.

Aos meus pais **Sálua** e **Jeferson,** que me proporcionaram uma vida digna, onde eu pudesse crescer, acreditando que tudo é possível, desde que sejamos honestos, íntegros e de caráter e tendo a convicção de que desistir nunca seja uma ação contínua em nossas vidas; que sonhar e concretizar os sonhos só dependerá da nossa vontade. Em especial, agradeço minha mãe por ser um exemplo de vida, guerreira e batalhadora; que me mostra sempre os melhores caminhos, que me dá os conselhos certos, que me ensina a ser sensata e que diariamente me faz perceber quão abençoada e privilegiada eu sou.

À minha irmã **Sâmia,** pelo incessante estímulo, incentivo, exemplo, experiência, enorme compreensão e companheirismo, com quem compartilhei todos os momentos e que tem sido uma constante benção em minha vida.

À amiga e minha orientadora **profa. Adriana Marcantonio,** a quem dedico admiração, gratidão e respeito; quem me ensinou os primeiros passos da pesquisa científica. Pela influência, direcionamento, grande amizade, apoio e eterna paciência. E por compartilhar comigo seu tempo e conhecimento.

À minha **Tia Salma** (*in memorian*), que em vida sempre apoiou as minhas decisões, me incentivou a fazer tudo o que eu tinha vontade, mostrou os caminhos do bem e me ensinou que bondade e caridade podem vir de pequenos gestos. Agradeço por compartilhar comigo tantos sonhos, conversas, conselhos, tempo e risadas. Sinto muito sua falta.

Agradeço aos meus familiares, em especial aos meus avós **Aparecida** e **Geraldo** e, minha prima **Sabrina Frasnelli** e minha **Tia Samira**, por entenderem minha ausência, falta de tempo e as visitas rápidas. Pelo apoio de sempre e o incentivo de continuar meu caminho com força de vontade e fé no futuro.

Á minha amiga querida **Livia Finoti**, a quem tenho grande admiração e apreço. Pessoa única, com coração imenso, a irmã mais nova que eu não tive! Sempre disposta a me ajudar com paciência e entusiasmo. Me ensinou coisas da vida e compartilhou muitos conhecimentos na pós-graduação. Me auxiliou na execução da metodologia da análise radiográfica tridimensional do estudo 2, juntamente com a amiga **Ane Polly**, que prontamente me socorreu com soluções certeiras para o desenvolvimento da avaliação.

Aos meus amigos do Laboratório de Genética Molecular: **Sâmia Corbi**, **Livia Finoti**, **Suzane Pigossi**, **Rafael Nepomuceno**, **Giovana Anovazzi**, **Romerito Lins**, **Guilherme Braido**, **Fernanda Coelho**, **profa. Raquel Caminaga** e **profa. Ticiana Capote** pelos momentos de diversão, alegria, risos e descontração.

Aos meus amigos de Doutorado: **Paula Macedo**, **Jackeline Tsurumaki**, **Cássio Rocha**, **Fernanda Florian**, **Vinícius Paiva**, **Adriana Cabrera**, **Tiago Fonseca**, **Lauriê Garcia**, **Patricia Maquera**, **Lélis Nícoli** e **Elton Pichotano**, por todos os momentos de aprendizados que passamos juntos.

Aos mestres e amigos: **Rubens Spin-Neto**, pelos ensinamentos desde minha iniciação científica e a quem tenho grande admiração e **Guilherme Oliveira**, pela paciência, explicações, dicas e por sempre estar acessível e à disposição para tirar dúvidas e sugerir ideias.

Aos meus amigos do Convescote: **Marina Manduca**, **Alessandro Alavarce**, **Tainara Alves, Aline dos Santos, Aylan Meneghini**, **Adriano Felicio, Bruna Bolsoni**, **Maria Angélica**, **Fernando Pavaneli** e **Paulo Zaccaro**, pelas tardes de brincadeiras, jogos, gargalhadas e muito conhecimento inútil.

Às minhas amigas do colegial: **Mariana Rozatto** e **Michele Valila**, pela amizade de sempre, apoio, conselhos, descobertas, cumplicidade e simplesmente por estarem na minha vida após tantos anos.

Aos amigos e funcionários que me ajudaram na realização deste trabalho: **Mikail**, no trabalho no biotério; **Luana**, no escaneamento das imagens do micro-CT; **Claudinha** e **Leandro**, pela enorme paciência nos procedimentos laboratoriais; **Isa** e **Suleima** na organização e funcionamento das clínicas de Periodontia. E também a **Thelma** e **Toninho**.

À **Faculdade de Odontologia de Araraquara (UNESP)**, na pessoa de sua Diretora, **Profa. Dra. Elaine Maria Sgavioli Massucato**, e do Vice-Diretor, **Prof. Dr. Edson Alves de Campos**, pelas condições oferecidas para a realização desta pesquisa.

À **profa. Janice Rodrigues Perussi**, ao **prof. Anderson Orzari Ribeiro** e **Cláudia Bernal**, que sintetizaram e cederam o fotossensibilizador ftalocianina de zinco tetracarboxi-N-metilglucamina utilizado neste trabalho.

Ao Coordenador atual do Programa de Pós-Graduação em Odontologia, Área de Periodontia, **Prof. Dr. Joni Augusto Cirelli** e ao anterior **Prof. Dr. Carlos Rossa Jr.** e a todos os docentes da disciplina de Periodontia, pela excelente formação, dedicação, competência e empenho em suas atividades.

Aos funcionários da Seção de Pós-Graduação, **José Alexandre** e **Cristiano**, pela gentileza, paciência e por sempre solucionarem dúvidas!

Aos funcionários da Biblioteca, **Ceres**, **Marley**, **Eliane**, **Maria Inês**, **Ana Cristina**, **Denise**, **Laudicélia** e **Maria Aparecida** pela atenção e correção desta tese.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – **CAPES,** por conceder bolsa de estudos e à Fundação de Amparo à Pesquisa do Estado de São Paulo – **FAPESP**, pelo auxílio pesquisa e pela utilização do equipamento de micrótomografo computadorizado nesta faculdade, processo: 2009/54080-0.

MUITO OBRIGADA!

"Passarinho quando aprende a voar, sabe mais sobre coragem, que de vôo!"

(Autor Desconhecido)

Corbi SCT. Terapia fotodinâmica com ftalocianina de zinco tetracarboxi-N-metilglucamina na doença periodontal induzida em ratos [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2017.

RESUMO

A Terapia Fotodinâmica Antimicrobiana (Antimicrobial Photodynamic Therapy – aPDT), tem sido utilizada como um tratamento complementar na doença periodontal (DP). Ela combina um fotossensibilizador (FS) com uma fonte de luz que induz a produção de espécies reativas de oxigênio que elimina células microbianas. O objetivo deste trabalho foi avaliar, in vivo, os efeitos da aPDT (com o FS ftalocianina de zinco tetracarboxi-N-metilglucamina – 10μg/mL e luz LED vermelho – 655mn, 0,45W de potência), coadjuvante a Raspagem e Alisamento Radicular (RAR) e como monoterapia, além de verificar as respostas e alterações teciduais da DP induzida em ratos, pelas avaliações: microtomográfica, histométrica, estereométrica e histológica. Ligaduras foram inseridas nos sulcos dos segundos molares superiores para indução da DP. No Estudo 1, as ligaduras permaneceram por 15 dias e foram removidas para aplicar os tratamentos e no Estudo 2, as ligaduras foram colocadas por 7 dias e continuaram em posição por todo o experimento. 40 animais foram utilizados no Estudo 1 e distribuídos em 4 grupos: DP (Somente indução da doença, sem tratamento); RAR (Indução e tratamento básico periodontal); aPDT (Indução e aplicação da aPDT – FS ftalocianina de zinco tetracarboxi-N-metilglucamina e luz LED vermelho); RAR+aPDT (Indução, tratamento básico periodontal e aplicação da aPDT). 42 animais foram utilizados no Estudo 2 e divididos também em 4 grupos: FS (Tratamento somente com a ftalocianina de zinco tetracarboxi-N-metilglucamina); Luz (Tratamento somente com irradiação de luz LED vermelha); aPDT (Tratamento com a terapia fotodinâmica – FS+Luz) E DP (Indução da doença, sem tratamento). No Estudo 1, um dia após a remoção das ligaduras, foi aplicado os tratamentos e os animais foram eutanasiados nos períodos de 7 e 30 dias. No estudo 2, os animais receberam a aplicação das terapias e foram eutanasiados nos períodos de 7 e 15 dias. Para ambos os estudos, foi aplicado o teste paramétrico ANOVA one way, seguida do pós-teste de Tukey. No Estudo 1: na histometria, não foram encontradas diferenças estatísticas; a análise microtomográfica mostrou diferenças significantes nos dois períodos entre o grupo DP e RAR+aPDT e em 7 dias para DP e aPDT na região de furca. Nas proximais não houve diferenças significantes e na histologia, mostrou que não houve danos aos tecidos. No Estudo 2, na

análise histométrica e radiográfica tridimensional, os resultados não mostraram diferenças estatisticamente significantes na região de furca e nas regiões interproximais, o perfil inflamatório demonstrou uma tendência a apresentar menor quantidade de células inflamatórias no grupo aPDT em 7 dias e na análise histológica não houve diferenças entre os grupos, indicando também que as terapias não causaram danos aos tecidos. Pode-se concluir que a aPDTcom ftalocianina de zinco tetracarboxi-N-metilglucamina foi efetiva no controle de perda óssea em DP induzida em ratos e que a aplicação da aPDT (com os componentes como monoterapias) e com a preservação da ligadura, favoreceu a permanência das bactérias no local e inibiu a ação dos tratamentos.

Palavras-chave: Doenças periodontais. Fotoquimioterapia. Microtomografia por raio-X.

Corbi SCT. Photodynamic therapy with zinc tetracarboxy-N-metylglucamine phthalocyanine in induced periodontal disease in rats [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2017.

ABSTRACT

Antimicrobial Photodynamic Therapy (PDT) is a minimally invasive method consisting in the application of a photosensitive dye, which is subsequently stimulated by a light source and reacts with oxygen, producing reactive species. The aim of this study was to evaluate in vivo, the aPDT effects (with the PS zinc tetracarboxy-N-metylglucamine phthalocyanine 10μg/mL, and red LED light with 655nm), as adjuvant treatment to Scaling Root and Planing (SRP) and as monotherapy and verify the responses and tissue changes after aPDT application in PD-induced rats by microtomographic, histometric, stereometric and histological evaluations. Ligatures were placed around the second maxillary molars for PD induction. In Study 1, the ligatures were placed for 15 days and then they were removed. On the following day the treatments were performed. In Study 2, the ligatures were placed for 7 days and remained in position throughout the periods. Forty animals were used in Study 1 and they were divided into 4 groups: PD (disease induction only, without treatment); SRP (induction and basic periodontal treatment); PDT (Induction and application of photodynamic therapy); SRP+PDT (induction, application of photodynamic therapy and basic periodontal treatment). Forty-two animals were used in Study 2 and they divided into 4 groups: PS (Treatment with zinc tetracarboxy-N-metylglucamine phthalocyanine only); Light (Treatment with red LED light irradiation only); aPDT (Treatment with photodynamic therapy – PS + Light) and PD (Periodontal disease induction, without treatment). In Study 1, the animals were euthanized after 7 and 30 days of treatment. In Study 2, the therapies were applied at zero period and the animals were euthanized at 7 and 15 days. For both studies, one-way ANOVA parametric test was applied, followed by Tukey's post-test. In Study 1, concerning histometry data, no statistical differences were observed between groups. The microtomographic analysis indicated significant differences in the two periods for the PD and SRP+PDT groups and, at 7 days, for the PD and PDT groups in the furcation area. No significant differences the interproximal regions were observed. Regarding the histological analyses, no tissue damage was observed. In Study 2, the three-dimensional radiographic and histometric analyses revealed no statistically

different results for the furcation and interproximal regions. The inflammatory profile presented a trend of lower amounts of inflammatory cells in the aPDT group at 7 days, while the histological analysis indicated no significant differences between the groups, indicating that the therapies did not cause tissue damage. Thus, these data indicate that PDT using zinc tetracarboxy-N-metylglucamine phthalocyanine was effective in maintaining bone loss in PD-induced in rats, although further studies are required to further elucidate PDT effects and the application of aPDT and its components as monotherapies in PD-induced in rats with the preservation of the ligatures, favored *in situ* bacteria permanence and inhibited treatment action.

Keywords: Periodontal disease. Photochemotherapy. Computadorized microtomography.

SUMÁRIO

1 INTRODUÇÃO

A Doença Periodontal (DP) é uma doença multifatorial, de caráter inflamatório, que acomete os tecidos de suporte do dente e caracteriza-se por inflamação gengival, perda do nível de inserção e reabsorção do osso alveolar (American et al.[2], 2001; Armitage[4], 2004). O fator etiológico primário da DP é a presença de microrganismos organizados em biofilmes que colonizam a superfície dos dentes. O biofilme é uma estrutura complexa composta por microcolônias de bactérias impregnadas em uma matrix extracelular que promove proteção celular e facilita a aderência aos dentes (Socransky, Haffajee[64], 2002; Socransky, Haffajee[65], 1991).

O padrão ouro no tratamento em Periodontia é a Raspagem e Alisamento Radicular (RAR), também conhecido como tratamento básico periodontal, onde a remoção mecânica do biofilme e do cálculo é realizada por um profissional em conjunto com o controle de placa feito pelo paciente (Tagge et al.[72], 1975). No entanto, em alguns casos, o debridamento mecânico pode ser falho ao remover alguns organismos patogênicos, seja por sua localização no tecido subgengival ou locais de difícil acesso, como regiões de furca, bolsas periodontais profundas e anatomias dentárias incomuns (Slots[61], 2002; Slots, Ting[62], 2002). Bonito et al.[7] (2005), mostraram que o tratamento básico periodontal pode não remover completamente as bactérias patogênicas. Além disso, a RAR isolada, reduz temporariamente a infecção bacteriana e pode resultar a um retorno aos níveis de pré-tratamento em menos de duas semanas (Adriaens et al.[1], 1988; Giuliana et al.[23], 1997).

Alguns pacientes continuam apresentando destruição do tecido periodontal após a realização da RAR. Estes pacientes, frequentemente, têm fatores de risco associados, tal como fumo, diabetes, condições hereditárias e doenças sistêmicas, que são acompanhadas por infecção persistente de uma ou mais bactérias periodontais patogênicas (Galler[19], 2000; Grossi et al.[26], 1994; Kuo et al.[33], 2008).

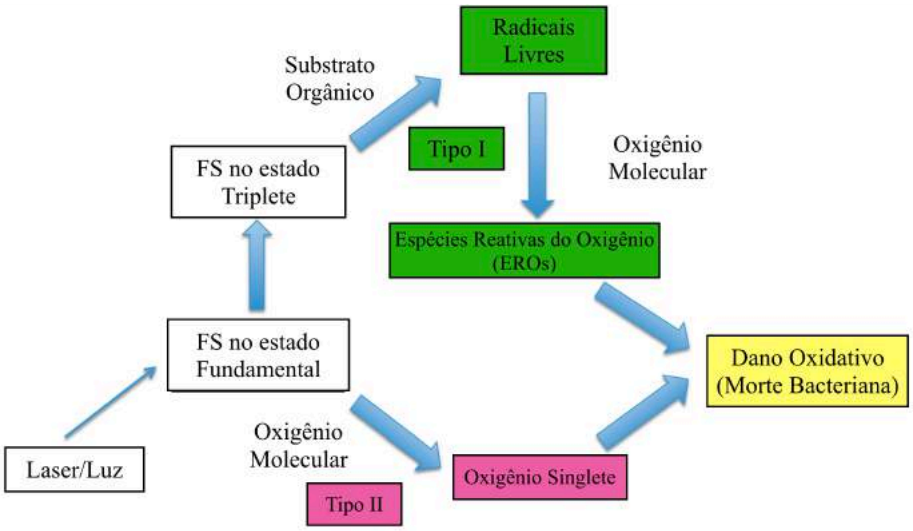
Assim, para contornar esta temática, o uso de antibióticos sistêmicos tem sido indicado. No entanto, a antibioticoterapia tem diminuído ao longo dos anos por causar efeitos colaterais, como problemas gastro-intestinais e o aumento de resistência bacteriana a estes medicamentos, já que em alguns casos, os pacientes abandonam o tratamento antes do término, facilitando assim, a seleção bacteriana (Slots[61], 2002; Walker[76], 1996).

Na última década, as limitações da terapia periodontal convencional e a

problemática da administração de antibióticos, resultaram nas tentativas de introduzir a Terapia Fotodinâmica ou Terapia Fotodinâmica Antimicrobiana (do inglês, *Antimicrobial Photodynamic Therapy – aPDT*), como um tratamento adjunto à periodontite crônica (Braun et al.[9], 2008; de Almeida et al.[13], 2008; de Almeida et al.[15], 2007; Meisel, Kocher[41], 2005). Além disso, a aPDT tem sido confirmada como sendo uma terapia antimicrobiana não antibiótica (Hamblin, Hasan[27], 2004; Wilson[77], 1993).

A aPDT combina luz visível de baixa intensidade (LASER/LED) e um fotossensibilizador (FS), corante não tóxico e fotossensível, que na presença de oxigênio, produz Espécies Reativas de Oxigênio (EROs) citotóxicos, tal como oxigênio singlete (Hamblin, Hasan[27], 2004; Huang et al.[29], 2012), sendo tóxico para bactérias. Assim resumidamente, a aPDT utiliza oxigênio singlete e radicais livres produzidos pelo FS ativado por luz, para eliminar bactérias. O processo fotoquímico é iniciado por uma fonte de luz de baixa intensidade com um comprimento de onda adequado e, desse modo, o FS no estado fundamental absorve luz e resulta em um estado singlete que pode perder energia por fluorescência para um estado triplete com longevidade. Este ultimo estágio leva à uma reação fotoquímica, que induz oxigênio singlete, radicais livres e superóxidos, que são citotóxicos, a causar a morte bacteriana (Kharkwal et al.[30], 2011), como ilustra a Figura 1. Curiosamente, enquanto a aPDT pode eliminar bactérias resistente à antibióticos (Maisch[37], 2009), não há informações sobre microrganismos desenvolvendo resistência a aPDT (Giuliani et al.[24], 2010).

Figura 1 – Mecanismo fotoquímico da Terapia Fotodinâmica.



Fonte: Elaboração própria. Adaptado de Kikuchi et al.[31](2015) para o português.

Vários estudos tem mostrado que bactérias periodontopatogênicas são susceptíveis à aPDT em culturas planctônicas (Bhatti et al.[5], 2002; Bhatti et al.[6], 1997; Chan, Lai[11], 2003; Klepac-Ceraj et al.[32], 2011; Matevski et al.[39], 2003; Nagahara et al.[45], 2013; Soukos et al.[70], 1998; Topaloglu et al.[74], 2013; Voos et al.[75], 2014; Wilson et al.[81], 1993) e biofilms (Klepac-Ceraj et al.[32], 2011; Voos et al.[75], 2014; Wilson et al.[80], 1992; Wood et al.[83], 1999) utilizando como FS azul de metileno (Chan, Lai[11], 2003; Dobson, Wilson[18], 1992; Wilson et al.[81], 1993), azul de metileno com nanopartículas (Klepac-Ceraj et al.[32], 2011), azul de toluidina O (Bhatti et al.[5], 2002; Bhatti et al.[6], 1997; Dobson, Wilson[18], 1992; Matevski et al.[39], 2003; Wilson et al.[81], 1993), ftalocianina (Dobson, Wilson[18], 1992; Wood et al.[83], 1999), hematoporfirina HCl (Dobson, Wilson[18], 1992), hematoporfirina ester (Dobson, Wilson[18], 1992), conjugado de poli-L-lisina com o FS clorina (e6) (Soukos et al.[70], 1998), indocianina verde (Topaloglu et al.[74], 2013), indocianina com nanoesferas (Nagahara et al.[45], 2013) e safranina O (Voos et al.[75], 2014). Porém, outros estudos tem demonstrado destruição incompleta de patógenos orais (Muller et al.[44], 2007; O'Neill et al.[47], 2002; Qin et al.[52], 2008; Soukos et al.[66], 2003; Soukos et al.[67], 2000).

A utilização de FS em aPDT, como porfirinas, ftalocianinas e fenotiazínicos (azul de metileno e azul de toluidina O) podem afetar tanto bactérias Gram-positivas como Gram-negativas por carregarem uma carga positiva (Merchat et al.[42], 1996; Merchat et al.[43], 1996; Wilson et al.[78], 1995), sugerindo que a aPDT pode ser útil para aplicações orais, especialmente para o tratamento periodontal (Passanezi et al.[50], 2015; Sgolastra et al.[57], 2013; Smiley et al.[63], 2015).

Bactérias Gram-negativas são amplamente resistentes à muitos FS utilizados na aPDT (Malik et al.[38], 1992), no entanto, algumas espécies, tal como bactérias pigmentadas com cor preta, contém FS naturais e são muito susceptíveis a aPDT. Foi demonstrado que o comprimento de onda variando de 380 a 520nm induz uma redução tripla de crescimento de *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, e *Prevotella melaninogenica* em amostras de biofilme dental obtidas de pacientes com periodontite crônica (Soukos et al.[68], 2005).

Além de estudos em bactérias, os efeitos da aPDT têm sido avaliados também por vários modelos de ligadura em ratos, com indução de periondontite experimental (Carvalho et al.[10], 2011; de Almeida et al.[15], 2007; Prates et al.[51], 2011). A ligadura leva ao acúmulo de biofilme, resultando em perda de inserção e e reabsorção de osso alveolar em 7 dias (Graves et al.[25], 2008). Resultados favoráveis com aPDT como uma

terapia adjuvante à RAR têm sido relatados em periodontite experimental em ratos (de Almeida et al.[13], 2008; de Almeida et al.[14], 2008; de Almeida et al.[15], 2007; Garcia et al.[22], 2014). A progressão da periodontite experimental foi substancialmente reduzida por aPDT em análises radiográfica e histológica (de Almeida et al.[15], 2007). Resultados positivos semelhantes também foram obtidos em áreas de furca (de Almeida et al.[14], 2008; Garcia et al.[21], 2013). Os ratos tratados com aPDT exibiram um número reduzido de células positivas ao ácido fosfatase resistente a tartarato, fraca imunoreatividade ao receptor-ativador do fator nuclear-κB, e forte imunoreactividade da osteoprotegerina (Garcia et al.[20], 2013; Garcia et al.[22], 2014). A eficácia da aPDT também foi confirmada na infecção periodontal em um modelo de cão da raça Beagle (de Oliveira et al.[17], 2011; Sigusch et al.[59], 2005). A melhora na cicatrização periodontal, associada à organização do colágeno, infiltração de células inflamatórias e perda óssea, com a adição da aPDT também tem sido relatada (Prates et al.[51], 2011).

Um receio para a aplicação clínica da aPDT é a potencial fotocitotoxicidade para as células hospedeiras. Contudo, foi demonstrado que as doses de luz necessárias para eliminar as bactérias na aPDT são muito mais baixas do que aquelas que são tóxicas para queratinócitos e fibroblastos (Soukos et al.[69], 1996). De fato, alguns efeitos benéficos da PDT foram relatados em células do ligamento periodontal, como inibição de mediadores inflamatórios, favorecendo a quimiotaxia celular e a promoção da vasodilatação local e a angiogênese (Houreld, Abrahamse[28], 2007). Em termos de modulação da imunidade inata, a PDT atua sobre neutrófilos e promove migração (Tanaka et al.[73], 2012). A PDT também inativa citocinas derivadas do hospedeiro, como o fator de necrose tumoral-α e a interleucina-1β, para inibir a ativação da E-selectina em células endoteliais (Braham et al.[8], 2009). A PDT afeta as células apresentadoras de antígenos, como macrófagos e células de Langerhans, reduzindo sua capacidade de ativar os linfócitos T e enfraquecendo a resposta inflamatória (Seguier et al.[56], 2010).

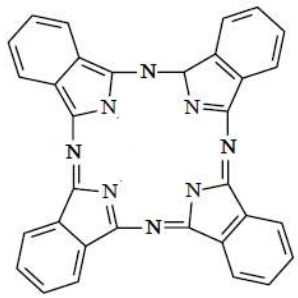
Duas vantagens principais são frequentemente citadas para a aPDT em comparação à outros tratamentos periodontais: Primeiro: na aPDT, um FS é colocado diretamente na bolsa periodontal e pode ser ativado através de uma fibra óptica colocada no mesmo local, o que ajuda a evitar danos aos tecidos circundantes (Qin et al.[53], 2008). Segundo: os efeitos da aPDT são iniciados pela exposição à uma fonte de luz e deste modo, as bactérias podem ser erradicadas em um curto período de tempo. Assim o desenvolvimento de resistência bacteriana é improvável (Maisch[36], 2007). Importante ressaltar que a erradicação de biofilmes e a inativação de citocinas

inflamatórias por aPDT provou ser eficaz e segura (Kikuchi et al.[31], 2015).

Nas últimas décadas, foram desenvolvidos compostos considerados como a segunda geração de corantes, com propósitos diagnóstico e terapêutico. Dentre eles, está as ftalocianinas, que são corantes sintéticos semelhantes às porfirinas e estruturalmente consideradas azaporfirinas (Spikes[71], 1986) e tem sido estudadas e avaliadas (Longo et al.[35], 2012; Nunes et al.[46], 2004; Ribeiro et al.[54], 2013). Estes compostos têm uma banda de absorção no espectro electromagnético que varia de 650 a 680nm, o que permite uma maior penetração da luz nos tecidos (Nunes et al.[46], 2004) e as suas propriedades fotofísicas dependem da composição, particularmente do íon metálico central. Entre as ftalocianinas, a ftalocianina de cloro-alumínio tem sido sugerida por possuir propriedades fotofísicas favoráveis para uso em aPDT, uma vez que produz altas quantidades de oxigênio singlete (Nunes et al.[46], 2004). A eficácia deste fotossensibilizador associado à luz LED foi comprovada em um estudo in vitro que avaliou o potencial fotodinâmico da ftalocianina de cloro-alumínio diluída em nanoemulsão catiônica para inativar as culturas planctônicas e de biofilmes formados por *Candida albicans* (Ribeiro et al.[54], 2013). Este FS foi também eficaz para a inativação de bactérias em pacientes com lesões cariosas (Longo et al.[35], 2012). Outros estudos (Dobson, Wilson[18], 1992; Oliveira et al.[49], 2006; Sibata et al.[58], 2004; Wilson, Dobson[79], 1993; Wilson et al.[81], 1993; Wilson, Pratten[82], 1995) têm corroborado a eficiência das ftalocianinas como agentes fotossensíveis na eliminação de microrganismos periodontopatogênicos com uso em aPDT. E também, as ftalocianinas de zinco (FcZn) estão entre os sensibilizadores promissores deste grupo (Anholt, Moan[3], 1992; Rosenthal[55], 1991; Spikes[71], 1986).

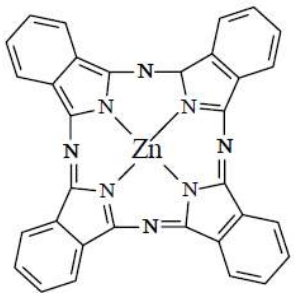
A ftalocianina (Figura 2) é um macrociclo simétrico composto por quatro unidades iminoisoindol com uma cavidade central de tamanho suficiente para acomodar vários íons metálicos e, este metal central possui influência considerável em sua propriedade fotossensibilizadora (Figura 3) (Anholt, Moan[3], 1992; Rosenthal[55], 1991; Spikes[71], 1986). O nome ftalocianina vem de uma combinação do prefixo phthal, originalmente do grego naphtha (óleo de rocha), para enfatizar a associação com seus vários precursores derivados do ácido ftálico, e o grego cyanine (azul) (Mckeown[40], 1998).

Figura 2 – Estrutura química da ftalocianina.



Fonte: Adaptado de de Melo[16] (2014).

Figura 3 – Estrutura química da ftalocianina acomodando o íon metálico zinco.



Fonte: de Melo[16] (2014).

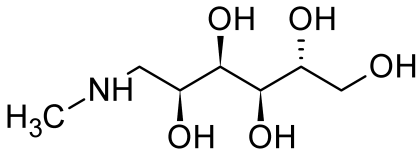
Um fato importante é quanto à hidrofobicidade que alguns fotossensibilizadores apresentam, pois essa propriedade em meio aquoso leva à auto-agregação e em muitos casos a uma subsequente precipitação, o que reduz drasticamente a capacidade do composto de gerar oxigênio singlete (Simplicio et al.[60], 2002). Desse modo é necessário que o princípio fotoativo apresente-se solúvel em meio aquoso para possível aplicação clínica.

Uma estratégia interessante para aumentar a solubilidade em meio aquoso de alguns FS envolve a formação de espécies supramoleculares hidrofílicas. Uma supramolécula é definida como uma espécie química constituída por duas ou mais moléculas unidas por interações intermoleculares. Nesse sentido a química supramolecular utiliza uma abordagem centrada na associação de moléculas, visando a obtenção de uma determinada propriedade ou funcionalidade (de Melo[16], 2014).

A meglumina, conhecida também como N-metilglucamina (Figura 4) é um aminocarboidrato derivado da glicose capaz de formar espécies supramoleculares

binárias hidrofílicas com compostos que possuam em sua estrutura átomos de hidrogênio ácidos(de Melo[16], 2014).

Figura 4 – Estrutura química da meglumina.



Fonte: de Melo[16] (2014).

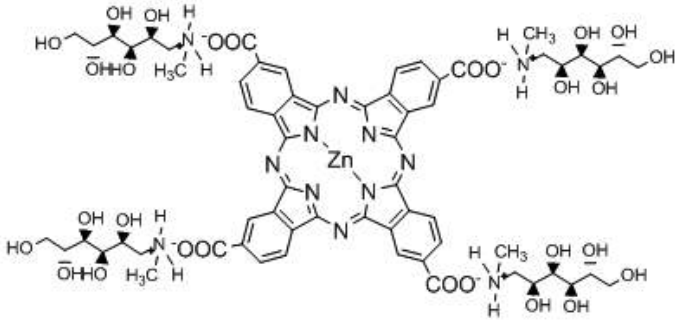
A formação dos compostos de meglumina envolve como condição uma reação ácido-base em que o hidrogênio ácido é transferido ao grupo amina do aminocarboidrato. Para viabilizar a formação de espécies supramoleculares entre a meglumina e as ftalocianinas, estas primeiramente são funcionalizadas com grupos carboxílicos como a ftalocianina de zinco tetracarboxilada (FcZnTc, Figura 5) (de Melo[16], 2014).

Figura 5 – Ftalocianina-Zn-tetracarboxilada.

Fonte: de Melo[16] (2014).

Posteriormente à inserção dos grupos ácidos, a reação com o aminocarboidrato pode ser conduzida produzindo espécies mais solúveis em água. Esse procedimento permite a inserção de até quatro moléculas de meglumina como é observado na Figura 6 para a ftalocianina de zinco tetracarboxi-N-metilglucamina (FcZnTcG). A presença de vários grupos hidroxila na supramolécula fornece os sítios onde as ligações de hidrogênio são estabelecidas aumentando a hidrofilicidade das ftalocianinas (de Melo[16], 2014).

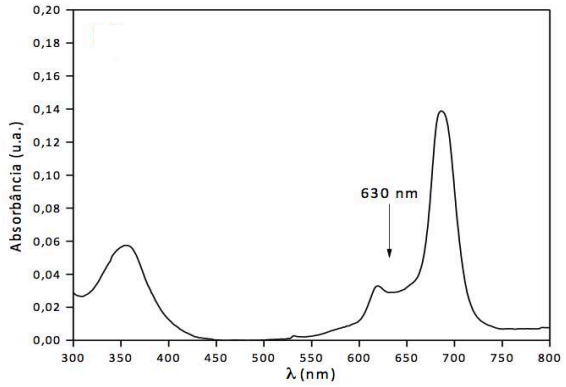
Figura 6 – Ftalocianina-Zn-tetracarboxi-N-metilglucamina.



Fonte: de Melo[16] (2014).

O espectro de absorção das ftalocianinas em solução consiste de duas bandas principais centradas em torno de 350 nm e 670 nm. A Figura 7 ilustra o espectro de absorção molecular da ftalocianina de zinco tetracarboxilada.

Figura 7 – Espectro de absorção molecular da ftalocianina de zinco tetracarboxilada em DMSO.



Fonte: de Melo[16] (2014).

Na literatura atual, há uma considerável quantidade de estudos em humanos e animais avaliando a aPDT com agentes fotossensibilizadores tradicionais (azul de metileno e azul de toluidina O), obtendo resutados satisfatórios utilizando diferentes metodologias. Os estudos não possuem padronização nos parâmetros de luz, dos tipos e concentrações dos fotossensibilizadores, tempos empregados, entre outros fatores que influenciam a ação da terapia. Alémd isso, existe uma escassez de estudos sobre corantes recém descobertos/descritos (como as ftalocianinas) e seu potencial na eliminação de microrganismos que mostra sua efetividade nas respostas e alterações teciduais da aplicação da aPDT na DP induzida em ratos.

2 PROPOSIÇÃO

Os objetivos deste estudo foram:

Objetivos gerais

Ambos os estudos tiveram como intuito observar perda óssea alveolar, verificar o grau de inflamação gengival e analisar características histológicas periodontais, utilizando o FS na sua forma mais solúvel, ftalocianina de zinco tetracarboxi-N-metilglucamina.

Objetivos Específicos

Publicação 1. Efeitos da aPDT como terapia associada ao tratamento da DP experimentalmente induzida em ratos.

Publicação 2. Respostas e alterações teciduais utilizando os componentes da aPDT como monoterapias também na DP induzida.

3 PUBLICAÇÕES

3.1 Publicação 1

Evaluation of Antimicrobial Photodynamic Therapy Effects Using Phthalocyanine-Glucamine Photosensitizer as an Adjunct Therapy in the Treatment of Induced Periodontal Disease in Rats*

Sâmara C. T. Corbi, Paula D. Macedo, Janice R. Perussi, Anderson O. Ribeiro, Rosemary A. C. Marcantonio

*Artigo submetido à revista Archives of Oral Biology em 08/11/2017 (Anexo B)
Manuscript ID: AOB-D-17-00667

Evaluation of Antimicrobial Photodynamic Therapy Effects Using Phthalocyanine-Glucamine Photosensitizer as an Adjunct Therapy in the Treatment of Induced Periodontal Disease in Rats

Sâmara Cruz Tfaile Corbi (SCT, Corbi) – PhD student in Periodontics[1]; email: sa_tfaile@yahoo.com.br

Paula Delello Macedo (Macedo, PD), PhD student in Periodontics[1]; email: paula_mac2@yahoo.com.br

Janice Rodrigues Perussi (Perussi, JR), Associate Professor in Chemistry[2]; e-mail: janice@iqsc.usp.br

Anderson Orzari Ribeiro (Ribeiro, AO), Adjunct Professor from UFABC[3], email: anderson.ribeiro@ufabc.edu.br

Rosemary Adriana Chiérici Marcantonio (Marcantonio, RAC), Associate Professor in Periodontics[1]; email: adriana@foar.unesp.br

[1]Sao Paulo State University (UNESP), School of Dentistry, Diagnosis and Surgery Department, Araraquara, SP, Brazil

[2]Sao Paulo University (USP), Chemistry and Molecular Physics Department, Sao Carlos, SP, Brazil

[3]Federal University of ABC (UFABC), Centre for Natural Sciences and Humanities, Santo Andre, SP, Brazil

[*]**Corresponding author:**

Rosemary Adriana C. Marcantonio, PhD

Department of Diagnosis and Surgery

Sao Paulo State University – School of Dentistry at Araraquara (UNESP)

CEP 14801-903, Araraquara, SP, Brasil

Tel: +55 16 3301-6376

E-mail: adriana@foar.unesp.br

ABSTRACT

Antimicrobial Photodynamic Therapy (PDT) is a minimally invasive method consisting in the application of a photosensitive dye, which is subsequently stimulated by a light source and reacts with oxygen, producing reactive species. The aim of the present study was to evaluate in vivo tissue responses to PDT using phthalocyanine-glucamine and red LED light in the treatment of induced periodontal disease (PD) in rats, through microtomographic, histometric and histological evaluations. Ligatures were placed into the sulcus of the second maxilarry molars for PD induction. The animals were divided into 4 groups: PD (disease induction only, without treatment); SRP (induction and basic periodontal treatment); PDT (Induction and application of photodynamic therapy); SRP+PDT (induction, application of photodynamic therapy and basic periodontal treatment). The ligatures were removed after 15 days and treatments were performed the following day. The animals were euthanized after 7 and 30 days of treatment. As all data were normaly distributed (Kolmogorov-Smirnov), the parametric ANOVA test was applied, followed by the Tukey test. Concerning histometry data, no statistical differences were observed between groups. The microtomographic analysis indicated significant differences in the two periods for the PD and SRP+PDT groups and, at 7 days, for the PD and PDT groups in the furcation area. No significant differences the interproximal regions were observed. Regarding the histological analyses, no tissue damage was observed. These data indicate that PDT using phthalocyanine-glucamine was effective in maintaining bone loss in PD-induced in rats, although further studies are required to further elucidate PDT effects.

Keywords: Periodontal diseases. Photochemotherapy. X-ray microtomography

INTRODUCTION

Periodontal disease (PD) is a multifactorial inflammatory disease that develops when the equilibrium between the host response and microbial challenge is altered (Andersen, Loebel, Hammond, & Wilson, 2007). This condition is clinically characterized by inflammation, bleeding on probing and pronounced loss of insertion, whose primary etiological factor is constituted by oral biofilm bacteria (de Almeida, Garcia, & Theodoro, 2006; de Almeida et al., 2007; Qin et al., 2008).

Scaling Root and Planing (SRP) is aimed at the removal of this oral biofilm, enabling health reinstatement, tissue regeneration and lessening clinical signs of inflammation (Qin et al., 2008). However, in some cases, this therapy does not seem to be able to repair or maintain periodontal health, which may lead to the permanence or recolonization of microorganisms (Drisko, 1998).

Studies have proposed the use of local and systemic antibiotics associated with SRP to aid in bacterial combat (Vergani, Silva, Vinholis, & Marcantonio, 2004). However, the regular and indiscriminate use of these drugs can cause several side effects, in addition to possible bacterial resistance (de Almeida et al., 2006; de Almeida et al., 2007; Jori et al., 2006; Pfitzner, Sigusch, Albrecht, & Glockmann, 2004; Qin et al., 2008). Therefore, the relevance of a search for a new technique or therapy compatible with conventional mechanical treatments is clear, allowing for greater efficiency in periodontal treatment.

Photodynamic Therapy (PDT), also called Antimicrobial Photodynamic Therapy (aPDT), because it affects microorganisms, is a new and thriving clinical treatment that basically employs the combination of the triad: oxygen, light source, and a photosensitizing agent (PS) (Maisch, 2007). Each of these isolated factors is not capable of causing damage, but, when combined, produce lethal cytotoxic agents that can selectively kill cells (Sharman, Allen, & van Lier, 1999).

The mechanism of action of PDT occurs due to the excitation of a non-toxic photosensitive dye (PS) which, when irradiated by visible light at a frequency resonant with the level of optical absorption of said substance, transfers energy to the surrounding molecules, generally molecular oxygen (O_2), and produces highly reactive species (ROS), such as free radicals and singlet oxygen and the latter, can modify the structures of plasma membranes or even DNA (Jori et al., 2006) and can also cause cell death through various mechanisms, including lipid peroxidation, inhibition of the enzymatic system, protein agglutination and reactions with other biological systems (Andersen et al., 2007). In periodontics, the success in eliminating microorganisms by PDT indicates that is as an adequate adjunct therapy in the fight against localized infections (Jori et al., 2006).

In vitro (Klepac-Ceraj et al., 2011) and in vivo (Andersen et al., 2007; de Almeida et al., 2007; Qin et al., 2008; Sigusch, Pfitzner, Albrecht, & Glockmann, 2005) studies have analyzed PDT effects on both periodontopathogenic and non-periodontopathogenic bacteria exposed to several PS and different wavelengths, resulting in an effective antimicrobial action of up to 99%. Clinically, a reduction in pocket depth, clinical insertion level and bleeding has been observed, thus documenting the effectiveness of PDT as a adjunct therapy in the treatment of PD (Qin et al., 2008). However, despite the benefits of this technique, some studies reveal difficulties in reaching microorganisms located in the deeper layers of oral biofilms. Even so, PDT produces higher bacterial death when compared to the use of systemic antibiotics (Fontana et al., 2009).

Phthalocyanines (FC) are a promising group of second generation

photosensitizers for PDT. They comprise organic compounds whose structure includes a ring formed by eight carbon atoms and eight nitrogen atoms joined by conjugated double bonds (Ryskova & Slezak, 2010). As a rule, FC display effective tissue penetration, since their most adequate light absorption region is between 600nm and 800nm (Ogunsipe et al., 2008). FC show high selectivity, low phototoxicity and are resistant to chemical or photochemical degradation (Garcia & Bentley, 2003). Their photophysical properties are strongly influenced by the presence and nature of the central metal ion, directly reflecting on the life time of the triplet excited state of metallophthalocyanines (Bonnet, 1995), providing a significant amount of singlet oxygen species that are able to remain in the triplet excited state for a longer period of time (Ryskova & Slezak, 2010).

Scarce studies are available on PS and their potential in eliminating microorganisms, mainly in dentistry, when applying PDT in the treatment of PD, as well as their placement associated with conventional periodontal therapy. In this context, the aim of the present study was to evaluate in vivo tissue responses after PDT using a phthalocyanine-glucamine as an adjunct therapy in the treatment of PD-induced in rats by means of three-dimensional radiographic (μCT), histometric and histological analyses.

MATERIAL AND METHODS*

Ethics comittee

This project was approved by the Ethics Committee on Animal Experimentation (No. 07/2012)[#].

Samples

Forty rats (*Rattus norvegicus*) of the *albinus* variation, *Holtzman*, adults, weighing between 300-330g were used. The animals were kept in plastic boxes, 5 animals per box, and treated with water and food *ad libitum* before and during the whole experimental period. The animals were maintained in an environment with controlled light, humidity and temperature.

Periodontal disease induction

The animals were anesthetized with a combination of ketamine (ketamine hydrochloride - Francotar 3% - Virbac do Brasil Ind. e Com. Ltda.) and xylazine (xylazine hydrochloride - Virbaxyl 2% - Virbac do Brasil Ind. e Com. Ltda.) at 0,08mL/100g and 0,04mL/100g body weight, respectively. The PD-induced hemimaxilae were chosen randomly (right or left). The ligatures were inserted in the

*Para metodologia completa, ver Apêndice 1. [#]Aprovação do Comitê de Ética, ver Anexo A.

subgingival region, into the sulcus and around the second maxillary molars using no. 24 cotton threads. The ligatures were removed after 15 days, and the treatments were performed one day after in each group (Figure 1).

Photodynamic Therapy

Photosensitizer preparation

Phthalocyanine-glucamine was prepared from a stock solution of zinc-tetracarboxy-phthalocyanine at 1,1mg/mL in DMSO and subsequently diluted in phosphate buffered saline (pH=7.2) to a final concentration of 10μg/mL. Soon after the preparation, the PS was stored in light-protected poliproylene tubes maintained in a refrigerated environment until use. For application, a blunt tip syringe containing 0,2mL of the PS was inserted into the gingival sulcus. The solution was applied around the entire tooth and, after 10 minutes of incubation time, light irradiation was performed.

Light source

The light source used to activate the phthalocyanine-glucamine PS corresponded to a wavelength of 655 nm, 0,45W power, 0,47W/cm^2 power density and 170,52J/cm^2 dose (red LED, 11mm diameter, DMC Equipamentos Ltda, São Carlos, Brazil), coinciding with the maximum absorption band of phthalocyanine-glucamine. The LED light was placed on the occlusal surface of the teeth and irradiation was maintained for 6 minutes.

Experimental groups

On the day after ligatures removal, the animals were randomly divided and treated according to their group (5 animals/group/period):

- *PD Group (PD):* disease induction only, without treatment.
- *Scaling Root and Planing Group (SRP)*: SRP performed with specific curettes (Gracey mine Five 5-6, HuFriedy).
- *Photodynamic Therapy Group (PDT):* Application of the PS followed by LED application.
- *SRP+PDT Group (SRP+PDT):* SRP (same as the SRP group) followed by PDT (same as the PDT group).

The animals were euthanized with an anesthetic overdose at 7 and 30 days after the treatments. The hemimaxillae were removed and fixed in 4% paraformol for 48h. Subsequently, the samples were washed in running water for 24h and placed in 70% alcohol, where they remained until the computerized microtomograph scanning.

Three-dimensional Radiographic Analysis (µCT)

The samples were scanned by means of X-ray beam scanning in a computerized microtomography system (Skyscan 1176, Aatselaar, Belgium, 2003). The parameters of the equipment were set as follows: Al 0.5mm filter; Voxel size: 17.48µm; Voltage 50KV and electric current 500µA. After scanning, the 3D images for each sample were obtained through the equipment software (NRecon 1.6.1.5 - SkyScan N. V., Belgium, 2003).

The images were rotated and repositioned in a standard orientation the Dataviewer software (SkyScan 1176, Aartselaar, Belgium, 2003) and a contrast threshold (ranging from 59 to 255) was established to distinguish mineralized tissues using the CTan/CTvol software (Skyscan 1176, Aatselaar, Belgium, 2003). The regions of interest (ROI) were positioned by measuring 3 regions of the second maxillary molar; a 1.26x1.15mm^2 furcation area and the mesial and distal proximal areas (1.26x0.56mm^2) from the cement-enamel junction (Figure 2). The data were expressed as a percentage of volume of bone tissue of each region.

Histological processing

After scanning, the samples were placed in a 7% EDTA solution, pH 7,2 (Synth, São Paulo, Brazil), buffered with sodium phosphate for decalcification. Following laboratory procedures, the samples were then included in paraffin. Semi-serial sections were made along the axis of the tooth, at 4µm thickness. For each hemimaxillae, approximately 30 sections were obtained, divided into slides containing 3 sections each.

Histometric analyses

For this analysis, a blind and calibrated examiner (Pearsons' Correlation, r=0.99) selected 2 slides from each hemimaxillae. The furcation area was delimited according to the methodology reported by da Silva et al., (2008).

Measurements were taken using the ImageJ Launcher imaging software, version 1.48b (National Institutes of Health, USA), evaluating the following areas:

- Furcation area: the area was defined, a 1000µm-zone under the furcation limited by the roots. Furcation and bone area were measured, thus obtaining the percentage of bone present in the furca region of each histological section.
- Interproximal region (mesial and distal): a linear measurement of the cement-enamel junction up to the top of the bone crest was performed, thus obtaining bone loss values.

Histological analyses

Using a DIASTAR (Leica Reichert & Jung products, Germany) optical microscope with 4,0-10,0-fold objective and 10 ocular magnifications, the images were captured and sent to a microcomputer with the aid of a DXC-1107A/107AP video camera (Sony Electronics Inc, Japan). The inflammatory reactions of the connective tissue, bone resorption processes and tissue neoformation in each experimental group were evaluated by an experienced, blind and calibrated examiner for the experimental groups.

Statistical analyses

The experimental data were tabulated using the Microsoft Excel for Mac 2011 software (Apple Inc, USA) and analyzed statistically with the aid of the GraphPad Prism 6.0 software (GraphPad Inc, USA).

The data were evaluated applying the central point theorem, to verify if their arrangement respected a normal distribution, using the Kolmogorov-Smirnov test. As all data were normally distributed, the parametric ANOVA (One Way) test was applied to verify the existence of statistical differences between the groups. Tukey's post-hoc test was subsequently applied, in order to detect differences among groups. For comparisons between the treatment periods, the ANOVA (Two Way) parametric test was applied. All tests were applied with a 95% confidence interval.

RESULTS

Three-dimensional radiographic analysis (µCT)

The images of this analysis are displayed in Figure 3. Regarding the furcation analysis, a statistically significant difference was observed between the PD and PDT groups concerning the percentage of bone tissue volume in the furca region of the second maxilarry molars of the hemimaxillae treated within the 7-day period ($^{*}p < 0.05$), as well as between the PD and SRP+PDT groups ($^{*}p < 0.05$). During the 30-day period, a statistical difference between the PD and SRP+PDT groups was observed ($^{\&}p<0.05$). The PD group displayed a lower percentage of bone volume when compared to the experimental groups in both periods, indicating bone loss. These data are displayed in Figure 3.1. On the other hand, no statistically significant differences concerning the percentage of bone tissue volume was observed in the proximal analysis. These data are displayed in Figure 3.2.

Histological analyses

In this analysis, a blind examiner (P.A.O.) evaluated the images and verified that the periodontal area of the animals of the PD group displayed several morphological alterations. At 7 days, an intense inflammatory process was observed in the interdental gingiva, which often appeared ulcerated; the gingival epithelium was adjacent to the surface of the acellular cement, in other words, located apically to the cement-enamel junction. A gradual but evident decrease in the inflammatory process was observed in subsequent periods. The SRP, PDT and SRP+PDT groups presented the same histological characteristics found in the PD group, but with additional migration of the epithelium, inflammatory processes, loss of the alveolar process and bone resorption in the furca region, albeit less pronounced. Thus, histological descriptions were similar for all experimental groups. The images illustrate the periodontal tissues of each treatment during the evaluated periods (Figure 4).

Histometric analysis

All data were statistically the same for the bone area in the furcation region (Figure 4.1) and for bone loss in the interproximal regions (Figure 4.2) of the second maxillary molars treated in the experimental groups.

DISCUSSION

The aim of this study was to evaluate *in vivo* tissue responses to PDT using the phthalocyanine-glucamine PS as an adjucant therapy in the treatment of PD-induced in rats.

It is known that PDT action is related to the experimental model used, in this case, ligature-induced periodontitis in rats, which has been widely applied to investigate PDT effects on PD (de Almeida et al., 2007; Garcia et al., 2011; Qin et al., 2008). Since PD is a multifactorial and polymicrobial disease, ligature placement may be the most representative method for its induction for periodontal treatment evaluation (Gaspersic, Stiblar-Martincic, Osredkar, & Skaleric, 2003). The study conducted by Graves et al. (2008) demonstrated that the ligature model is simple, versatile and inexpensive, and, when applied to rats, allows for considerable information of the immune system, due to the availability of a wide range of genetically modified strains and immunochemistry and cellular reagents.

In addition to the experimental model, the applied PS should also be evaluated, since the formation of aggregates between PS molecules can hinder the process of reactive oxygen species generation. Such aggregates tend to be formed as the concentration of the photosensitizing agent is increased or when the hydrophobic agent

is in an aqueous medium (da Silva et al., 2009). In the present study, a PS concentration that would be effective in PD treatment was used.

Light irradiation time should also be taken into account when evaluating PDT effects. This concerns the maximum amount of light possible at the maximum absorption of the PS, without significant thermal effects. In this context, an efficient heat dissipation system is required, in order to minimize the thermal effects of the heat generated by the light source on the irradiated tissue and its surroundings. For this reason, a LED light was used. Finally, the availability of essential oxygen must also be considered, to obtain a successful therapy.

The histological results observed herein indicated no differences between the groups in the evaluated periods, demonstrating that PDT did not cause tissue damage. Kaestner et al. (2003) applied zinc phthalocyanine to the skin of mice and demonstrated satisfactory responses regarding absorption, since the dye penetrates only to the epidermis, and not the dermal layers, which are rich in blood capillaries, in topical applications. Therefore, this dye should pose no risks regarding either photosensitivity or phototoxicity.

de Almeida et al. (2007) evaluted the effects of PDT on the evolution of PD-induced in rats by histological and radiographical methods. The PDT group in that study was treated with methylene blue, irradiated with a low intensity laser and displayed lower bone loss compared to the control group at 5 and 15 days, with no statistical difference at 30 days. After 15 days, the histological results indicated a statistically significant difference in the extent of the inflammatory reaction in the gingival tissue. The authors concluded that PDT can, thus, reduce periodontal bone destruction in a transient manner. In the following year, de Almeida et al. (2008) developed an experiment, also in rats, with the purpose of histologically evaluating the influence of PDT applied with methylene blue on bone loss in furcation areas. The PDT group showed significantly lower bone loss compared to the other groups in a 7-day period. After 15 days, the dye-only and PDT groups presented significantly lower bone loss when compared to the other groups.

The histometric results of the present study indicated no statistically significant differences between the groups in the evaluated periods. However, the PDT group maintained the same pattern of bone loss when compared to the SRP group, in both the interproximal and furcation areas. The results of the three-dimensional radiographic analysis indicate that, at the 7 and 30 day evaluations, the PDT group presented higher bone volume in the interproximal and furcation regions when compared to the other groups. The PD group exhibited higher bone loss at all periods, but was statistically significant at the furcation region at 7- and 30-day evaluations. The results of these

analyses, evaluating the same regions, differ due to the concepts of each method.

Traditionally, quantitative histological techniques are considered the standard in the evaluation of trabecular and cortical bone architecture. Although histological analyses provide unique information on cellularity and dynamic indices of bone remodeling, they display limitations regarding the evaluation of bone microarchitecture, since the evaluated structural parameters are derived from stereometry of only a few 2D sections, generally assuming that the underlying structure is overlaid on the image (Parfitt et al., 1987). In comparison, 3D high-resolution imaging techniques, such as three-dimensional radiography directly measure bone microarchitecture without relying on stereological models. Introduced by Feldkamp and colleagues (Feldkamp, Goldstein, Parfitt, Jesion, & Kleerekoper, 1989) in the late 1980s, micro-CT has now become the gold standard for the evaluation of bone morphology and microarchitecture in mice and other small *ex vivo* animal models.

Some animal studies (Qin et al., 2008; Sigusch et al., 2005) have demonstrated that PDT is effective in reducing periodontopathogenic microorganisms, while others (de Almeida et al., 2008; Garcia et al., 2011) have indicated that, when PDT is combined with conventional periodontal treatment, alveolar bone loss is lower when compared to the isolated intervention. One of the factors that may affect PDT results in PD is the presence of gingival fluid, blood and inflammatory exudate (Matevski et al., 2003), as these conditions may reflect or absorb light, promoting a "protective effect" for the bacteria present in the area. This may explain the results observed herein, although not significant, of the lower amount of bone tissue formed in the SRP+PDT group, since scraping prior to the application of PDT may have generated bleeding that inhibited the PS from acting on the microorganisms.

The PDT mechanism of action seems to be very clear, so there is no dispute about its bactericidal capacity. Studies are unanimous in affirming that the excitation of the photosensitizer caused by the light source triggers the appearance of molecules with toxic effects on the microorganisms (Sigusch et al., 2005). Several studies (Pfitzner et al., 2004; Qin et al., 2008; Sigusch et al., 2005) have proven the high rate of microorganism destruction in samples submitted to PDT. On the other hand, certain bacterial species, such as *Aggregatibacter actinomycetemcomitans*, are more resistant to this therapy when compared to others, such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum* (Pfitzner et al., 2004). Gram-positive bacteria are generally susceptible to photoinactivation, while gram-negative bacteria are often resistant (Soukos & Goodson, 2011). This resistance is attributed to the outer membrane of these organisms, which acts as a barrier to the penetration of the PDT-produced compounds. The use of a cationic dye shows greater photodynamic activity against

gram-negative bacteria, since the positive charge promotes an electrostatic bond to the external surface of the cell, inducing an initial damage that favors dye penetration (Merchat, Spikes, Bertoloni, & Jori, 1996).

Although this study did not directly evaluate PDT effects on microorganisms, Persson et al. (2001) demonstrated that, when a decrease in the amount of bacteria present in periodontitis or peri-implantitis is observed, tissue repair or re-osseointegration is subsequently observed. In vitro (Klepac-Ceraj et al., 2011) and in vivo(de Almeida et al., 2006; de Almeida et al., 2008; Qin et al., 2008; Sigusch et al., 2005) studies have shown favorable results using PDT principles. A study performed in humans (Andersen et al., 2007) verified that PDT was effective in PD treatment, reducing clinical insertion level and probing depth. Thus, this therapy has been proposed as a complementary alternative, especially in areas of difficult access to manual instruments, such as furcation regions, concavities and deep pockets (de Almeida et al., 2007). This therapy may also be an alternative method to reduce the use of antibiotics, avoiding the development of resistant organisms (Maisch, 2007).

As mentioned previously, the limitations of this study are due to the fact that different variables were present, such as the experimental animal model, dye concentration, tissue retention period, time for biological response, irradiation time, light energy and wavelength, pH of the site (tissue/tooth/interface), presence of exudate, blood and gingival fluid and frequency and mode of application of the dye, all of which may influence biological responses to PDT (de Almeida et al., 2006). Thus, further studies evaluating these factors are required in order to better understand the photoinactivation action of the local microorganisms.

CONCLUSIONS

With the results obtained herein, we can conclude that PDT applied with phthalocyanine-glucamine as the PS, both as a monotherapy and associated to SRP, is effective in bone loss control in PD-induced rats, similar to the conventional mechanical treatment. Thus, phthalocyanine-glucamine may be considered a promising photosensitizer. However, further in vitro and in vivo studies are required to elucidate PDT action and the effect of this PS as an adjunct therapy in the treatment of PD.

ACKNOWLEDMENTS

We would like to thank Capes and CNPq for financial support and Prof. Dr. Anderson O. Ribeiro from the Federal University of ABC for synthesize the solution of the photosensitizer phtalocyanine-glucamine used in this study.

CONFLICT OF INTEREST AND SOURCES OF FUNDING STATEMENT

The authors declare that there are no conflicts of interest.

REFERENCES

Andersen, R., Loebel, N., Hammond, D., & Wilson, M. (2007). Treatment of periodontal disease by photodisinfection compared to scaling and root planing. *The Journal of Clinical Dentistry,* 18, 34-38.

Bonnet, R. (1995). Photosensitizers of the porphyrin and phthalocyanine series for photodynamic therapy. *Chemical Society Reviews,* 24, 19-33.

da Silva, A. R., Inada, N. M., Rettori, D., Baratti, M. O., Vercesi, A. E., & Jorge, R. A. (2009). In vitro photodynamic activity of chloro(5,10,15,20-tetraphenylporphyrinato)indium(III) loaded-poly(lactide-co-glycolide) nanoparticles in LNCaP prostate tumour cells. *Journal of Photochemistry and Photobiology B: Biology,* 94, 101-112.

da Silva, V. C., Cirelli, C. C., Ribeiro, F. S., Leite, F. R., Benatti Neto, C., Marcantonio, R. A., & Cirelli, J. A. (2008). Intrusion of teeth with class III furcation: a clinical, histologic and histometric study in dogs. *Journal of Clinical Periodontology,* 35, 807-816.

de Almeida, J. M., Garcia, V. G., & Theodoro, L. H. (2006). Terapia fotodinâmica: uma opção na terapia periodontal. *Arquivos do Centro de Estudos do Curso de Odontologia da Universidade Federal de Minas Gerais,* 42, 199-210.

de Almeida, J. M., Theodoro, L. H., Bosco, A. F., Nagata, M. J., Oshiiwa, M., & Garcia, V. G. (2007). Influence of photodynamic therapy on the development of ligature-induced periodontitis in rats. *Journal of Periodontology,* 78, 566-575.

de Almeida, J. M., Theodoro, L. H., Bosco, A. F., Nagata, M. J., Oshiiwa, M., & Garcia, V. G. (2008). In vivo effect of photodynamic therapy on periodontal bone loss in dental furcations. *Journal of Periodontology,* 79, 1081-1088.

Drisko, C. H. (1998). The use of locally delivered doxycycline in the treatment of periodontitis. Clinical results. *Journal of Clinical Periodontology,* 25, 947-952; discussion 978-949.

Feldkamp, L. A., Goldstein, S. A., Parfitt, A. M., Jesion, G., & Kleerekoper, M. (1989). The direct examination of three-dimensional bone architecture in vitro by computed tomography. *Journal of Bone and Mineral Research,* 4, 3-11.

Fontana, C. R., Abernethy, A. D., Som, S., Ruggiero, K., Doucette, S., Marcantonio, R. C., & Soukos, N. S. (2009). The antibacterial effect of photodynamic therapy in dental plaque-derived biofilms. *Journal of Periodontal Research,* 44, 751-759.

Garcia, F. S., & Bentley, M. V. (2003). Phthalocyanine dyes: importance and future directives for use in photodynamic therapy of cancer. *Trends in Photochemistry & Photobiology,* 10, 77-82.

Garcia, V. G., Fernandes, L. A., Macarini, V. C., de Almeida, J. M., Martins, T. M., Bosco, A. F., & Theodoro, L. H. (2011). Treatment of experimental periodontal disease with antimicrobial photodynamic therapy in nicotine-modified rats. *Journal of Clinical Periodontology,* 38, 1106-1114.

Gaspersic, R., Stiblar-Martincic, D., Osredkar, J., & Skaleric, U. (2003). Influence of subcutaneous administration of recombinant TNF-alpha on ligature-induced periodontitis in rats. *Journal of Periodontal Research,* 38, 198-203.

Graves, D. T., Fine, D., Teng, Y. T., Van Dyke, T. E., & Hajishengallis, G. (2008). The use of rodent models to investigate host-bacteria interactions related to periodontal diseases. *Journal of Clinical Periodontology,* 35, 89-105.

Jori, G., Fabris, C., Soncin, M., Ferro, S., Coppellotti, O., Dei, D., & Roncucci, G. (2006). Photodynamic therapy in the treatment of microbial infections: basic principles and perspective applications. *Lasers in Surgery and Medicine,* 38, 468-481.

Kaestner, L., Cesson, M., Kassab, K., Christensen, T., Edminson, P. D., Cook, M. J., & Jori, G. (2003). Zinc octa-n-alkyl phthalocyanines in photodynamic therapy: photophysical properties, accumulation and apoptosis in cell cultures, studies in erythrocytes and

topical application to Balb/c mice skin. *Photochemical and Photobiological Sciences,* 2, 660-667.

Klepac-Ceraj, V., Patel, N., Song, X., Holewa, C., Patel, C., Kent, R., & Soukos, N. S. (2011). Photodynamic effects of methylene blue-loaded polymeric nanoparticles on dental plaque bacteria. *Lasers in Surgery and Medicine,* 43, 600-606.

Maisch, T. (2007). Anti-microbial photodynamic therapy: useful in the future? *Lasers in Medical Science,* 22, 83-91.

Matevski, D., Weersink, R., Tenenbaum, H. C., Wilson, B., Ellen, R. P., & Lepine, G. (2003). Lethal photosensitization of periodontal pathogens by a red-filtered Xenon lamp in vitro. *Journal of Periodontal Research,* 38, 428-435.

Merchat, M., Spikes, J. D., Bertoloni, G., & Jori, G. (1996). Studies on the mechanism of bacteria photosensitization by meso-substituted cationic porphyrins. *Journal of Photochemistry and Photobiology B: Biology,* 35, 149-157.

Ogunsipe, A., Durmusß, M., Atilla, D., Gürek, A. G., Ahsen, V., & Nyokong, T. (2008). Synthesis, photophysical and photochemical studies on long chain zinc phythalocyanines. *Synthetic Metals,* 158, 839-847.

Parfitt, A. M., Drezner, M. K., Glorieux, F. H., Kanis, J. A., Malluche, H., Meunier, P. J., & Recker, R. R. (1987). Bone histomorphometry: standardization of nomenclature, symbols, and units. *Journal of Bone and Mineral Research,* 2, 595-610.

Persson, L. G., Berglundh, T., Lindhe, J., & Sennerby, L. (2001). Re-osseointegration after treatment of peri-implantitis at different implant surfaces. An experimental study in the dog. *Clinical Oral Implants Research,* 12, 595-603.

Pfitzner, A., Sigusch, B. W., Albrecht, V., & Glockmann, E. (2004). Killing of periodontopathogenic bacteria by photodynamic therapy. *J Periodontol,* 75, 1343-1349.

Qin, Y. L., Luan, X. L., Bi, L. J., Sheng, Y. Q., Zhou, C. N., & Zhang, Z. G. (2008). Comparison of toluidine blue-mediated photodynamic therapy and conventional scaling treatment for periodontitis in rats. *Journal of Periodontal Research,* 43, 162-167.

Ryskova, L., & Slezak, R. (2010). Photodynamic antimicrobial therapy. *Central European Journal of Biology,* 5, 400-406.

Sharman, W. M., Allen, C. M., & van Lier, J. E. (1999). Photodynamic therapeutics: basic principles and clinical applications. *Drug Discovery Today,* 4, 507-517.

Sigusch, B. W., Pfitzner, A., Albrecht, V., & Glockmann, E. (2005). Efficacy of photodynamic therapy on inflammatory signs and two selected periodontopathogenic species in a beagle dog model. *Journal of Periodontology,* 76, 1100-1105.

Soukos, N. S., & Goodson, J. M. (2011). Photodynamic therapy in the control of oral biofilms. *Periodontology 2000,* 55, 143-166.

Vergani, S. A., Silva, E. B., Vinholis, A. H., & Marcantonio, R. A. (2004). Systemic use of metronidazole in the treatment of chronic periodontitis: a pilot study using clinical, microbiological, and enzymatic evaluation. *Brazilian Oral Research,* 18, 121-127.

Figure1 – Experimental design of the present study.

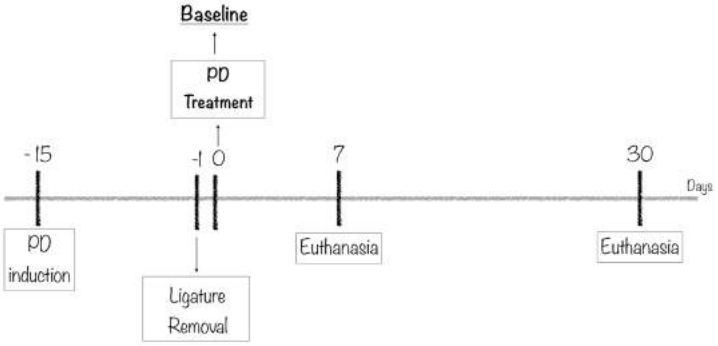


Figure 2 – µ-CT images obtained of the second maxillary molar region, where the ROIs were obtained. A: Furcation region with a 1.26x1.15mm^2 area; B: proximal regions (mesial and distal) with a 1.26x0.56mm^2 area from the cement-enamel junction.

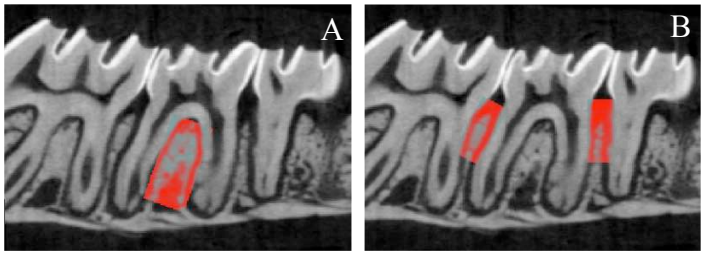


Figure 3 – Photograph of the 3D models obtained from the scanning of the hemimaxillae in the µ-CT, palatal view, indicating the analyzed regions (distal, mesial and furcation) of the experimental groups: A, B, C and D (7 day period) and E , F, G and H (30 day period); In sequence, the PD, SRP, PDT and SRP+PDT groups. The graphs display the means and standard deviations of the percentage of bone volume (%) of the furcation area (3A) and proximal regions (3B), of the second maxillary molar of the treated hemimaxillae of the experimental groups. $^{*, \#, \&}$ indicate statistically significant differences between groups.

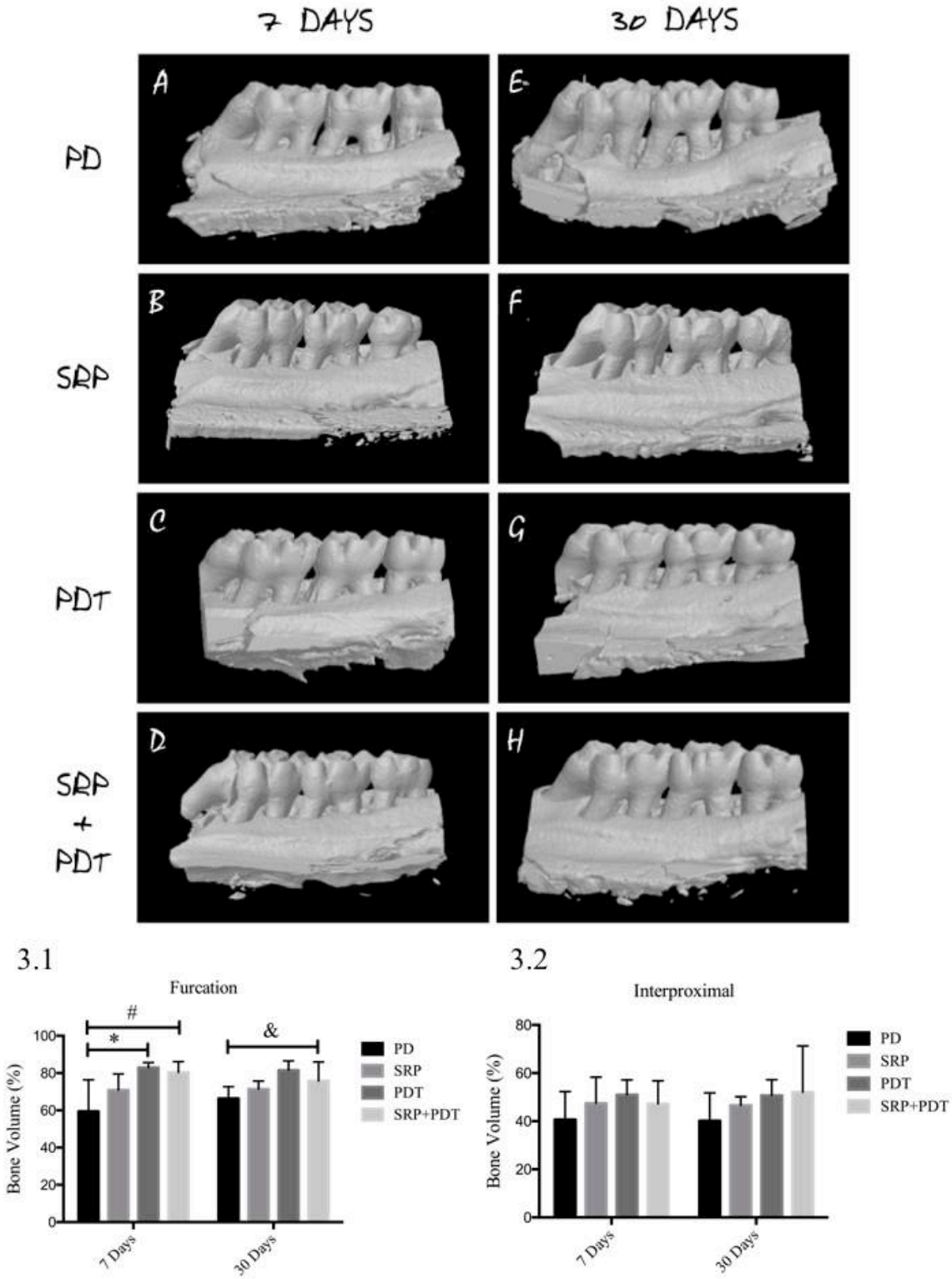
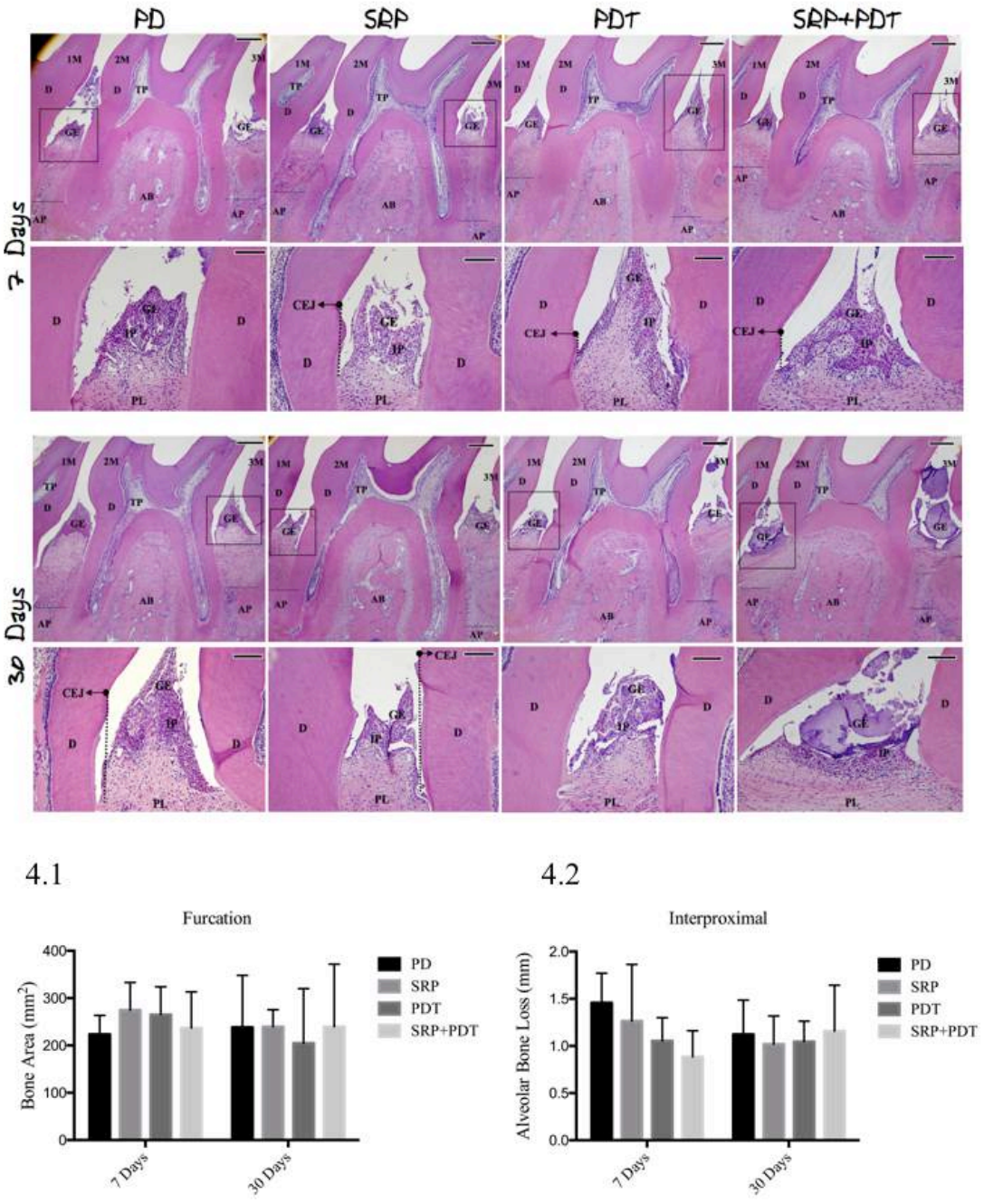


Figure 4 – Photomicrographs of the sagittal sections of rat hemimaxillae of the experimental groups, in the 7 and 30 day periods, at 4x magnification, showing the alveolar process (AP) formed by irregular bone trabeculae, located between the 1st (1M) and 2nd (2M) and between the 2nd (2M) and 3rd (3M) molars. The cervical portion of the alveolar process (AP) is located near the middle and apical third of the 2M roots. Reabsorption of alveolar bone (AB) was observed in the furcation region. At 10x magnification, the gingival epithelium (GE) is located apically at the cement-enamel junction (CEJ), and the gingival mucosa exhibits an inflammatory process (IP) evident in the PD group and the destroyed interdental papilla. In the other groups, the inflammatory process (IP) is less pronounced than the PD group. CEJ, cement-enamel junction; GE, gingival epithelium; D, dentin; P, Tooth pulp; PL, periodontal ligament; AB, alveolar bone of the furcation region. HE. X10 - Bar: 80. x4 - Bar: 330. Graph 4A displays the means and standard deviation of the bone area in square millimeters (mm^2) of the furcation region and figure 4B displays the means and standard deviations of bone linear loss in millimeters (mm) of the proximal regions of the second maxillary molar of the treated hemimaxillae of the experimental groups.



3.2 Publicação 2

Tissue responses of aPDT with phthalocyanine-tetracarboxyglucamine photosensitizer in ligature-induced periodontal disease in rats*

Sâmara C. T. Corbi, Paula D. Macedo, Janice R. Perussi, Anderson O. Ribeiro, Rosemary A. C. Marcantonio

*Artigo a ser submetido à revista Jornal of Applied Oral Science.

Tissue responses of aPDT with phthalocyanine-tetracarboxyglucamine photosensitizer in ligature-induced periodontal disease in rats

Sâmara Cruz Tfaile Corbi (SCT, Corbi) – PhD student in Periodontics[1]; email: sa_tfaile@yahoo.com.br

Paula Delello Macedo (Macedo, PD), PhD student in Periodontics[1]; email: paula_mac2@yahoo.com.br

Janice Rodrigues Perussi (Perussi, JR), Associate Professor in Chemistry[2]; e-mail: janice@iqsc.usp.br

Anderson Orzari Ribeiro (Ribeiro, AO), Adjunct Professor from UFABC[3], email: anderson.ribeiro@ufabc.edu.br

***Rosemary Adriana Chiérici Marcantonio (Marcantonio, RAC),** Associate Professor in Periodontics[1]; email: adriana@foar.unesp.br

[1]Sao Paulo State University (UNESP), School of Dentistry, Diagnosis and Surgery Department, Araraquara, SP, Brazil

[2]Sao Paulo University (USP), Chemistry and Molecular Physics Department, Sao Carlos, SP, Brazil

[3]Federal University of ABC (UFABC), Centre for Natural Sciences and Humanities, Santo Andre, SP, Brazil

[*]**Corresponding author:**

Rosemary Adriana C. Marcantonio, PhD

Department of Diagnosis and Surgery

Sao Paulo State University – School of Dentistry at Araraquara (UNESP)

CEP 14801-903, Araraquara, SP, Brasil

Tel: +55 16 3301-6376

E-mail: adriana@foar.unesp.br

ABSTRACT

Antimicrobial Photodynamic Therapy (aPDT) has been used as an adjuvant treatment in periodontal disease (PD). This technique combines a photosensitizer (PS) with a light source to induce the production of reactive oxygen species (ROS), eliminating microbial cells. The aim of this study was to evaluate in vivo responses and tissue changes after aPDT application (with the PS phthalocyanine-tetracarboxyglucamine - 10μg/mL, and red LED light with 655nm) in PD-induced rats by microtomographic, histometric, stereometric and histological evaluations. Ligatures were placed around the second maxillary molars in both sides for PD induction for 7 days and remained in position throughout the experiment. Forty-two animals were divided into 4 groups: PS (treatment with phthalocyanine-tetracarboxyglucamine only); Light (Treatment with red LED light irradiation only); aPDT (Treatment with photodynamic therapy – PS + Light) and PD (periodontal disease induction, without treatment). At the baseline, the animals were treated and euthanized at 7 and 15 days. A one-way ANOVA parametric test was applied, followed by Tukey's post-test. The three-dimensional radiographic and histometric analyses revealed no statistically different results for the furcation and interproximal regions. The inflammatory profile presented a trend of lower amounts of inflammatory cells in the aPDT group at 7 days, while the histological analysis indicated no significant differences between the groups, indicating that the therapies did not cause tissue damage. Thus, the application of aPDT and its components as monotherapies in PD-induced in rats with the preservation of the ligatures, favored *in situ* bacteria permanence and inhibited treatment action.

Keywords: Periodontal diseases, photochemotherapy, X-ray microtomography.

INTRODUCTION

Periodontal Disease (PD) is an oral infection affecting the gingiva, cementum, periodontal ligament and alveolar bone, and its primary etiological factor is the presence of pathogenic bacteria in biofilm, which adhere to the teeth surface of susceptible host. Pathogens release enzymes, endotoxins and cytotoxic factors that can, in turn, trigger inflammation mechanisms and cause various changes in the periodontium[36].

The standard therapy for PD treatment is Scaling Root and Planing (SRP)[56], which aims to remove microorganisms found on all teeth in both the supra and subgingival region. SRP benefits can be verified by decreases in marginal bleeding, bleeding at probing and probing depth, as well as stabilization of clinical insertion levels[9]. These clinical improvements are due to well-executed SRP, which influences the amount and composition of the oral microbiota[11]. However, this therapy does not

seem to be responsive to mechanical treatment in some sites, which makes it less effective

To overcome this difficulty, some therapeutic alternatives have been applied, such as the use of local and systemic antibiotics. However, it is known that this type of treatment has undesirable side effects and contributes to the development of bacterial resistance to drugs[29, 35, 72]. Thus, alternative methods that target bacterial reduction, such as the use of lasers and associated dyes, known as Antimicrobial Photodynamic Therapy (aPDT), could be a complementary feature to conventional periodontal treatment.

PDT has emerged in recent years as a new, noninvasive, therapeutic modality for the treatment of various bacterial, fungal, and viral infections[27]. This therapy is defined as an oxygen-dependent photochemical reaction that occurs after the activation of a light-mediated photosensitizing compound, leading to the generation of cytotoxic reactive oxygen species (ROS), predominantly singlet oxygen[44]. This therapy can be topically applied in the periodontal pocket, thus avoiding overdoses. Other aPDT advantages include, reducing the likelihood of side effects associated with the systemic administration of antimicrobial agents and minimizing bacterial resistance[22, 73].

Several in vitro[8, 10, 21, 31, 37, 41, 59, 70, 74] and in vivo experimental studies[20, 24, 30, 61, 62] have obtained satisfactory results through aPDT, applying protocols comprising different types of dyes and wavelengths to eliminate several types of oral bacteria, such as periodontopathogenic and non-pathogenic bacteria.

In the last decades, certain compounds with diagnostic and therapeutic purposes, considered second-generation dyes, have been developed, such as phthalocyanines, which are synthetic dyes similar to porphyrins and structurally classified as azaporphyrins[34, 55, 67]. The name phthalocyanine originates from a combination of the prefix phthal, originally from the Greek word naphtha (rock oil), to emphasize the association with its various precursors derived from phthalic acid, and the Greek word cyanine (blue)[38]. Phthalocyanines are symmetric macrocycles composed of four iminoisoindole (Figure 1A) units with a central cavity that accommodates different metal ions, such as chlorine, aluminum and zinc. When inserted, these metals present considerable influence on photosensitizing properties (Figure 1B)[3, 57, 67]. These compounds display an absorption band in the electromagnetic spectrum, ranging from 650 to 680nm, which allows greater light penetration into tissues[40], and their photophysical properties depend on their composition, particularly that of the central metal ion. Among phthalocyanines, a chloro-aluminum phthalocyanine has been suggested as displaying photophysical properties favorable for use in aPDT, as it produces high amounts of singlet oxygen[40]. The effectiveness of

this photosensitizer associated with LED light was confirmed in an in vitro study that evaluated the photodynamic potential of a chloro-aluminum phthalocyanine diluted in a cationic nanoemulsion to inactivate planktonic cultures and biofilms formed by *Candida albicans*[55]. This PS was also shown to be effective for bacteria inactivation in patients with carious lesions[34]. Other studies[16, 47, 63, 74-76] have supported the efficiency of phthalocyanines as photosensitive agents in the elimination of periodontopathogenic microorganisms with applications in aPDT. Another phthalocyanine routinely applied as a PS is zinc phthalocyanine (FCZn)[3, 57, 67].

The hydrophobicity of some PS is an important fact, since this property in aqueous media leads to molecule self-aggregation and, in most cases, to a subsequent precipitation, drastically reducing the ability of the compound to generate singlet oxygen[64]. Thus, the photoactive principle must be soluble in aqueous media for possible clinical applications. An interesting strategy applied to increase the aqueous solubility of some PS involves the formation of hydrophilic supramolecular species. A supramolecule is defined as a chemical species made up of two or more molecules linked by intermolecular interactions. In this sense, supramolecular chemistry uses an approach centered on molecule association, aiming at obtaining a certain property or functionality[14].

Meglumine, also known as N-methylglucamine (Figure 1C), is an aminocarbohydrate derived from glucose capable of forming hydrophilic binary supramolecular species with compounds containing acidic hydrogen atoms in their structure[14]. The formation of meglumine compounds involves an acid-base reaction, in which the acidic hydrogen is transferred to the amine group of the aminocarbohydrate. To enable the formation of supramolecular species between meglumine and phthalocyanines, the latter are primarily functionalized with carboxylic groups, such as tetracarboxylated zinc phthalocyanine (FtZnTc, Figure 1D)[14].

Subsequently to the insertion of the acid groups, a reaction with the aminocarbohydrate can be conducted, producing species that are more soluble in water. This procedure allows for the insertion of up to four meglumine molecules, as observed in Figure 1E for zinc phthalocyanine tetracarboxy-N-methylglucamine (FtZnTcG). The presence of several hydroxyl groups in the supramolecule provides the sites where the hydrogen bonds are established by increasing the hydrophilicity of the phthalocyanines[14]. The absorption spectrum of phthalocyanines in solution consists of two main bands centered at 350nm and 670nm. Figure 2 illustrates the molecular absorption spectrum of the tetracarboxylated zinc phthalocyanine.

Phthalocyanines have been used in aPDT in cancer treatments[3, 4, 54, 71, 77], cutaneous e subcutaneous lesions[6], in eradication of neoplasitic processes[57, 67] and

encapsulated virus[7], due to its high selectivity and low phototoxicity characteristics and promotes an appreciable amount of ROS, capable of remaining in the triplet excited state for a longer period of time[58]. In this sense, the aim of this study was to evaluate in vivo the responses and tissue changes of aPDT application and its components as monotherapies, using phthalocyanine-tetracarboxyglucamine PS in PD-induced in rats.

MATERIAL AND METHODS*

Ethics comittee

This project was approved by the Ethics Committee on Animal Experimentation (No. 07/2012)[#].

Samples

Forty-two rats (*Rattus norvegicus*) of the *albinus* variation, *Holtzman*, adults, weighing between 300-330g were used. The animals were kept in plastic boxes, 3/4 animals per box, and treated with water and food *ad libitum* before and during the whole experimental period. The animals were maintained in an environment with controlled light, humidity and temperature.

Periodontal disease induction

The animals were anesthetized with a combination of ketamine (ketamine hydrochloride - Francotar 3% - Virbac do Brasil Ind. e Com. Ltda.) and xylazine (xylazine hydrochloride - Virbaxyl 2% - Virbac do Brasil Ind. e Com. Ltda.) at 0,08mL/100g and 0,04mL/100g body weight, respectively. The ligatures were placed around the second maxillary molars on both sides. The hemimaxilae that received the therapies was chosen randomly (right or left). The PD was induced for 7 day before the beginning the therapies application of each group/period and 0-day period was considered the baseline. The ligatures remained in position throughout the experiment (Figure 3).

Photodynamic Therapy

Photosensitizer preparation

Phthalocyanine-tetracarboxyglucamine was prepared from a stock solution of zinc-tetracarboxy-phthalocyanine at 1,1mg/mL in DMSO and subsequently diluted in

phosphate buffered saline (pH=7.2) to a final concentration of 10µg/mL, in ointment consistency. Soon after the preparation, the PS was stored in light-protected poliproylene tubes maintained in a refrigerated environment until use.

*Para metodologia completa, ver Apêndice 1. [#]Aprovação do Comitê de Ética, ver Anexo A.

Photosensitizer Application

At the time of application, the animals' mouth and the PS were light-protected. A blunt tip syringe containing 0,2mL of the PS was inserted into the gingival sulcus. The solution was applied around the entire second maxillary molars and, after 10 minutes of incubation time in the dark, the light irradiation was performed. This application was done only once.

Light source

The light source used to activate the phthalocyanine-tetracarboxyglucamine PS corresponded to a wavelength of 655 nm, 0,45W power density and 34J/cm^2 dose (red LED, 11mm diameter, DMC Equipamentos Ltda, São Carlos, Brazil), coinciding with the maximum absorption band of phthalocyanine-tetracarboxyglucamine. The LED light was placed on the occlusal surface of the second maxillary molars and irradiation was maintained for 72 seconds.

Experimental groups

After 7 days of PD-induced, the animals were randomly divided the therapies application were performed according to their group (7animals/group/period):

- Photosensitizer Group (PS): PS was applied around the entire second maxillary molars, into the sulcus (10 minutes of incubation time in the dark) and after, the PS was removed with cotton swabs.
- Red LED Light Group (LED): The LED light (655nm; 34J/cm^2) was placed on the occlusal surface of the second maxillary molars and irradiation was maintained for 72 seconds.
- Photodynamic Therapy Group (aPDT): Application of the PS followed by LED application.
- PD Group (PD): Disease induction only, without treatment.

The ligatures remained in position and they were removed at 7- and 15-days periods. Soon after, the animals were euthanized with an anesthetic overdose. The hemimaxillae were removed and fixed in 4% paraformol for 48h. Subsequently, the samples were washed in running water for 24h and placed in 70% alcohol, where they remained until the computerized microtomograph scanning.

Histological processing

After scanning, the samples were placed in a 7% EDTA solution, pH 7,2 (Synth, São Paulo, Brazil), buffered with sodium phosphate for decalcification. Following

laboratory procedures, the samples were then included in paraffin. Semi-serial sections were made along the axis of the tooth, at 4µm thickness. For each hemimaxillae, approximately 30 sections were obtained, divided into slides containing 3 sections each. Two slides from each hemimaxillae were stained by hematoxilin-eosin technique (HE) and used for, histometric, stereometric and histologic analysis.

Three-dimensional Radiographic Analysis (µCT)

The samples were scanned by means of X-ray beam scanning in a computerized microtomography system (Skyscan 1176, Aatselaar, Belgium, 2003). The parameters of the equipment were set as follows: Al 0.5mm filter; Voxel size: 17.48µm; Voltage 50KV and electric current 500µA. After scanning, the 3D images for each sample were obtained through the equipment software (NRecon 1.6.1.5 - SkyScan N. V., Belgium, 2003). The images were rotated and repositioned in a standard orientation with the two-dimensional Dataviewer software (SkyScan 1176, Aartselaar, Belgium, 2003). for better viewing. At each 28 scans, a 18x18x18µm matrix was reconstructed and 3D images were generated for each sample. Subsequently, a volumetric analysis was performed by a trained examiner with no knowledge of the experimental groups.

For this analysis, a specific software (ITK-SNAP 3.6.0 - Pennsylvania, USA) was used, where the examiner delimited a three-dimensional region of interest (ROI), of 217x193x269, driven by the morphometry of the samples. To maximize bone quantification and minimize teeth and root inclusion, the ROI was delimited from the distal first molar root to the mesial third molar root, and from the furcation and interproximals region of the second molar to the end of the cortical bone, thus making sure that all the bone area in the furcation and interproximal region of the second molar were encompassed by the ROI, as displayed in Figure 4. After the ROI delineation, a binary image was generated and a single threshold was established for each sample, analyzing tissue similarity. Subsequently, the roots were digitally removed so as not to influence the results. The analyses and the resulting values are given in cubic millimeters (mm^3).

Histometric analyses

For this analysis, a blind and calibrated examiner (Pearson's correlation, r=0.93) selected 2 slides from each hemimaxillae. The furcation area was delimited according to the methodology reported by da Silva et al.,[12] (2008). Measurements were taken using the Fiji ImageJ Launcher imaging software (National Institutes of Health, USA), evaluating the following areas:

- Furcation area: the area was defined, a 1000µm-zone under the furcation limited by the roots. The furcation and bone area were measured, thus obtaining the percentage of bone present in the furca region of each histological section.
- Interproximal region (mesial and distal): a linear measurement of the cement-enamel junction up to the top of the bone crest was performed, thus obtaining alveolar bone loss values.

The furcation region measurements were obtained in percentage and interproximals region in micrometers.

Stereometric Analysis

This analysis was performed using a Leica DMLS light microscope (Leica - Reichert Diastar Products & Jung, Wetzlar, Germany) to select 2 sections per tooth, with intervals ranging from 40 to 50µm between them, and a Leica DFC 300 digital camera FX (Leica - Reichert Diastar Products & Jung, Wetzlar, Germany) to capture the furcation and interproximal region images at a 200x magnification. In a two-dimensional plane, a 75x75 pixel area grid with 252 intersection points was placed in the regions of interest (supra-alveolar connective tissue at the furcation ceiling and papillae, in both mesial and distal sides) using the software Fiji ImageJ (National Institutes of Health, USA). Subsequently, the point counting technique was performed to analyze the proportion of tissue constituents (bone tissue, extracellular matrix, blood vessels, fibroblasts, inflammatory cells) at the angles of the grid. For the graphical representation of the stereometry, a percentage measurement of each tissue constituent corresponding to the total number of points was performed, based on Liu et al.[33] (2006) and Odze et al.[45] (1996).

Histological analyses

Using a DIASTAR (Leica Reichert & Jung products, Germany) optical microscope with 4,0-10,0-fold objective and 10 ocular magnifications, the images were captured and sent to a microcomputer with the aid of a DXC-1107A/107AP video camera (Sony Electronics Inc, Japan). The inflammatory reactions of the connective tissue, bone resorption processes and tissue neoformation in each experimental group were evaluated by an experienced, blind and calibrated examiner for the experimental groups.

Statistical analyses

The experimental data were tabulated using the Microsoft Excel for Mac 2011 software (Apple Inc, USA) and analyzed statistically with the aid of the GraphPad Prism 6.0 software (GraphPad Inc, USA).

The data were evaluated applying the central point theorem, to verify if their arrangement respected a normal distribution, using the Shapiro-Wilk test. As all data were normally distributed, the parametric ANOVA (One Way) test was applied to verify the existence of statistical differences between the groups. Tukey's post-test was subsequently applied, in order to detect differences among groups. For comparisons between the treatment periods, the ANOVA (Two Way) parametric test was applied. All tests were applied with a 95% confidence interval.

RESULTS

Three-dimensional Radiographic Analysis (µCT)

No statistically significant difference was detected for bone volume in the region of the second maxillary molars of the treated hemimaxils. These data are displayed in Figure 5.

Histometric Analysis

The results of the furcation and interproximal analyses indicate that all the data were statistically the same for the bone area and bone loss, respectively, for region of the second maxillary molars molars of the treated hemimaxils of the experimental groups, as displayed in Figure 6.

Stereometric Analysis

Significant differences were observed in the percentage of bone tissue at 7 days between the PS and LED light groups (*$p<0.05$). The percentage of blood vessels was also significantly different in the PD group between the 7 and 15 day groups ($^{\#}p<0.05$). The percentages of extracellular matrix and inflammatory cells were not significantly different. Regarding fibroblasts, a statistically significant difference was detected in the PD group comparing the 7 and 15 day periods ($^{a}p<0.05$), the photosensitized group was statistically different compared to the PD, LED and aPDT groups ($^{bcd}p <0.05$). These data are displayed in Figure 7.

Histological Analysis

In this evaluation, the morphological aspects of the gingival mucosa and bone tissue of the second maxillary molars of the rats were analyzed in the different groups.

The animals periodontium in the PD group exhibited changes at 7 days: An exacerbated inflammatory process in the lamina propria of the interdental papilla, was observed, frequently ulcerated. The junctional epithelium was located apically to the cement-enamel junction. Bone in the furcation region showed moderate resorption. At the 15-day period, a decrease in the inflammatory process and a slight bone resorption in the furcation were observed. The PS, light and aPDT groups presented microscopic characteristics similar to those described for the PD group, but less intense. Thus, histological descriptions were similar for the experimental groups. Figure 6 illustrates the periodontal tissues of the different periods.

DISCUSSION

The aim of this study was to evaluate in vivo alterations and tissue responses after the application of aPDT and its components as monotherapies using the phthalocyanine-tetracarboxyglucamine PS in rats presenting induced PD.

The inflammatory profiles were not statistically different and indicated a trend of lower amounts of inflammatory cells in the aPDT group at 7 days. The histological analysis indicated no significant differences between the groups within the analyzed periods, suggesting that the applied therapies did not cause tissue damage. These findings are in agreement with the study by Al Habashneh et al.,[2] (2015), that mentioned that PS application without a light source does not cause damage to healthy tissues, and that the bacterial destruction process occurs only when PS is used in conjunction with a light emitter. In the study conducted by Melo et al.,[14] (2014) several conditions were analyzed for the selective photoinactivation of *S. aureus* and *E. coli*, in plankton cultures and biofilms, by using the PSs hypericin and zinc phthalocyanine and new synthesized hydrosoluble derivatives, such as phthalocyanine tetracarboxyglucamine (FcZnTcG), at various concentrations, incubation times and light densities, among others. The authors verified that the employed photoinactivation parameters contributed to the inhibition of 100% of the bacterial cells without damaging the cells used as the host model (VERO epithelial cell line). This indicates that the PS concentrations and incubation time should be low in order to facilitate selective accumulation in microorganisms, faster in bacteria than in epithelial cells, so that low dose irradiation leads to damage only to microorganisms and not healthy tissue. In the present study, a PS concentration of 10 μg/mL and an incubation time of 10 minutes were used. These parameters were defined based on the results reported by Melo et al.,[14] (2014) in both cell and microorganism cultures. The absence of differences between the experimental groups could be related to these parameters, which were not sufficient to achieve any effect on the microorganisms located in the subgingival

ligature.

The results of the three-dimensional radiographic and histometric analyses indicate no differences between the groups and analyzed periods, indicating that the applied therapies did not interfere in PD progression, since the ligatures were placed around the second molars on both sides of the maxillae and remained in position throughout the experimental period, simulating a clinical situation of PD progression in patients. Contrarily to other studies that evaluated aPDT associated with for PD treatment in animals[5, 15, 19, 50], the present study analyzed aPDT with the PS phthalocyanine-tetracarboxyglucamine under disease conditions without other treatments. With the applied methodology, the ligature placed to induce PD was not removed, thus maintaining a large amount of biofilm in the region. This condition may have interfered in the results, suggesting that aPDT may have acted only on the superficial layer of the biofilm, and was not able to completely eliminate the bacteria present in the area.

Fontana et al.,[18] (2009) investigated the effects of aPDT on bacteria derived from human dental biofilms under planktonic conditions and in in vitro biofilms. The objective was to compare the susceptibility of bacteria to aPDT after sensitization with methylene blue and exposure to red light at 665nm. The results indicate that aPDT eliminated about 63% of the bacteria in the planktonic phase, whereas the light effect resulted in much lower decrease in microorganism counts (31% were killed) in biofilms derived from the same samples. Thus, biofilm bacteria presented resistance to the aPDT, with elimination not exceeding 32% in relation to the controls. The authors concluded that, although aPDT was less effective in treating bacteria in biofilms than in planktonic cultures, the difference was only two-fold, while another study[60] demonstrated that antibiotics are approximately 250 times less effective under these conditions. Müller et al.,[39] (2007) reported less than 1 $\log_{10}$ bacterial destruction in oral biofilms comprising six species developed on bovine enamel disks after sensitization with methylene blue followed by 665nm red light irradiation. The authors reported the incomplete destruction of bacteria in *A. naeslundii* biofilms[65, 66], as well as those developed in agar in 24-well plates using human saliva as inoculum[46]. In these studies, the limited biofilm susceptibility to aPDT was attributed to reduced penetration of the PS. O'Neill et al.,[43] (2002) analyzed microscopic laser scanning images of confocal biofilms derived from saliva and revealed that the photodamage occurred predominantly in the outer layer of biofilm agglomerates after exposure to toluidine blue dye at 25ug/mL and 31.5J of a red light dose. It has been suggested that water channels can carry solutes inside or outside the depths of a biofilm, but do not guarantee access to the interior of cell clusters[68], whose diameter can range from 20 to

600µm[53].

It is worth mentioning that a SRP group (PD induction and conventional mechanical treatment) was not included in the present study due to a methodological limitation, since the ligatures were not removed and SRP could not be performed. In an earlier study (unpublished results), the effects of aPDT and SRP applying phthalocyanine-tetracarboxyglucamine, at the same concentrations and using the parameters of the present study were analyzed in vivo, and the results indicated that aPDT, either as a monotherapy or associated, was effective in reducing bone loss in PD-induced in rats, similar to conventional mechanical treatment.

Although PD induction by ligature in rats is widely applied to reproduce non-human periodontitis, scarce studies have described the microbiota associated with the periodontal destruction induced by this method. Duarte et al.,[17] (2010) placed ligtures around the first mandibular molars in 12 Wistar rats and the contralateral side was considered the control. Forty-two days later, ligatures and biofilm samples were collected and analyzed by checkerbord DNA-DNA hybridization for 40 different types of human periodontal bacterial species. The authors concluded that none of the 40 bacterial species were detected in the unligated teeth, while 25 bacterial species were found in the biofilm around the ligatures. One or more of the tested species, especially those detected in the ligatures, could probably be present in the oral biofilm of teeth without ligatures, but at levels below the detection limit of the method (10^4 cells). Complementing this information, the study by Isogai et al.,[26] (1985), applied the culture technique and examined changes in oral biofilm in non-genetically-engineered rats for 12 months. The authors observed that early biofilms were predominantly composed of *Streptococcus* species. As the plaque developed, there was a decrease in the proportions of *Streptococcus* species and a concomitant increase in bacterial and *Fusobacterium* species.

It is important to note that aPDT presents an antimicrobial effect on both gram-positive and gram-negative bacteria. Gram-positive bacteria readily absorb molecules like PS and can be photoinactivated by most of the dyes used in aPDT. On the other hand, gram-negative bacteria are relatively impermeable, due to their highly specific and negatively charged surface[28]. Thus, gram-negative periodontopathogenic bacteria hinder photochemical inactivation by presenting lipoproteins and lipopolysaccharides in their cellular composition[70]. Pfitzner et al.,[49] (2004) demonstrated that the bacterium *Aggregatibacter actynomycetencomitans* presents greater resistance to therapy effects. Considering this fact, studies are being conducted in order to increase membrane permeability of gram-negative bacteria, using special membrane-activating substances or positively charged PSs that bind more spontaneously to the bacterial

membrane[49, 52]. In the present study, no microbiological analyses were performed, but one of the factors that may have influenced the results is the PS capacity to affect the bacteria present in the ligature.

aPDT involves the use of a light source at a wavelength appropriate to eliminate previously treated microorganisms with a photosensitive dye, which has become interesting in the periodontics area due to its beneficial effects on chronic inflammations, accelerated tissue repair, short-term bactericidal effects and minimal side effects and systemic[25]. However, it is worth mentioning that PD is not cured exclusively by aPDT. Some studies indicate this method as an alternative to antibiotic therapy and evidence it as an effective adjuvant in conventional mechanical treatment[1]. Thus, aPDT may potentiate SRP effects in deep periodontal pockets, promoting a decrease in periodontopathogenic bacteria, as the PS can penetrate into epithelial and connective tissues and reach microorganisms that can infiltrate through the epithelial barrier[31]. This makes it possible to apply aPDT as an efficient therapy in the maintenance phase of periodontal treatment.

Among the various PS existing classes, phthalocyanines have become particularly attractive due to their interesting photophysical properties[42], since they exhibit a strong absorption band with a high molar absorption coefficient in the red region of the electromagnetic spectrum[48], allowing for deep tissue penetration, high photostability and ease of chemical and physical modifications, providing association to several metals, increasing their phototoxicity and ionic characteristics[69]. In addition, their organic composition presents high selectivity, low phototoxicity[58], and more efficient production of singlet oxygen, with greater oxidative power compared to compounds derived from hematoporphyrin and phenothiazine. In addition to their high photodynamic activity, they are easy to synthesize, are quickly eliminated with minimal adverse effects and are commercially available[32]. Despite the general favorable characteristics of this PS, it is possible that the PD model and methodology used in the present study did not provide adequate conditions for proper effects of the dye alone and in conjunction with aPDT.

Scientific studies seek to evaluate the effectiveness of aPDT as an adjunctive therapy to non-surgical periodontal treatment and describe different methodologies. However, variations in protocols, such as type and absorption of the PS, incubation time, light source model (LASER/LED), intensity, energy density, power, wavelength, light exposure time and amount applied indicate limitations and can influence the outcomes, as well as the presence of gingival crevicular fluid, blood and biofilm structure[51]. Almeida et al.,[13] (2007) emphasize the concentration of PS, pH, presence

of exudate and saliva and Harris et al.,[23] (2005) mention another type of diluent or protein content, such as root canals and dental surfaces.

Limitations of this study include the fact that the PS applied herein (phthalocyanine-tetracarboxyglucamine), both as an isolated therapy and in conjunction with aPDT, was applied only once, in a single concentration and always following the same incubation time. In addition, aPDT was applied without association with SRP, which may have influenced the results. Normalization of the applied parameters is necessary to reduce methodological insufficiencies and, thus, increase the reliability of studies that propose to evaluate the efficiency of aPDT for PD treatment. Thus, to better accommodate the technique, new in vivo and in vitro studies, both controlled and randomized, are required, in order to achieve a better understanding of the effects of this PS on microorganisms.

CONCLUSION

The results of the present study indicate that the application of aPDT with phthalocyanine-tetracarboxyglucamine as PS and its components as monotherapies in rats presenting induced PD did not cause tissue damage and did not alter the inflammatory profile.

ACKNOWLEDMENTS

We would like to thank Capes and CNPq for financial support and Prof. Dr. Anderson O. Ribeiro from the Federal University of ABC for synthesize the solution of the photosensitizer hypericin-glucamine used in this study.

CONFLICT OF INTEREST AND SOURCES OF FUNDING STATEMENT

The authors declare that there are no conflicts of interest.

REFERÊNCIAS

1. Akram Z, Hyder T, Al-Hamoudi N, Binshabaib MS, Alharthi SS, Hanif A. Efficacy of photodynamic therapy versus antibiotics as an adjunct to scaling and root planing in the treatment of periodontitis: A systematic review and meta-analysis. Photodiagnosis Photodyn Ther. 2017;19:86-92.
2. Al Habashneh R, Asa'ad FA, Khader Y. Photodynamic therapy in periodontal and peri-implant diseases. Quintessence Int. 2015;46:677-90.
3. Anholt H, Moan J. Fractionated treatment of CaD2 tumors in mice sensitized with aluminium phthlocyanine tetrasulfonate. Cancer Lett. 1992;61:263-7.
4. Bachor R, Flotte TJ, Scholz M, Dretler S, Hasan T. Comparison of intravenous and intravesical administration of chloro-aluminum sulfonated phthalocyanine for photodynamic treatment in a rat bladder cancer model. J Urol. 1992;147:1404-10.

5. Barin LM, Barcelos RCS, Vey LT, Pillusky FM, Palma VM, Kantorski KZ, et al. Role of the adjunctive antimicrobial photodynamic therapy to periodontal treatment at plasmatic oxidative stress and vascular behavior. J Photochem Photobiol B. 2017;173:538-44.
6. Baron ED, Malbasa CL, Santo-Domingo D, Fu P, Miller JD, Hanneman KK, et al. Silicon phthalocyanine (Pc 4) photodynamic therapy is a safe modality for cutaneous neoplasms: results of a phase 1 clinical trial. Lasers Surg Med. 2010;42:728-35.
7. Ben-Hur E, Horowitz B. Virus inactivation in blood. AIDS. 1996;10:1183-90.
8. Bhatti M, MacRobert A, Meghji S, Henderson B, Wilson M. Effect of dosimetric and physiological factors on the lethal photosensitization of Porphyromonas gingivalis in vitro. Photochem Photobiol. 1997;65:1026-31.
9. Carvalho LH, D'Avila GB, Leao A, Haffajee AD, Socransky SS, Feres M. Scaling and root planing, systemic metronidazole and professional plaque removal in the treatment of chronic periodontitis in a Brazilian population. I. clinical results. J Clin Periodontol. 2004;31:1070-6.
10. Chan Y, Lai CH. Bactericidal effects of different laser wavelengths on periodontopathic germs in photodynamic therapy. Lasers Med Sci. 2003;18:51-5.
11. Colombo AP, Teles RP, Torres MC, Rosalem W, Mendes MC, Souto RM, et al. Effects of non-surgical mechanical therapy on the subgingival microbiota of Brazilians with untreated chronic periodontitis: 9-month results. J Periodontol. 2005;76:778-84.
12. da Silva VC, Cirelli CC, Ribeiro FS, Leite FR, Benatti Neto C, Marcantonio RA, et al. Intrusion of teeth with class III furcation: a clinical, histologic and histometric study in dogs. J Clin Periodontol. 2008;35:807-16.
13. de Almeida JM, Theodoro LH, Bosco AF, Nagata MJ, Oshiiwa M, Garcia VG. Influence of photodynamic therapy on the development of ligature-induced periodontitis in rats. J Periodontol. 2007;78:566-75.
14. de Melo WC. Fotoinativação seletiva dos microrganismos: Escherichia coli e Staphylococcus aureus [tese de doutorado]. São Carlos: Escola de Engenharia de São Carlos/ Faculdade de Medicina de Ribeirão Preto/ Instituto de Química de São Carlos da Universidade de São Paulo; 2014.
15. de Oliveira PG, Silveira ESAM, Novaes AB, Jr., Taba M, Jr., Messora MR, Palioto DB, et al. Adjunctive effect of antimicrobial photodynamic therapy in induced periodontal disease. Animal study with histomorphometrical, immunohistochemical, and cytokine evaluation. Lasers Med Sci. 2016;31:1275-83.
16. Dobson J, Wilson M. Sensitization of oral bacteria in biofilms to killing by light from a low-power laser. Arch Oral Biol. 1992;37:883-7.
17. Duarte PM, Tezolin KR, Figueiredo LC, Feres M, Bastos MF. Microbial profile of ligature-induced periodontitis in rats. Arch Oral Biol. 2010;55:142-7.
18. Fontana CR, Abernethy AD, Som S, Ruggiero K, Doucette S, Marcantonio RC, et al. The antibacterial effect of photodynamic therapy in dental plaque-derived biofilms. J Periodontal Res. 2009;44:751-9.
19. Garcia VG, Longo M, Fernandes LA, Gualberto EC, Jr., Santinoni Cdos S, Bosco AF, et al. Treatment of experimental periodontitis in rats using repeated adjunctive antimicrobial photodynamic therapy. Lasers Med Sci. 2013;28:143-50.
20. Haas R, Baron M, Dortbudak O, Watzek G. Lethal photosensitization, autogenous bone, and e-PTFE membrane for the treatment of peri-implantitis: preliminary results. Int J Oral Maxillofac Implants. 2000;15:374-82.
21. Haas R, Dortbudak O, Mensdorff-Pouilly N, Mailath G. Elimination of bacteria on different implant surfaces through photosensitization and soft laser. An in vitro study. Clin Oral Implants Res. 1997;8:249-54.
22. Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem Photobiol Sci. 2004;3:436-50.

23. Harris F, Chatfield LK, Phoenix DA. Phenothiazinium based photosensitisers--photodynamic agents with a multiplicity of cellular targets and clinical applications. Curr Drug Targets. 2005;6:615-27.
24. Hayek RR, Araujo NS, Gioso MA, Ferreira J, Baptista-Sobrinho CA, Yamada AM, et al. Comparative study between the effects of photodynamic therapy and conventional therapy on microbial reduction in ligature-induced peri-implantitis in dogs. J Periodontol. 2005;76:1275-81.
25. Ishikawa I, Aoki A, Takasaki AA, Mizutani K, Sasaki KM, Izumi Y. Application of lasers in periodontics: true innovation or myth? Periodontol 2000. 2009;50:90-126.
26. Isogai E, Isogai H, Sawada H, Kaneko H, Ito N. Microbial ecology of plaque in rats with naturally occurring gingivitis. Infect Immun. 1985;48:520-7.
27. Jori G. Photodynamic therapy of microbial infections: state of the art and perspectives. J Environ Pathol Toxicol Oncol. 2006;25:505-19.
28. Jori G, Brown SB. Photosensitized inactivation of microorganisms. Photochem Photobiol Sci. 2004;3:403-5.
29. Kapoor A, Malhotra R, Grover V, Grover D. Systemic antibiotic therapy in periodontics. Dent Res J (Isfahan). 2012;9:505-15.
30. Komerik N, Curnow A, MacRobert AJ, Hopper C, Speight PM, Wilson M. Fluorescence biodistribution and photosensitising activity of toluidine blue o on rat buccal mucosa. Lasers Med Sci. 2002;17:86-92.
31. Komerik N, Nakanishi H, MacRobert AJ, Henderson B, Speight P, Wilson M. In vivo killing of Porphyromonas gingivalis by toluidine blue-mediated photosensitization in an animal model. Antimicrob Agents Chemother. 2003;47:932-40.
32. Kyriazi M, Alexandratou E, Yova D, Rallis M, Trebst T. Topical photodynamic therapy of murine non-melanoma skin carcinomas with aluminum phthalocyanine chloride and a diode laser: pharmacokinetics, tumor response and cosmetic outcomes. Photodermatol Photoimmunol Photomed. 2008;24:87-94.
33. Liu R, Bal HS, Desta T, Krothapalli N, Alyassi M, Luan Q, et al. Diabetes enhances periodontal bone loss through enhanced resorption and diminished bone formation. J Dent Res. 2006;85:510-4.
34. Longo JP, Leal SC, Simioni AR, de Fatima Menezes Almeida-Santos M, Tedesco AC, Azevedo RB. Photodynamic therapy disinfection of carious tissue mediated by aluminum-chloride-phthalocyanine entrapped in cationic liposomes: an in vitro and clinical study. Lasers Med Sci. 2012;27:575-84.
35. Lopez NJ, Gamonal JA, Martinez B. Repeated metronidazole and amoxicillin treatment of periodontitis. A follow-up study. J Periodontol. 2000;71:79-89.
36. Malik R, Manocha A, Suresh DK. Photodynamic therapy--a strategic review. Indian J Dent Res. 2010;21:285-91.
37. Matevski D, Weersink R, Tenenbaum HC, Wilson B, Ellen RP, Lepine G. Lethal photosensitization of periodontal pathogens by a red-filtered Xenon lamp in vitro. J Periodontal Res. 2003;38:428-35.
38. Mckeown NB. Phthalocyanines materials: synthesis, structure and function. Cambridge: Cambridge University Press; 1998.
39. Muller P, Guggenheim B, Schmidlin PR. Efficacy of gasiform ozone and photodynamic therapy on a multispecies oral biofilm in vitro. Eur J Oral Sci. 2007;115:77-80.
40. Nunes SM, Sguilla FS, Tedesco AC. Photophysical studies of zinc phthalocyanine and chloroaluminum phthalocyanine incorporated into liposomes in the presence of additives. Braz J Med Biol Res. 2004;37:273-84.
41. Nussbaum EL, Lilge L, Mazzulli T. Effects of 630-, 660-, 810-, and 905-nm laser irradiation delivering radiant exposure of 1-50 J/cm2 on three species of bacteria in vitro. J Clin Laser Med Surg. 2002;20:325-33.
42. Nyokong T. Effects of substituents on the photochemical and photophysical properties of main group metal phthalocyanines. Coordination chemistry reviews. 2007;251:1707-22.

43. O'Neill JF, Hope CK, Wilson M. Oral bacteria in multi-species biofilms can be killed by red light in the presence of toluidine blue. Lasers Surg Med. 2002;31:86-90.
44. Ochsner M. Photophysical and photobiological processes in the photodynamic therapy of tumours. J Photochem Photobiol B. 1997;39:1-18.
45. Odze RD, Marcial MA, Antonioli D. Gastric fundic gland polyps: a morphological study including mucin histochemistry, stereometry, and MIB-1 immunohistochemistry. Hum Pathol. 1996;27:896-903.
46. Ogura M, Abernethy AD, Blissett RD, Ruggiero K, Som S, Goodson JM, et al. Photomechanical wave-assisted molecular delivery in oral biofilms. World Journal of Microbiology and Biotechnology. 2007;23:1637-46.
47. Oliveira DM, Lacava ZG, Lima EC, Morais PC, Tedesco AC. Zinc phthalocyanine/magnetic fluid complex: a promising dual nanostructured system for cancer treatment. J Nanosci Nanotechnol. 2006;6:2432-7.
48. Owens JW, Smith R, Robinson R, Robins M. Photophysical properties of porphyrins, phthalocyanines, and benzochlorins. Inorganica Chimica Acta. 1998;279:226-31.
49. Pfitzner A, Sigusch BW, Albrecht V, Glockmann E. Killing of periodontopathogenic bacteria by photodynamic therapy. J Periodontol. 2004;75:1343-9.
50. Pillusky FM, Barcelos RCS, Vey LT, Barin LM, de Mello Palma V, Maciel RM, et al. Antimicrobial photodynamic therapy with photosensitizer in ethanol improves oxidative status and gingival collagen in a short-term in periodontitis. Photodiagnosis Photodyn Ther. 2017;19:119-27.
51. Prates RA, Yamada AM, Jr., Suzuki LC, Eiko Hashimoto MC, Cai S, Gouw-Soares S, et al. Bactericidal effect of malachite green and red laser on Actinobacillus actinomycetemcomitans. J Photochem Photobiol B. 2007;86:70-6.
52. Raghavendra M, Koregol A, Bhola S. Photodynamic therapy: a targeted therapy in periodontics. Aust Dent J. 2009;54 Suppl 1:S102-9.
53. Rani SA, Pitts B, Stewart PS. Rapid diffusion of fluorescent tracers into Staphylococcus epidermidis biofilms visualized by time lapse microscopy. Antimicrob Agents Chemother. 2005;49:728-32.
54. Reddi E, Lo Castro G, Biolo R, Jori G. Pharmacokinetic studies with zinc(II)-phthalocyanine in tumour-bearing mice. Br J Cancer. 1987;56:597-600.
55. Ribeiro AP, Andrade MC, da Silva Jde F, Jorge JH, Primo FL, Tedesco AC, et al. Photodynamic inactivation of planktonic cultures and biofilms of Candida albicans mediated by aluminum-chloride-phthalocyanine entrapped in nanoemulsions. Photochem Photobiol. 2013;89:111-9.
56. Rodrigues RM, Goncalves C, Souto R, Feres-Filho EJ, Uzeda M, Colombo AP. Antibiotic resistance profile of the subgingival microbiota following systemic or local tetracycline therapy. J Clin Periodontol. 2004;31:420-7.
57. Rosenthal I. Phthalocyanines as photodynamic sensitizers. Photochem Photobiol. 1991;53:859-70.
58. Ryskova L, Buchta V, Slezak R. Photodynamic antimicrobial therapy. Central European Journal of Biology. 2010;5:400-6.
59. Sarkar S, Wilson M. Lethal photosensitization of bacteria in subgingival plaque from patients with chronic periodontitis. J Periodontal Res. 1993;28:204-10.
60. Sedlacek MJ, Walker C. Antibiotic resistance in an in vitro subgingival biofilm model. Oral Microbiol Immunol. 2007;22:333-9.
61. Shibli JA, Martins MC, Nociti FH, Jr., Garcia VG, Marcantonio E, Jr. Treatment of ligature-induced peri-implantitis by lethal photosensitization and guided bone regeneration: a preliminary histologic study in dogs. J Periodontol. 2003;74:338-45.
62. Shibli JA, Martins MC, Ribeiro FS, Garcia VG, Nociti FH, Jr., Marcantonio E, Jr. Lethal photosensitization and guided bone regeneration in treatment of peri-implantitis: an experimental study in dogs. Clin Oral Implants Res. 2006;17:273-81.

63. Sibata MN, Tedesco AC, Marchetti JM. Photophysicals and photochemicals studies of zinc(II) phthalocyanine in long time circulation micelles for photodynamic therapy use. Eur J Pharm Sci. 2004;23:131-8.
64. Simplicio FI, Maionchi F, Hioka N. Terapia fotodinâmica: aspectos farmacológicos, aplicações e avanços recentes no desenvolvimento de medicamentos. Quim Nova. 2002;25:801-7.
65. Soukos NS, Mulholland SE, Socransky SS, Doukas AG. Photodestruction of human dental plaque bacteria: enhancement of the photodynamic effect by photomechanical waves in an oral biofilm model. Lasers Surg Med. 2003;33:161-8.
66. Soukos NS, Socransky SS, Mulholland SE, Lee S, Doukas AG. Photomechanical drug delivery into bacterial biofilms. Pharm Res. 2000;17:405-9.
67. Spikes JD. Phthalocyanines as photosensitizers in biological systems and for the photodynamic therapy of tumors. Photochem Photobiol. 1986;43:691-9.
68. Stewart PS. Diffusion in biofilms. J Bacteriol. 2003;185:1485-91.
69. Tapajos EC, Longo JP, Simioni AR, Lacava ZG, Santos MF, Morais PC, et al. In vitro photodynamic therapy on human oral keratinocytes using chloroaluminum-phthalocyanine. Oral Oncol. 2008;44:1073-9.
70. Usacheva MN, Teichert MC, Biel MA. Comparison of the methylene blue and toluidine blue photobactericidal efficacy against gram-positive and gram-negative microorganisms. Lasers Surg Med. 2001;29:165-73.
71. van Bruggen N, Chan WS, Syha J, Marshall JF, Proctor E, Williams SR, et al. Cell and tissue responses of a murine tumour to phthalocyanine-mediated photodynamic therapy. Eur J Cancer. 1992;28:42-6.
72. van Winkelhoff AJ, Rams TE, Slots J. Systemic antibiotic therapy in periodontics. Periodontol 2000. 1996;10:45-78.
73. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). J Antimicrob Chemother. 1998;42:13-28.
74. Wilson M. Photolysis of oral bacteria and its potential use in the treatment of caries and periodontal disease. J Appl Bacteriol. 1993;75:299-306.
75. Wilson M, Dobson J. Lethal photosensitization of oral anaerobic bacteria. Clin Infect Dis. 1993;16 Suppl 4:S414-5.
76. Wilson M, Pratten J. Lethal photosensitisation of Staphylococcus aureus in vitro: effect of growth phase, serum, and pre-irradiation time. Lasers Surg Med. 1995;16:272-6.
77. Wohrle D, Iskander N, Graschew G, Sinn H, Friedrich EA, Maier-Borst W, et al. Synthesis of positively charged phthalocyanines and their activity in the photodynamic therapy of cancer cells. Photochem Photobiol. 1990;51:351-6.

Figure 1 – Modifications of the phthalocyanine molecule. A) Phthalocyanine structure with empty central cavity. B) Phthalocyanine structure accommodating the metallic ion zinc. C), Chemical structure of meglumine. D) Phthalocyanine-Zn-tetracarboxylated structure. E) Phthalocyanine-Zn-tetracarboxy-N-methylglucamine structure.

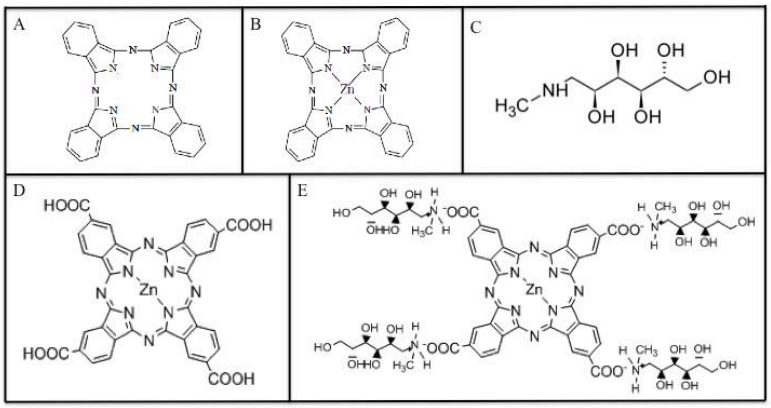


Figure 2 – Molecular absorption spectrum of tetracarboxylated zinc phthalocyanine in DMSO.

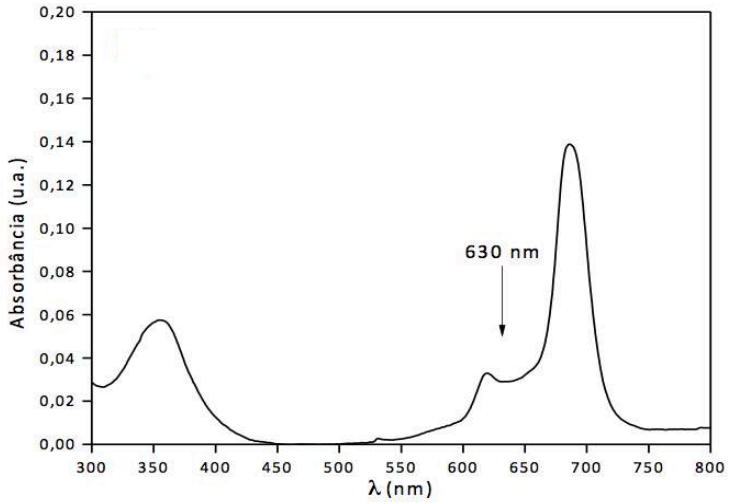


Figure 3 – Experimental design of the present study.

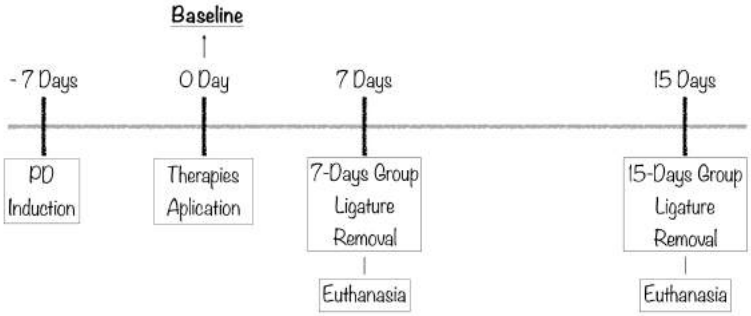
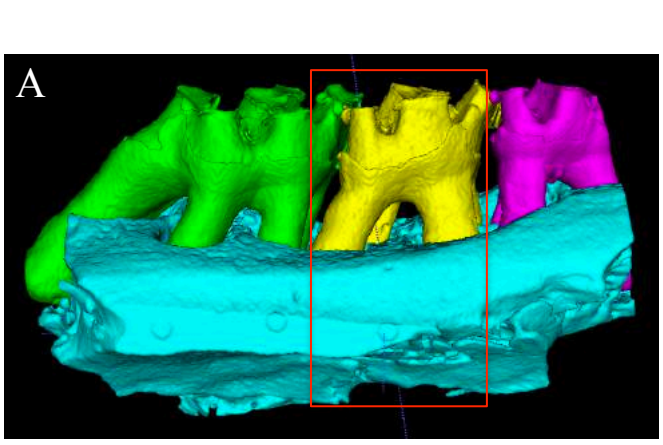


Figure 4 – A) Photograph of the 3D model made from the scans in the μ-CT: maxilla in blue; first maxillary molar in green; second maxillary molar in yellow, third maxillary molar in pink. B) The analyzed area delimited by the ROI. Roots were removed digitally.



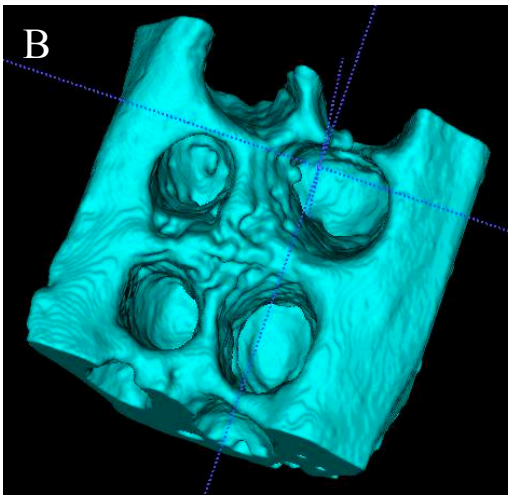


Figure 5 – Photograph of the 3D models obtained from sample scanning for the three-dimensional radiographic analysis, with the ROI selection. Teeth were removed digitally, indicating the analyzed region. A, B, C and D display the 7-day models of the PD, PS, LED and aPDT groups, respectively while E, F, G and H display 15-day models of the PD, PS, LED and aPDT groups, respectively. Data is presented as means and standard deviation of the bone volume (mm^3) of the second maxillary molar region of the treated hemimaxils of the experimental groups.

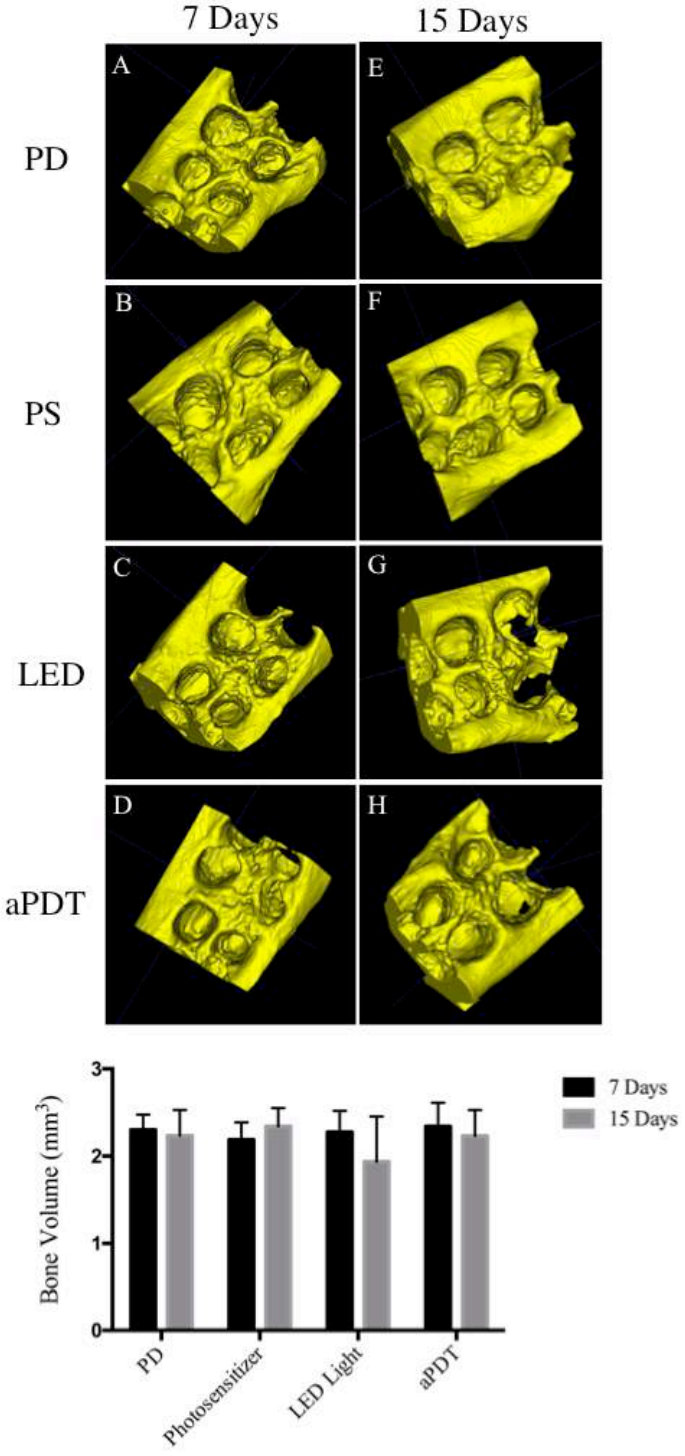


Figure 6 – Photomicrographs of sagittal sections of rat maxillae. At 2.5x magnification: A, B, C and D display the samples at 7 days; and M, N, O and P at 15 days, for the PD, PS, LED Light and aPDT groups, respectively, indicating the alveolar process (AP) formed by irregular bone trabeculae, between the 1st (1M) and 2nd (2M) and 2nd (2M) and 3rd (3M) molars. The cervical portion of the alveolar process (AP) is located near the apical third of the 2M roots. Resorption of the alveolar bone (AB) was observed in the furcation region. At a 20x magnification: E, F, G, H and I, J, K, L display the interproximal and furcation regions, respectively, at 7 days; and Q, R, S, T and U, V, W, X display the interproximals and furcation regions, respectively, at 15 days. The gingival epithelium (GE) is located apically at the cementum-enamel junction (CEJ), the gingival mucosa exhibits an inflammatory process (IP) and the interdental papilla is destroyed. GE, gingival epithelium; D, dentin; DP, dental pulp; AB, alveolar bone of the furcation region. HE. x2,5 Scale bar:100μm. x20 Scale bar: on the images. Data is displayed as means and standard deviation of the bone percentage (%) of the furcation region and alveolar bone loss (μm) of the proximal regions of the second maxillary molar of the treated hemimaxils of the experimental groups.

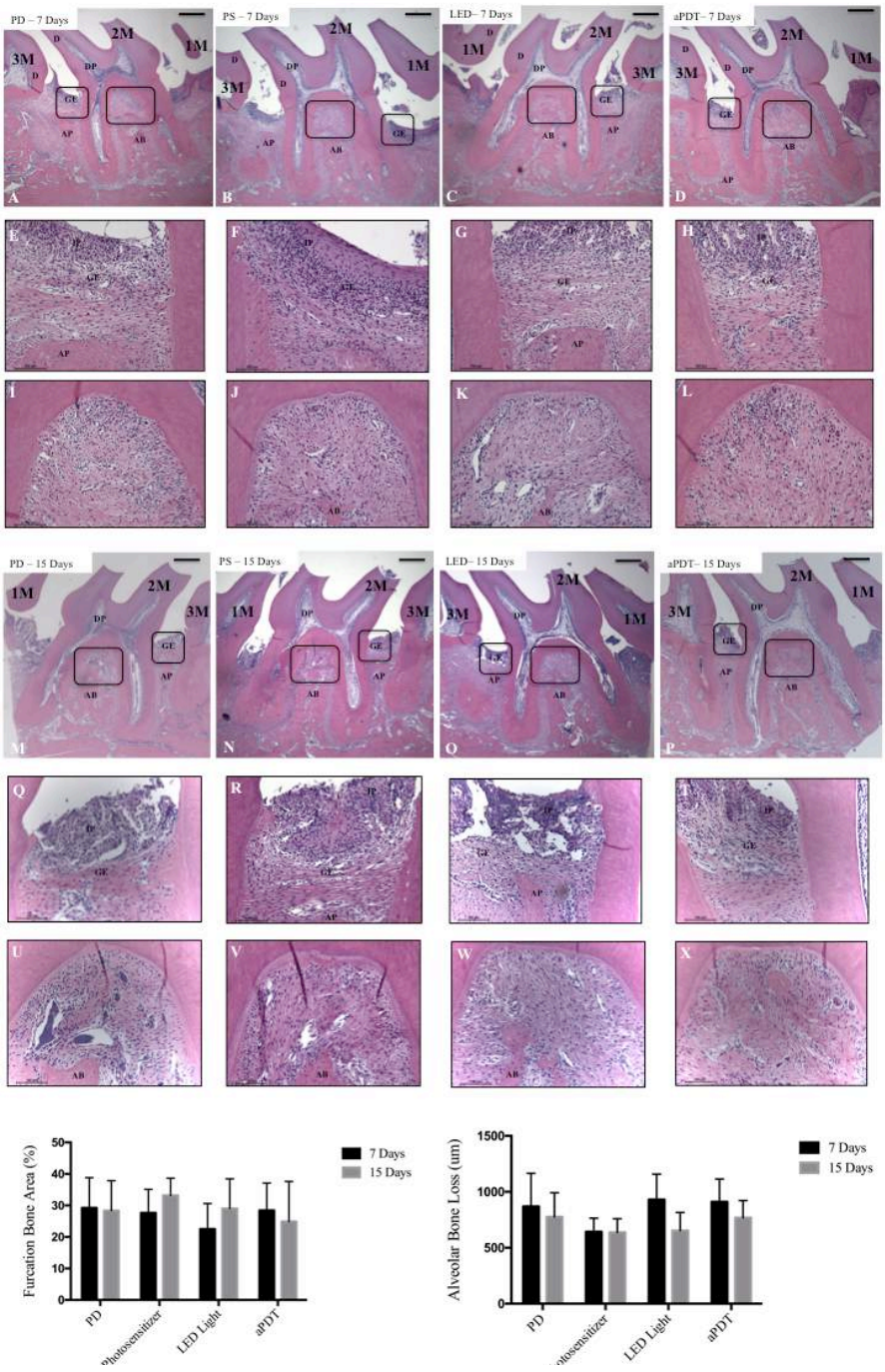
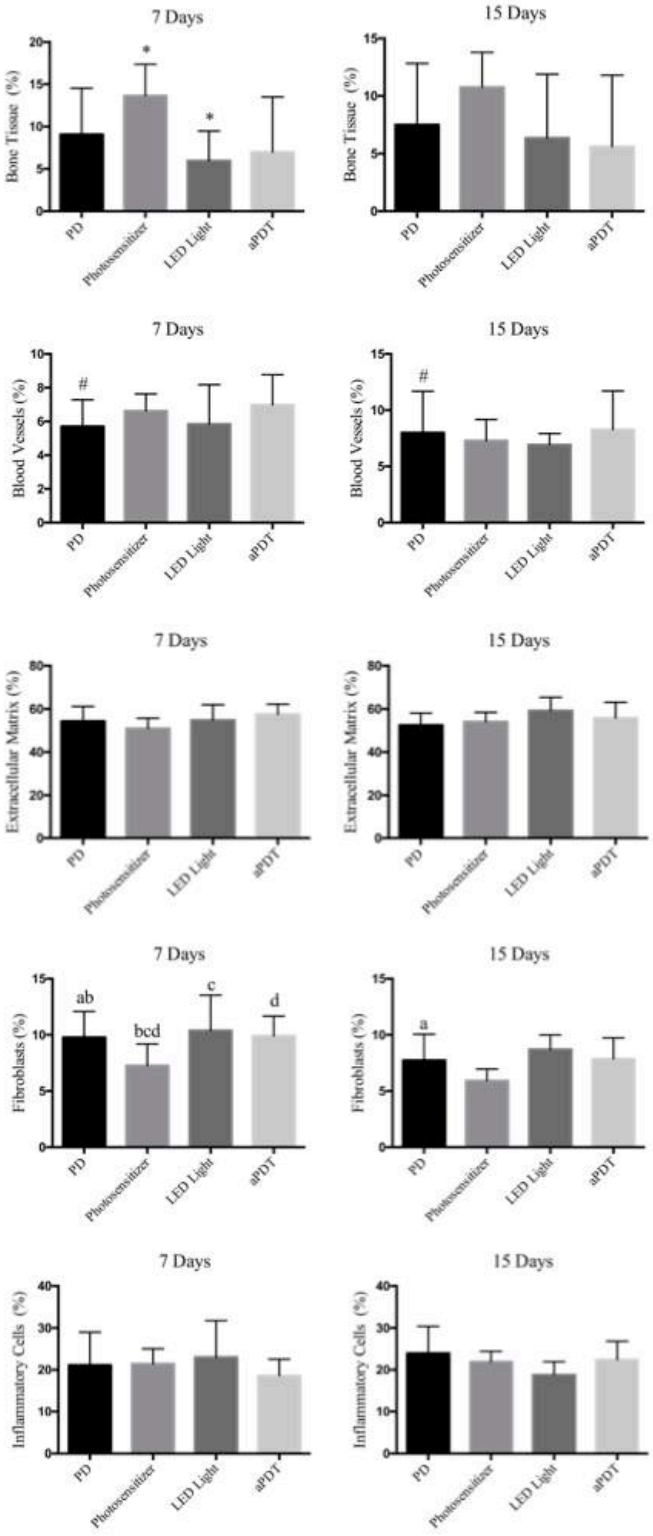


Figure 7 – Quantification graphics of bone tissue, blood vessels, extracellular matrix, inflammatory cells and fibroblasts. All data are presented as percentage (%) with means and standard deviation of the second maxillary molar region of the treated hemimaxils of the experimental groups, at the different timeframes. *, #, a, b, c, d indicate statistically significant differences between groups.



4 CONCLUSÃO

Com os resultados obidos pode-se concluir que:

Publicação 1: A aPDT com ftalocianina de zinco tetracarboxi-N-metilglucamina, como monoterapia ou associada a RAR, foi efetiva no controle de perda óssea em DP induzida em ratos, similarmente ao tratamento mecânico convencional.

Publicação 2: A aplicação da aPDT e de seus componentes como monoterapias na DP induzida não acarretou danos aos tecidos e não alterou o perfil inflamatório. E a permanência da ligadura durante o período experiemental inibiu a ação das terapias sobre as bactérias e a resposta do hospedeiro frente ao tratamento tornou-se comprometida com a metodologia utilizada.

Assim, a ftalocianina de zinco tetracarboxi-N-metilglucamina pode ser considerada um promissor fotossensibilizador. No entanto, mais estudo in vitro e in vivo são necessários para elucidar a ação da aPDT e o efeito deste FS como terapia adjunta no tratamento da DP.

REFERÊNCIAS*

1. Adriaens PA, Edwards CA, De Boever JA, Loesche WJ. Ultrastructural observations on bacterial invasion in cementum and radicular dentin of periodontally diseased human teeth. J Periodontol. 1988; 59(8): 493-503.
2. American, Academy, Periodontology o. Treatment of plaque-induced gingivitis, chronic periodontitis, and other clinical conditions. J Periodontol. 2001; 72(12): 1790-800.
3. Anholt H, Moan J. Fractionated treatment of CaD2 tumors in mice sensitized with aluminium phthlocyanine tetrasulfonate. Cancer Lett. 1992; 61(3): 263-7.
4. Armitage GC. Periodontal diagnoses and classification of periodontal diseases. Periodontol 2000. 2004; 34: 9-21.
5. Bhatti M, MacRobert A, Henderson B, Wilson M. Exposure of Porphyromonas gingivalis to red light in the presence of the light-activated antimicrobial agent toluidine blue decreases membrane fluidity. Curr Microbiol. 2002; 45(2): 118-22.
6. Bhatti M, MacRobert A, Meghji S, Henderson B, Wilson M. Effect of dosimetric and physiological factors on the lethal photosensitization of Porphyromonas gingivalis in vitro. Photochem Photobiol. 1997; 65(6): 1026-31.
7. Bonito AJ, Lux L, Lohr KN. Impact of local adjuncts to scaling and root planing in periodontal disease therapy: a systematic review. J Periodontol. 2005; 76(8): 1227-36.
8. Braham P, Herron C, Street C, Darveau R. Antimicrobial photodynamic therapy may promote periodontal healing through multiple mechanisms. J Periodontol. 2009; 80(11): 1790-8.
9. Braun A, Dehn C, Krause F, Jepsen S. Short-term clinical effects of adjunctive antimicrobial photodynamic therapy in periodontal treatment: a randomized clinical trial. J Clin Periodontol. 2008; 35(10): 877-84.
10. Carvalho AS, Napimoga MH, Coelho-Campos J, Silva-Filho VJ, Thedei G. Photodynamic therapy reduces bone resorption and decreases inflammatory response in an experimental rat periodontal disease model. Photomed Laser Surg. 2011; 29(11): 735-40.
11. Chan Y, Lai CH. Bactericidal effects of different laser wavelengths on periodontopathic germs in photodynamic therapy. Lasers Med Sci. 2003; 18(1): 51-5.
12. da Silva VC, Cirelli CC, Ribeiro FS, Leite FR, Benatti Neto C, Marcantonio RA, et al. Intrusion of teeth with class III furcation: a clinical, histologic and histometric study in dogs. J Clin Periodontol. 2008; 35(9): 807-16.
13. de Almeida JM, Theodoro LH, Bosco AF, Nagata MJ, Bonfante S, Garcia VG. Treatment of experimental periodontal disease by photodynamic therapy in rats with diabetes. J Periodontol. 2008; 79(11): 2156-65.
14. de Almeida JM, Theodoro LH, Bosco AF, Nagata MJ, Oshiiwa M, Garcia VG. In vivo effect of photodynamic therapy on periodontal bone loss in dental furcations. J Periodontol. 2008; 79(6): 1081-8.

*De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das normas Vancouver. Disponível no site da biblioteca: http://www.foar.unesp.br/ - biblioteca/manual.

15. de Almeida JM, Theodoro LH, Bosco AF, Nagata MJ, Oshiiwa M, Garcia VG. Influence of photodynamic therapy on the development of ligature-induced periodontitis in rats. J Periodontol. 2007; 78(3): 566-75.

16. de Melo WC. Fotoinativação seletiva dos microrganismos: Escherichia coli e Staphylococcus aureus [tese de doutorado]. São Carlos: Escola de Engenharia de São Carlos/ Faculdade de Medicina de Ribeirão Preto/ Instituto de Química de São Carlos da Universidade de São Paulo; 2014.

17. de Oliveira RR, Novaes AB, Jr., Garlet GP, de Souza RF, Taba M, Jr., Sato S, et al. The effect of a single episode of antimicrobial photodynamic therapy in the treatment of experimental periodontitis. Microbiological profile and cytokine pattern in the dog mandible. Lasers Med Sci. 2011; 26(3): 359-67.

18. Dobson J, Wilson M. Sensitization of oral bacteria in biofilms to killing by light from a low-power laser. Arch Oral Biol. 1992; 37(11): 883-7.

19. Galler C. Periodontal summary score. A new and relevant periodontal assessment guide. J Ont Dent Assoc. 2000; 1: 21-8.

20. Garcia VG, Gualberto Junior EC, Fernandes LA, Bosco AF, Hitomi Nagata MJ, Casatti CA, et al. Adjunctive antimicrobial photodynamic treatment of experimentally induced periodontitis in rats with ovariectomy. J Periodontol. 2013; 84(4): 556-65.

21. Garcia VG, Longo M, Fernandes LA, Gualberto EC, Jr., Santinoni Cdos S, Bosco AF, et al. Treatment of experimental periodontitis in rats using repeated adjunctive antimicrobial photodynamic therapy. Lasers Med Sci. 2013; 28(1): 143-50.

22. Garcia VG, Longo M, Gualberto Junior EC, Bosco AF, Nagata MJ, Ervolino E, et al. Effect of the concentration of phenothiazine photosensitizers in antimicrobial photodynamic therapy on bone loss and the immune inflammatory response of induced periodontitis in rats. J Periodontal Res. 2014; 49(5): 584-94.

23. Giuliana G, Ammatuna P, Pizzo G, Capone F, D'Angelo M. Occurrence of invading bacteria in radicular dentin of periodontally diseased teeth: microbiological findings. J Clin Periodontol. 1997; 24(7): 478-85.

24. Giuliani F, Martinelli M, Cocchi A, Arbia D, Fantetti L, Roncucci G. In vitro resistance selection studies of RLP068/Cl, a new Zn(II) phthalocyanine suitable for antimicrobial photodynamic therapy. Antimicrob Agents Chemother. 2010; 54(2): 637-42.

25. Graves DT, Fine D, Teng YT, Van Dyke TE, Hajishengallis G. The use of rodent models to investigate host-bacteria interactions related to periodontal diseases. J Clin Periodontol. 2008; 35(2): 89-105.

26. Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG, Machtei EE, et al. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. J Periodontol. 1994; 65(3): 260-7.

27. Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem Photobiol Sci. 2004; 3(5): 436-50.

28. Houreld N, Abrahamse H. In vitro exposure of wounded diabetic fibroblast cells to a helium-neon laser at 5 and 16 J/cm2. Photomed Laser Surg. 2007; 25(2): 78-84.

29. Huang L, Xuan Y, Koide Y, Zhiyentayev T, Tanaka M, Hamblin MR. Type I and Type II mechanisms of antimicrobial photodynamic therapy: an in vitro study on gram-negative and gram-positive bacteria. Lasers Surg Med. 2012; 44(6): 490-9.

30. Kharkwal GB, Sharma SK, Huang YY, Dai T, Hamblin MR. Photodynamic therapy for infections: clinical applications. Lasers Surg Med. 2011; 43(7): 755-67.

31. Kikuchi T, Mogi M, Okabe I, Okada K, Goto H, Sasaki Y, et al. Adjunctive application of antimicrobial photodynamic therapy in nonsurgical periodontal treatment: a review of literature. Int J Mol Sci. 2015; 16(10): 24111-26.

32. Klepac-Ceraj V, Patel N, Song X, Holewa C, Patel C, Kent R, et al. Photodynamic effects of methylene blue-loaded polymeric nanoparticles on dental plaque bacteria. Lasers Surg Med. 2011; 43(7): 600-6.

33. Kuo LC, Polson AM, Kang T. Associations between periodontal diseases and systemic diseases: a review of the inter-relationships and interactions with diabetes, respiratory diseases, cardiovascular diseases and osteoporosis. Public Health. 2008; 122(4): 417-33.

34. Liu R, Bal HS, Desta T, Krothapalli N, Alyassi M, Luan Q, et al. Diabetes enhances periodontal bone loss through enhanced resorption and diminished bone formation. J Dent Res. 2006; 85(6): 510-4.

35. Longo JP, Leal SC, Simioni AR, de Fatima Menezes Almeida-Santos M, Tedesco AC, Azevedo RB. Photodynamic therapy disinfection of carious tissue mediated by aluminum-chloride-phthalocyanine entrapped in cationic liposomes: an in vitro and clinical study. Lasers Med Sci. 2012; 27(3): 575-84.

36. Maisch T. Anti-microbial photodynamic therapy: useful in the future? Lasers Med Sci. 2007; 22(2): 83-91.

37. Maisch T. A new strategy to destroy antibiotic resistant microorganisms: antimicrobial photodynamic treatment. Mini Rev Med Chem. 2009; 9(8): 974-83.

38. Malik Z, Ladan H, Nitzan Y. Photodynamic inactivation of Gram-negative bacteria: problems and possible solutions. J Photochem Photobiol B. 1992; 14(3): 262-6.

39. Matevski D, Weersink R, Tenenbaum HC, Wilson B, Ellen RP, Lepine G. Lethal photosensitization of periodontal pathogens by a red-filtered Xenon lamp in vitro. J Periodontal Res. 2003; 38(4): 428-35.

40. Mckeown NB. Phthalocyanines materials: synthesis, structure and function. Cambridge: Cambridge University Press; 1998.

41. Meisel P, Kocher T. Photodynamic therapy for periodontal diseases: state of the art. J Photochem Photobiol B. 2005; 79(2): 159-70.

42. Merchat M, Bertolini G, Giacomini P, Villanueva A, Jori G. Meso-substituted cationic porphyrins as efficient photosensitizers of gram-positive and gram-negative bacteria. J Photochem Photobiol B. 1996; 32(3): 153-7.

43. Merchat M, Spikes JD, Bertoloni G, Jori G. Studies on the mechanism of bacteria photosensitization by meso-substituted cationic porphyrins. J Photochem Photobiol B. 1996; 35(3): 149-57.

44. Muller P, Guggenheim B, Schmidlin PR. Efficacy of gasiform ozone and photodynamic therapy on a multispecies oral biofilm in vitro. Eur J Oral Sci. 2007; 115(1): 77-80.

45. Nagahara A, Mitani A, Fukuda M, Yamamoto H, Tahara K, Morita I, et al. Antimicrobial photodynamic therapy using a diode laser with a potential new photosensitizer, indocyanine green-loaded nanospheres, may be effective for the clearance of Porphyromonas gingivalis. J Periodontal Res. 2013; 48(5): 591-9.

46. Nunes SM, Sguilla FS, Tedesco AC. Photophysical studies of zinc phthalocyanine and chloroaluminum phthalocyanine incorporated into liposomes in the presence of additives. Braz J Med Biol Res. 2004; 37(2): 273-84.

47. O'Neill JF, Hope CK, Wilson M. Oral bacteria in multi-species biofilms can be killed by red light in the presence of toluidine blue. Lasers Surg Med. 2002; 31(2): 86-90.

48. Odze RD, Marcial MA, Antonioli D. Gastric fundic gland polyps: a morphological study including mucin histochemistry, stereometry, and MIB-1 immunohistochemistry. Hum Pathol. 1996; 27(9): 896-903.

49. Oliveira DM, Lacava ZG, Lima EC, Morais PC, Tedesco AC. Zinc phthalocyanine/magnetic fluid complex: a promising dual nanostructured system for cancer treatment. J Nanosci Nanotechnol. 2006; 6(8): 2432-7.

50. Passanezi E, Damante CA, de Rezende ML, Greghi SL. Lasers in periodontal therapy. Periodontol 2000. 2015; 67(1): 268-91.

51. Prates RA, Yamada AM, Suzuki LC, Franca CM, Cai S, Mayer MP, et al. Histomorphometric and microbiological assessment of photodynamic therapy as an adjuvant treatment for periodontitis: a short-term evaluation of inflammatory periodontal conditions and bacterial reduction in a rat model. Photomed Laser Surg. 2011; 29(12): 835-44.

52. Qin Y, Luan X, Bi L, He G, Bai X, Zhou C, et al. Toluidine blue-mediated photoinactivation of periodontal pathogens from supragingival plaques. Lasers Med Sci. 2008; 23(1): 49-54.

53. Qin YL, Luan XL, Bi LJ, Sheng YQ, Zhou CN, Zhang ZG. Comparison of toluidine blue-mediated photodynamic therapy and conventional scaling treatment for periodontitis in rats. J Periodontal Res. 2008; 43(2): 162-7.

54. Ribeiro AP, Andrade MC, da Silva Jde F, Jorge JH, Primo FL, Tedesco AC, et al. Photodynamic inactivation of planktonic cultures and biofilms of Candida albicans mediated by aluminum-chloride-phthalocyanine entrapped in nanoemulsions. Photochem Photobiol. 2013; 89(1): 111-9.

55. Rosenthal I. Phthalocyanines as photodynamic sensitizers. Photochem Photobiol. 1991; 53(6): 859-70.

56. Seguier S, Souza SL, Sverzut AC, Simioni AR, Primo FL, Bodineau A, et al. Impact of photodynamic therapy on inflammatory cells during human chronic periodontitis. J Photochem Photobiol B. 2010; 101(3): 348-54.

57. Sgolastra F, Petrucci A, Severino M, Graziani F, Gatto R, Monaco A. Adjunctive photodynamic therapy to non-surgical treatment of chronic periodontitis: a systematic review and meta-analysis. J Clin Periodontol. 2013; 40(5): 514-26.

58. Sibata MN, Tedesco AC, Marchetti JM. Photophysicals and photochemicals studies of zinc(II) phthalocyanine in long time circulation micelles for photodynamic therapy use. Eur J Pharm Sci. 2004; 23(2): 131-8.

59. Sigusch BW, Pfitzner A, Albrecht V, Glockmann E. Efficacy of photodynamic therapy on inflammatory signs and two selected periodontopathogenic species in a beagle dog model. J Periodontol. 2005; 76(7): 1100-5.

60. Simplicio FI, Maionchi F, Hioka N. Terapia fotodinâmica: aspectos farmacológicos, aplicações e avanços recentes no desenvolvimento de medicamentos. Quim Nova. 2002; 25: 801-7.

61. Slots J. Selection of antimicrobial agents in periodontal therapy. J Periodontal Res. 2002; 37(5): 389-98.

62. Slots J, Ting M. Systemic antibiotics in the treatment of periodontal disease. Periodontol 2000. 2002; 28: 106-76.

63. Smiley CJ, Tracy SL, Abt E, Michalowicz BS, John MT, Gunsolley J, et al. Systematic review and meta-analysis on the nonsurgical treatment of chronic periodontitis by means of scaling and root planing with or without adjuncts. J Am Dent Assoc. 2015; 146(7): 508-24 e5.

64. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. Periodontol 2000. 2002; 28: 12-55.

65. Socransky SS, Haffajee AD. Microbial mechanisms in the pathogenesis of destructive periodontal diseases: a critical assessment. J Periodontal Res. 1991; 26(3 Pt 2): 195-212.

66. Soukos NS, Mulholland SE, Socransky SS, Doukas AG. Photodestruction of human dental plaque bacteria: enhancement of the photodynamic effect by photomechanical waves in an oral biofilm model. Lasers Surg Med. 2003; 33(3): 161-8.

67. Soukos NS, Socransky SS, Mulholland SE, Lee S, Doukas AG. Photomechanical drug delivery into bacterial biofilms. Pharm Res. 2000; 17(4): 405-9.

68. Soukos NS, Som S, Abernethy AD, Ruggiero K, Dunham J, Lee C, et al. Phototargeting oral black-pigmented bacteria. Antimicrob Agents Chemother. 2005; 49(4): 1391-6.

69. Soukos NS, Wilson M, Burns T, Speight PM. Photodynamic effects of toluidine blue on human oral keratinocytes and fibroblasts and Streptococcus sanguis evaluated in vitro. Lasers Surg Med. 1996; 18(3): 253-9.

70. Soukos NS, Ximenez-Fyvie LA, Hamblin MR, Socransky SS, Hasan T. Targeted antimicrobial photochemotherapy. Antimicrob Agents Chemother. 1998; 42(10): 2595-601.

71. Spikes JD. Phthalocyanines as photosensitizers in biological systems and for the photodynamic therapy of tumors. Photochem Photobiol. 1986; 43(6): 691-9.

72. Tagge DL, O'Leary TJ, El-Kafrawy AH. The clinical and histological response of periodontal pockets to root planing and oral hygiene. J Periodontol. 1975; 46(9): 527-33.

73. Tanaka M, Mroz P, Dai T, Huang L, Morimoto Y, Kinoshita M, et al. Photodynamic therapy can induce a protective innate immune response against murine bacterial arthritis via neutrophil accumulation. PLoS One. 2012; 7(6): e39823.

74. Topaloglu N, Gulsoy M, Yuksel S. Antimicrobial photodynamic therapy of resistant bacterial strains by indocyanine green and 809-nm diode laser. Photomed Laser Surg. 2013; 31(4): 155-62.

75. Voos AC, Kranz S, Tonndorf-Martini S, Voelpel A, Sigusch H, Staudte H, et al. Photodynamic antimicrobial effect of safranine O on an ex vivo periodontal biofilm. Lasers Surg Med. 2014; 46(3): 235-43.

76. Walker CB. The acquisition of antibiotic resistance in the periodontal microflora. Periodontol 2000. 1996; 10: 79-88.

77. Wilson M. Photolysis of oral bacteria and its potential use in the treatment of caries and periodontal disease. J Appl Bacteriol. 1993; 75(4): 299-306.

78. Wilson M, Burns T, Pratten J, Pearson GJ. Bacteria in supragingival plaque samples can be killed by low-power laser light in the presence of a photosensitizer. J Appl Bacteriol. 1995; 78(5): 569-74.

79. Wilson M, Dobson J. Lethal photosensitization of oral anaerobic bacteria. Clin Infect Dis. 1993; 16 Suppl 4: S414-5.

80. Wilson M, Dobson J, Harvey W. Sensitization of oral bacteria to killing by low-power laser radiation. Curr Microbiol. 1992; 25(2): 77-81.

81. Wilson M, Dobson J, Sarkar S. Sensitization of periodontopathogenic bacteria to killing by light from a low-power laser. Oral Microbiol Immunol. 1993; 8(3): 182-7.

82. Wilson M, Pratten J. Lethal photosensitisation of Staphylococcus aureus in vitro: effect of growth phase, serum, and pre-irradiation time. Lasers Surg Med. 1995; 16(3): 272-6.

83. Wood S, Nattress B, Kirkham J, Shore R, Brookes S, Griffiths J, et al. An in vitro study of the use of photodynamic therapy for the treatment of natural oral plaque biofilms formed in vivo. J Photochem Photobiol B. 1999; 50(1): 1-7.

APÊNDICE

Apêndice A – Material e Método

Comitê de Ética

O projeto foi encaminhado e aprovado pelo Comitê de Ética em Experimentação Animal da Faculdade de Odontologia de Araraquara – UNESP (CEUA no. 07/2012) (Anexo), dentro dos regulamentos exigidos pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA).

Amostra

Neste estudo foram utilizados 82 ratos machos adultos *(Rattus Norvegicus),* variação *albinus, Holtzman*, com peso variando entre 300-330 gramas, provenientes do Biotério da Faculdade de Odontologia de Araraquara (FOAr) – UNESP. Os animais foram mantidos no Biotério da FOAr, em caixas plásticas coletivas com 3/4 animais cada, e tratados com água e ração *ad libitum* antes e durante todo o período experimental, mantidos em ambiente com luz, umidade e temperatura controladas.

Indução da Doença Periodontal

Os animais foram anestesiados por uma combinação de Quetamina (Cloridrato de Quetamina – Francotar 3% – Virbac do Brasil Ind. e Com. Ltda.) com Xilazina (Cloridrato de Xilazina - Virbaxyl 2% - Virbac do Brasil Ind. e Com. Ltda.), na proporção 0,08 ml/100g e 0,04 ml/100g de massa corporal, respectivamente. Logo em seguida, os animais foram colocados em posição supina na mesa operatória e para ter livre acesso aos dentes posteriores da maxila, cada animal teve a boca encaixada em argolas de metal, afastando mandíbula e língua para facilitar a abertura.

Estudo 1

Em 40 animais a hemimaxila que recebeu a indução da DP, foi escolhida de forma randômica (direito ou esquerdo), como demonstra a Figura A1B .

As ligaduras foram colocadas com fios de algodão nº 24, e foram inseridos na região subgengival ao redor dos segundos molares superiores com auxílio de sonda e pinça específicas. O nó cirúrgico manteve-se voltado para a face vestibular da boca do animal. Após um período de 15 dias, as ligaduras foram removidas e um dia após,

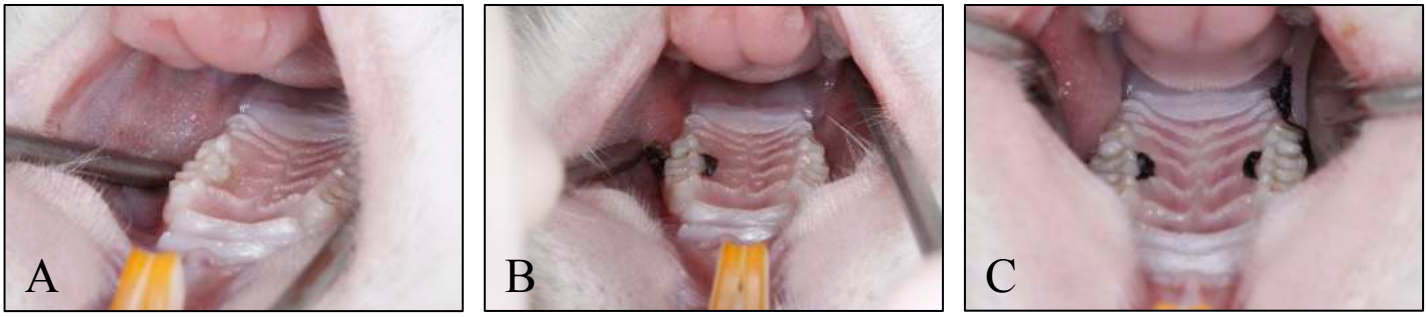
foram realizados os tratamentos em cada grupo, como mostra o desenho experimental Estudo 1.

Estudo 2

Em 42 animais, as ligaduras foram colocadas em ambas hemimaxila, como mostra a Figura A1C. A hemimaxila que recebeu o tratamento foi escolhida de forma randômica (direito ou esquerdo) e o lado oposto foi considerado o controle negativo.

As ligaduras foram inseridas igualmente como no Estudo 1. A DP foi induzida por 7 dias antes do início dos tratamentos de cada grupo/período e 0 dias foi considerado o *baseline*. As ligaduras se mantiveram em posição durante todos os períodos e foram removidas em 7 e 15 dias, como ilustrado no desenho experimental Estudo 2.

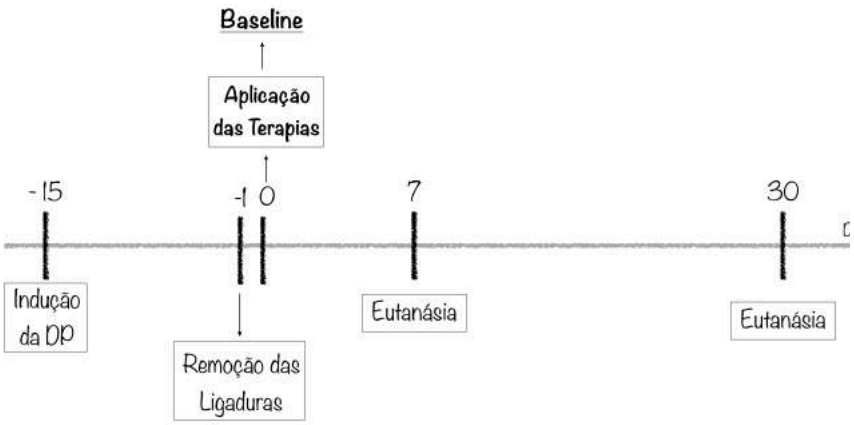
Figura A1 – Indução da DP com ligadura. Em A, maxila antes da colocação da ligadura. Em B (Estudo 1) a ligadura foi inserida somente em um lado da maxila e em C (Estudo 2) em ambos os lados.



Fonte: Elaboração própria.

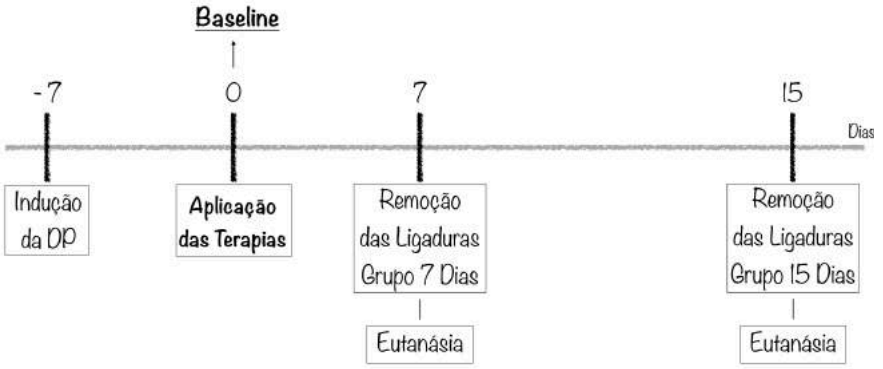
Desenho Experimental

Estudo 1



Fonte: Elaboração própria.

Estudo 2



Fonte: Elaboração própria.

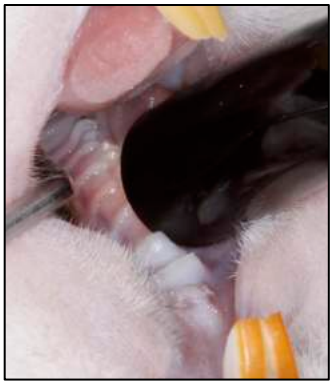
Preparo do Fotossensibilizador (FS) - aPDT

A ftalocianina de zinco tetracarboxi-N-metilglucamina foi preparada no Instituto de Química de São Carlos, Universidade de São Paulo (USP), de acordo com as especificações: foi preparada solução de estoque de zinco-tetracarboxi-ftalocianina na concentração 1,1 mg/mL em DMSO e posteriormente diluída em salina tamponada com fosfato (pH=7,2) para a concentração final de 10μg/mL, na composição líquida para o Estudo 1 e na forma farmacêutica de pomada para o Estudo 2. Logo após o preparo, o FS foi armazenado em falcons protegidos da luz com papel alumínio e estes, permaneceram em ambiente refrigerado até o momento da aplicação.

Fonte de Luz

Para o Estudo 1, a fonte de luz utilizada para ativar o FS ftalocianina de zinco tetracarboxi-N-metilglucamina correspondeu ao comprimento de onda de 655 nm, com potência de 0,45W, densidade de potência de 0.47W/cm^2 e dose de 170,52J/cm^2 (LED vermelho, diâmetro 11mm, DMC Equipamentos Ltda, São Carlos, Brasil), coincidindo com a banda de absorção máxima da ftalocianina de zinco tetracarboxi-N-metilglucamina. O LED foi colocado na face oclusal dos dentes a serem tratados (perpendicular ao longo eixo), ficando 6 minutos em contato com toda a superfície ao redor dos dentes. Para o Estudo 2, a fonte de luz correspondeu ao comprimento de onda de 655 nm, com potência de 0,45W e dose de 34J/cm^2, contabilizando 72 segundos em contato com o dente (Figura A2).

Figura A2 – Aplicação da fonte de luz (LED) na face oclusal dos dentes a serem tratados após o tempo de incubação do FS (10 minutos) no tratamento aPDT em ambos os estudos.



Fonte: Elaboração própria.

Aplicação do Fotossensibilizador

Em ambos os estudos, no momento de aplicação, a boca do animal e o FS estavam em ambiente protegidos da luz. O material, que estava acondicionado em um *falcon* envolto com lâmina de papel alumínio, foi transferido para uma seringa com uma agulha de ponta romba e foi posicionada no sulco gengival. O FS foi inserido por todas as faces do dente a ser tratado e manteve-se dentro da bolsa periodontal por 10 minutos no escuro (tempo de incubação) e logo após, a luz vermelha foi irradiada. Essa aplicação foi realizada apenas uma vez.

Especificação dos Grupos Experimentais

Estudo 1

No dia seguinte à remoção das ligaduras, os animais foram divididos aleatoriamente e tratados de acordo com os grupos (5 animais/grupo nos períodos de 7 e 30 dias):

Grupo DOENÇA PERIODONTAL (DP): somente indução da DP, não houve tratamento.

Grupo RASPAGEM E ALISAMENTO RADICULAR (RAR): os animais foram submetidos à RAR com curetas específicas (Gracey mine Five 5-6, HuFriedy).

Grupo TERAPIA FOTODINÂMICA (aPDT): os animais foram submetidos à Terapia Fotodinâmica: Aplicação do FS e em seguida irradiação com LED.

Grupo RAR+aPDT: os animais foram submetidos à RAR (igual ao tratamento do grupo RAR) em seguida, aplicação da Terapia Fotodinâmica (igual ao tratamento do grupo aPDT).

Os animais foram eutanaziados nos períodos experimentais de 7 e 30 dias após os tratamentos.

Estudo 2

Após 7 dias de indução da DP, os tratamentos foram realizados de acordo com os grupos abaixo, compostos por 7 animais/grupo nos períodos de 7 e 15 dias:

Grupo DOENÇA PERIODONTAL (DP): Os animais receberam ligaduras em ambas hemimaxilas, porém em uma delas, não recebeu tratamento.

Grupo FOTOSSENSIBILIZADOR (FS): os animais fora submetidos à aplicação do FS dentro do sulco gengival ao redor de todo o dente (10 minutos de incubação no escuro) e após esse tempo, o FS foi removido com *swabs* de algodão.

Grupo LUZ LED (LED): os animais foram submetidos à irradiação com LED vermelho (655nm; 34J/cm^2) ao redor do 2^o molar superior durante 72 segundos.

Grupo TERAPIA FOTODINÂMICA (aPDT): os animais foram submetidos à Terapia Fotodinâmica: Aplicação do FS dentro do sulco gengival (10 minutos de incubação no escuro) e em seguida, irradiação com LED vermelho (655nm; 34J/cm^2) ao redor do 2^o molar superior durante 72 segundos, e após a aPDT, o FS foi removido com *swabs* de algodão.

As ligaduras foram removidas nos determinados períodos experimentais de 7 e 15 dias e os animais eutanasiados.

Obtenção das Peças Cirúrgicas

Após os períodos correspondentes, os animais foram eutanaziados com sobredose dos anestésicos utilizados (Quetamina e Xilazina). As hemimaxilas foram removidas, imersas em paraformol 4% durante 48 horas. Após esse período, as peças foram lavadas em água corrente por 24 horas e colocadas em álcool 70%, onde permaneceram até a realização do escaneamento no microtomógrafo computadorizado para a avaliação radiográfica tridimensional.

Processamento Histológico

Após a análise de radiografia tridimensional, as peças foram colocadas em solução de EDTA (Marca: Synth 7%; pH=7,2; tamponado de fosfato de sódio monobásico e dibásico) para descalcificação por um período de 8 semanas, com 3 trocas semanais. Posteriormente foram lavadas e desidratadas em álcool, diafanizadas em xilol e incluídas em parafina. Os cortes foram realizados no sentido mésio-distal ao longo eixo do dente com 4µm de espessura, utilizando o micrótomo manual (Marca: Microm; Modelo: HM 360). Foram obtidos aproximadamente 30 cortes seriados de cada bloco, divididos em lâminas com 3 cortes cada. Duas lâminas de cada bloco com intervalos de 40 a 50µm foram coradas pela técnica de hematoxilina-eosina (HE) e usadas para a avaliação histométrica e histológica para os Estudos 1 e 2 e estereométrica para o Estudo 2.

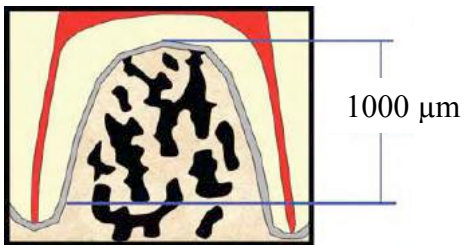
Análise Histométrica

Para realização da histometria de ambos os estudos, um examinador cego e calibrado (Correlação de Pearson, r=0.93), selecionou duas lâminas de cada grupo e examinou a região de furca e as regiões interproximais. As mensurações foram feitas utilizando um software de imagens Fiji ImageJ (National Institutes of Health, USA). Foram realizadas duas análises:

Região de Furca

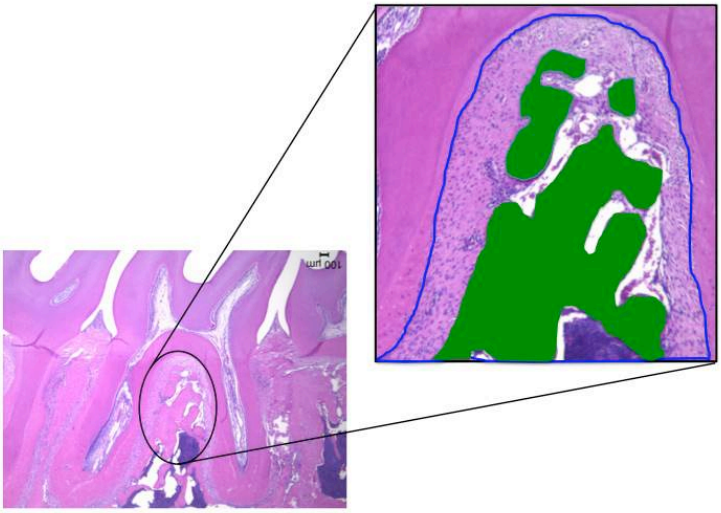
Para a área da região de furca foi considerada a metodologia de acordo com da Silva et al.[12] (2008), onde foi mensurado 1000µm a partir do teto da furca delimitado pelas raízes, e medido a área de furca e a de osso, obtendo assim, a quantidade de osso presente na região de furca de cada corte histológico, como demonstra a Figura A3 e como foi realizado na Figura A4.

Figura A3 – Metodologia mostrando a delimitação da área de furca utilizada para a análise histométrica.



Fonte: da Silva et al.[12] (2008).

Figura A4 – Fotografia de corte corado com HE, na região de furca do 2º molar superior, com aumento de 2,5x. Em destaque, a área analisada para histometria de região de furca, com aumento de 5x. Em azul, delimitação da furca e em verde, área de osso presente.



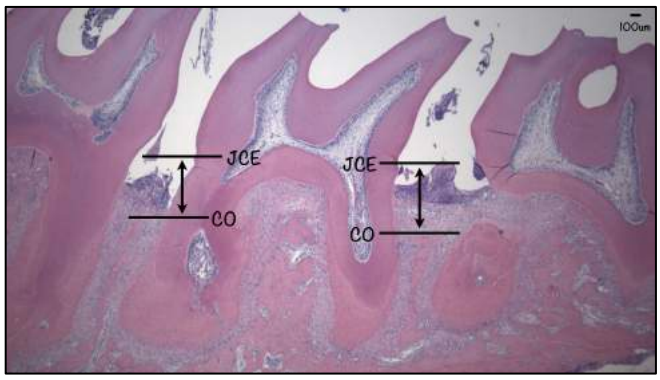
Fonte: Elaboração própria.

Regiões Interproximais

Para a avaliação das regiões interproximais (Mesial e Distal) foi averiguado a Perda Óssea Alveolar: foi medida pela distância entre a junção cemento-esmalte e o topo da crista alveolar.

A ilustração destes dois índices está na Figura A5.

Figura A5 – Fotografia de corte corado com HE, observando o segundo molar superior, as estruturas adjacentes das regiões interproximais e os 3 pontos utilizados nesta análise, com aumento de 2,5x.



Fonte: Elaboração própria.

As medidas da região de furca foram obtidas em porcentagem e da perda óssea alveolar em micrômetros.

Análise Radiográfica Tridimensional (µCT)

As hemimaxilas foram escaneadas através de uma varredura de feixe de raios-X em um sistema de microtomografia computadorizada (Skyscan 1176, Aatselaar, Belgium, 2003). Os parâmetros utilizados para este procedimento foram: filtro de Al 0.5mm; tamanho do voxel: 17,48µm; voltagem de 50KV e corrente elétrica de 500µA. Utilizando o software específico (NRecon 1.6.1.5 – SkyScan, Belgium, 2003), as peças foram reconstruídas e com auxílio do software bidimensional (DataViewer 1.4.3.1 – SkyScan, Belgium, 2003) as imagens foram rotacionadas e reposicionadas em uma orientação padrão, para melhor visualização. A cada 28 *scan* (varredura) foi reconstruído uma matriz de 18x18x18µm e as imagens 3D foram geradas para cada amostra e, após esta etapa foi realizada a análise volum.

Estudo 1

Nesta análise, foi estabelecido um limite de contraste (Threshold variando de 59 a 255) para distinguir tecidos mineralizados utilizando os softwares CTan/CTvol (Skyscan 1176, Aatselaar, Belgium, 2003). A região de interesse (ROI) foi posicionada medindo-se 3 regiões no 2º molar superior: região de furca com uma área de 1,26x1,15mm^2, e as proximais (mesial e distal) com uma área 1,26x0,56mm^2 a partir da junção cemento-esmalte (Figura A6). Os dados foram obtidos em porcentagem de volume de tecido ósseo de cada região.

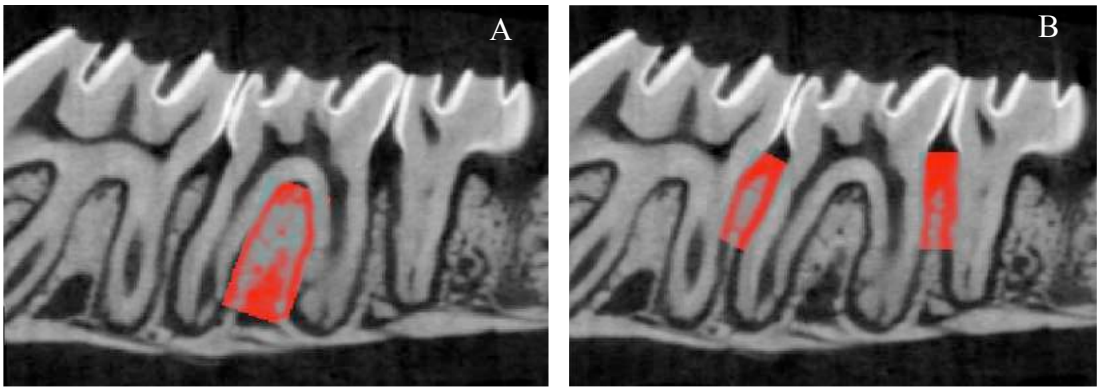
Estudo 2

Para esta análise, utilizou-se o software específico (ITK-SNAP 3.6.0 – Pennsylvania, USA), o examinador desenhou uma área de interesse tridimensional (ROI – Region Of Interest) conduzido pela morfometria da peça.

Para maximizar a quantificação de osso e minimizar a inclusão de dentes e raízes, o ROI foi delimitado no sentido mesio-distal a partir da raiz distal do primeiro molar superior até a raiz mesial do terceiro molar superior. E no sentido cervico-apical, a partir do teto da furca do segundo molar até o término do osso cortical, confiando assim que toda a área óssea na região de furca e interproximais do segundo molar foram

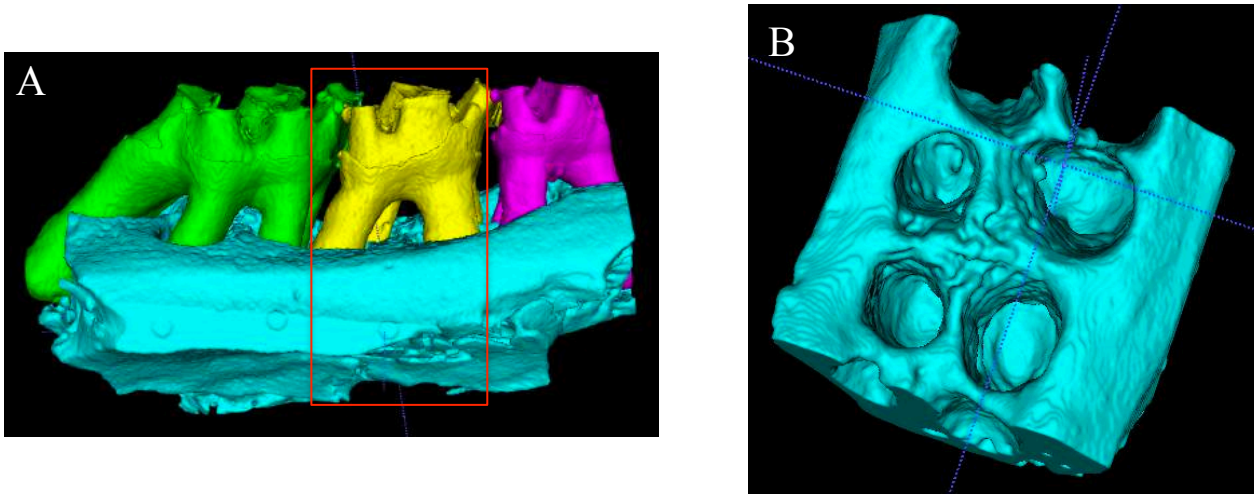
englobadas pelo ROI desenhado, como demonstra a Figura A7. Após a delimitação do ROI, uma imagem binária foi gerada e para cada peça e foi estabelecido um threshold único analisando a similaridade tecidual. Os valores obtidos nesta análise foram dados em milímetros cúbicos (mm^3). As amostras foram processadas e analisadas por um examinador cego e calibrado no laboratório de Microscopia in vivo da Faculdade de Odontologia de Araraquara – UNESP.

Figura A6 – Imagens obtidas no microCt da região de 2º molar superior, onde foram obtidos os ROIs. A: Região de furca com uma área e 1,26x1,15mm^2 e B: proximais (mesial e distal) com uma área 1,26x0,56mm^2 a partir da junção cemento-esmalte.



Fonte: Elaboração própria.

Figura A7 – Em A, fotografia do modelo em 3D confeccionado a partir do escaneamento das peças no μ-CT, mostrando em azul, a maxila; em verde, o primeiro molar superior; em amarelo, segundo molar superior e em rosa, terceiro molar superior. Em B, está a área delimitada pelo ROI e que foi analisada.

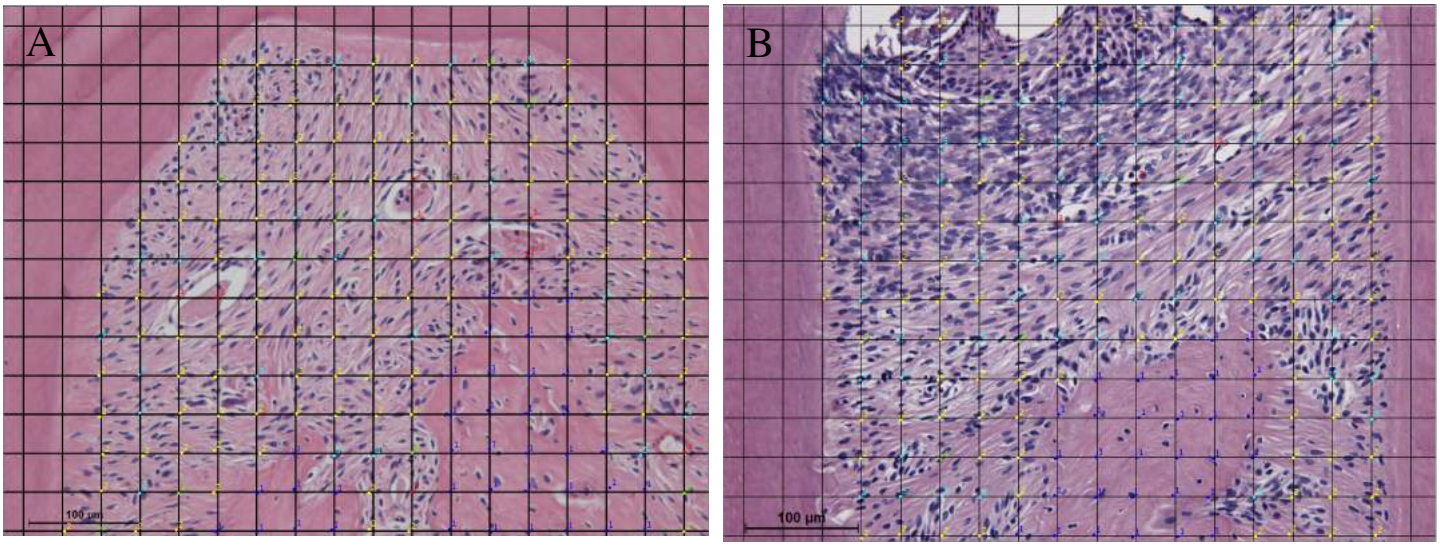


Fonte: Elaboração própria.

Análise Estereométrica

Esta análise foi realizada para o Estudo 2 com o auxílio de um microscópio de luz Leica DMLS (Leica - Reichert Diastar Products & Jung, Wetzlar, Germany) para selecionar 2 cortes por dente, com intervalos variando de 40 a 50µm entre si e uma câmera digital Leica DFC 300 FX (Leica - Reichert Diastar Products & Jung, Wetzlar, Germany) para capturar as imagens da região de furca e interproximais, na magnificação de 200x. Em plano bidimensional, uma grade de 75x75 pixels de área e 252 pontos de intersecção, foi posicionada nas regiões de interesse (tecido conjuntivo supra-alveolar no teto da furca e papilas, na mesial e distal), utilizando o software de imagens Fiji ImageJ (National Institutes of Health, USA) e então foi realizada a técnica de contagem de pontos para analisar a proporção de constituintes teciduais (tecido ósseo, matriz extracelular, vasos sanguíneos, fibroblastos, células inflamatórias) nos ângulos da grade, permitindo assim, uma avaliação quantitativa da inflamação presente ao redor da agressão, como ilustra a Figura A8. Em seguida, para representação gráfica da estereometria, realizou-se uma mensuração percentual de cada constituinte tecidual correspondente ao número total de pontos, baseados nos trabalhos de Liu et al.[34] (2006) e de Odze et al.[48] (1996).

Figura A8 – Imagens histológicas representativas da metodologia utilizada para a análise estereométrica de contagem de constituintes teciduais. Observar posicionamento supra-ósseo da grade na região de furca em A e interproximal em B. H/E. Aumento 100x.



Fonte: Elaboração própria.

Análise Histológica

Após o processamento histológico, as peças foram cortadas em micrótomo e coradas com HE. Utilizando-se um microscópio óptico DIASTAR (Leica Reichert & Jung products, Germany) com objetiva para aumento de 4.0/10 vezes e oculares com aumento de 10 vezes, as imagens foram capturadas e enviadas para um microcomputador, com o auxílio de uma câmera de vídeo DXC-1107A/107AP (Sony Eletronics Inc, Japão). Na análise histológica, foram avaliadas as reações inflamatórias do tecido conjuntivo em cada grupo experimental, processos de reabsorção óssea e neoformação tecidual.

Delineamento Estatístico

Os dados experimentais foram tabulados utilizando o programa Microsoft Excel para Mac 2011 (Apple Inc, USA) e analisados estatisticamente com o auxílio do programa GraphPad Prism 6.0 (GraphPad Inc, USA).

Os dados foram avaliados em relação ao teorema do ponto central, para verificar se a disposição dos mesmos respeitava a distribuição normal. Para tal, foi o utilizado o teste de Shapiro-Wilk com intervalo de confiança de 95%. Como todos os dados apresentaram-se normais, aplicou-se o teste paramétrico ANOVA (One Way) para analisar a existência de diferenças estatísticas entre os grupos. A complementação da análise foi realizada com o teste de Tukey com a finalidade de detectar entre quais grupos ocorreram às diferenças. Para comparação entre os períodos, foi utilizado o teste paramétrico ANOVA (OneWay with repetead measures). Os testes foram aplicados com 95% de intervalo de confiança.

ANEXO

Anexo A – Certificado do Comitê de Ética

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araraquara
FACULDADE DE ODONTOLOGIA

Proc. CEUA nº 07/2012

Araraquara, 13 de setembro de 2017.

Senhor Pesquisador:

A Comissão de Ética no Uso de Animal - CEUA desta Faculdade, procedeu a análise do Relatório Parcial do projeto de pesquisa de sua responsabilidade intitulado ***"AVALIAÇÃO DOS EFEITOS DA TERAPIA FOTODINÂMICA COM FTALOCIANINA E HIPERICINA-GLUCAMINA NO TRATAMENTO DA DOENÇA PERIODONTAL. ESTUDO EM RATOS"*** (Proc. CEUA nº 07/2012), e considerou-o APROVADO, bem como sua solicitação de prorrogação no prazo da pesquisa.

Lembramos que o Relatório Final deste projeto deverá ser entregue em **DEZEMBRO/2019.**

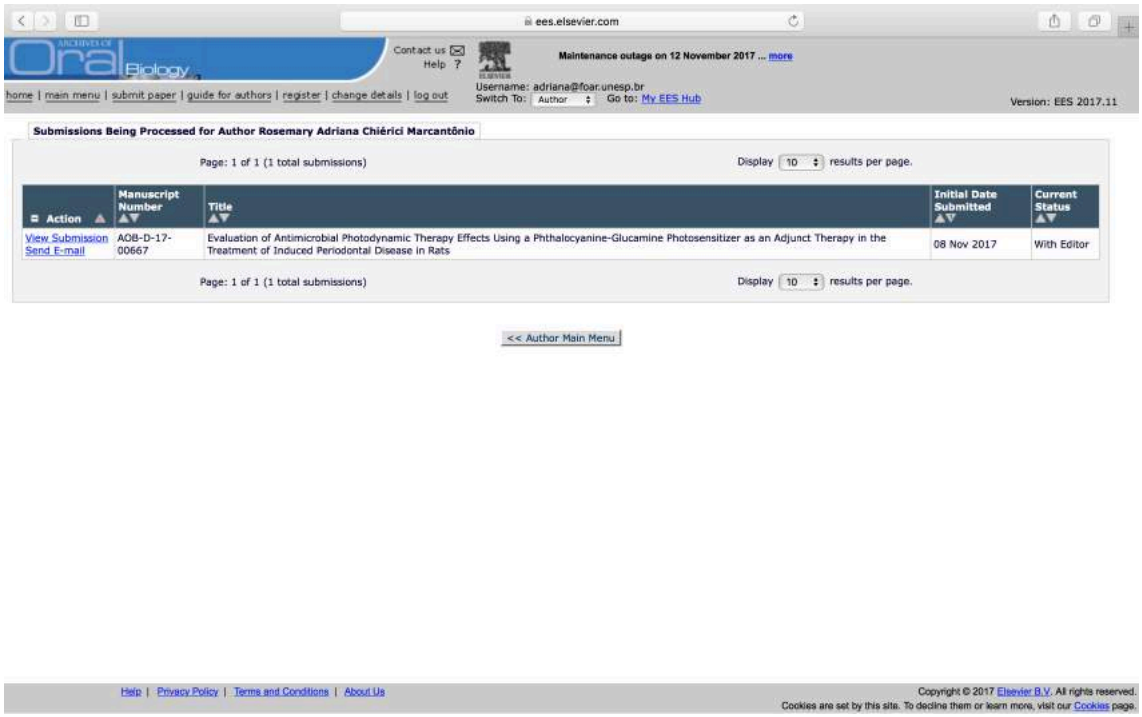
Atenciosamente.

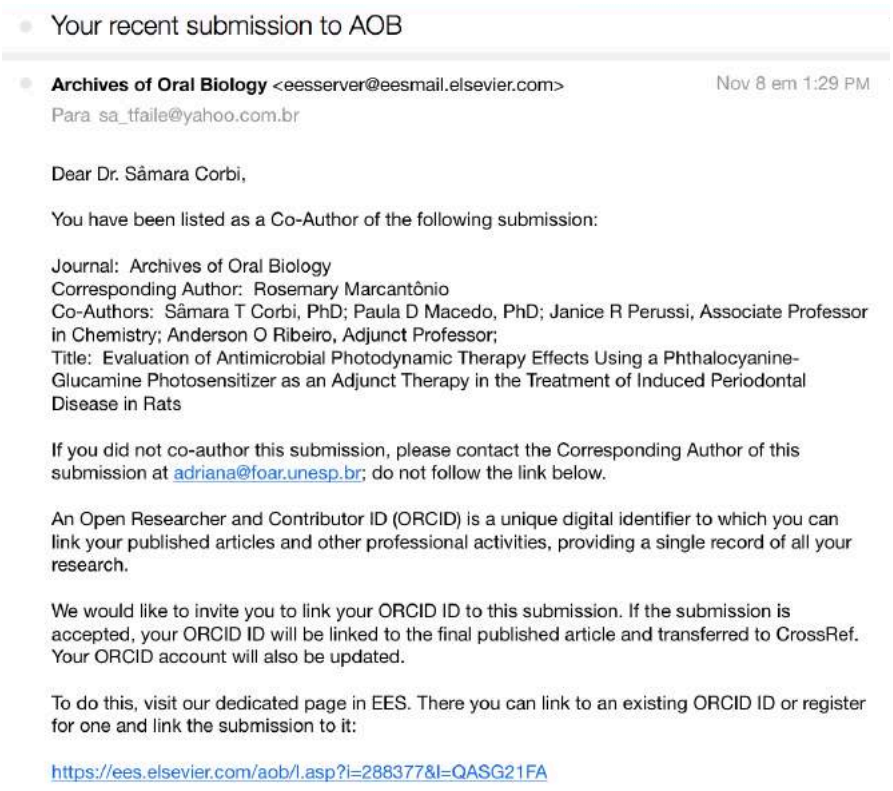
Prof. Dr. PAULO SÉRGIO CERRI
Vice-Coordenador da CEUA

À
Profa. Dra. ROSEMARY ADRIANA CHIERICI MARCANTONIO
DD. Pesquisador Responsável
Departamento de Diagnóstico e Cirurgia

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) 3301-6432

Anexo B – Documentos Comprobatórios





Your recent submission to AOB

Archives of Oral Biology <eesserver@eesmail.elsevier.com> Nov 8 em 1:29 PM

Para sa_tfaile@yahoo.com.br

Dear Dr. Sâmara Corbi,

You have been listed as a Co-Author of the following submission:

Journal: Archives of Oral Biology
Corresponding Author: Rosemary Marcantônio
Co-Authors: Sâmara T Corbi, PhD; Paula D Macedo, PhD; Janice R Perussi, Associate Professor in Chemistry; Anderson O Ribeiro, Adjunct Professor;
Title: Evaluation of Antimicrobial Photodynamic Therapy Effects Using a Phthalocyanine-Glucamine Photosensitizer as an Adjunct Therapy in the Treatment of Induced Periodontal Disease in Rats

If you did not co-author this submission, please contact the Corresponding Author of this submission at adriana@foar.unesp.br; do not follow the link below.

An Open Researcher and Contributor ID (ORCID) is a unique digital identifier to which you can link your published articles and other professional activities, providing a single record of all your research.

We would like to invite you to link your ORCID ID to this submission. If the submission is accepted, your ORCID ID will be linked to the final published article and transferred to CrossRef. Your ORCID account will also be updated.

To do this, visit our dedicated page in EES. There you can link to an existing ORCID ID or register for one and link the submission to it:

https://ees.elsevier.com/aob/l.asp?i=288377&l=QASG21FA

ARARAQUARA, 07 DEZEMBRO DE 2017

SÂMARA CRUZ TFAILE CORBI