UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS ODONTOLÓGICAS

Victor de Mello Palma

INFLUÊNCIA DA TERAPIA FOTODINÂMICA ANTIMICROBIANA SOBRE O COLÁGENO E O STATUS OXIDATIVO GENGIVAL EM MODELO EXPERIMENTAL DE PERIODONTITE EM RATOS

Santa Maria, RS
2019

Victor de Mello Palma

INFLUÊNCIA DA TERAPIA FOTODINÂMICA ANTIMICROBIANA SOBRE O COLÁGENO E O STATUS OXIDATIVO GENGIVAL EM MODELO EXPERIMENTAL DE PERIODONTITE EM RATOS

Dissertação apresentada ao Curso de Mestrado do Programa de Pós Graduação em Ciências Odontológicas, Área de Concentração em Odontologia, Ênfase em Patologia Bucal, da Universidade Federal de Santa Maria (UFSM-RS), como requisito parcial para obtenção do título de **Mestre em Ciências Odontológicas.**

Orientadora: Profª Drª Cristiane Cademartori Danesi

Coorientadora: Profª Drª Kívia Linhares Ferrazzo

Santa Maria, RS

2019

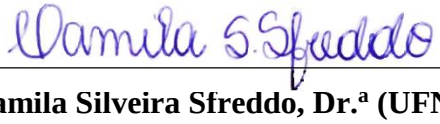
Victor de Mello Palma

INFLUÊNCIA DA TERAPIA FOTODINÂMICA ANTIMICROBIANA SOBRE O COLÁGENO E O STATUS OXIDATIVO GENGIVAL EM MODELO EXPERIMENTAL DE PERIODONTITE EM RATOS

Dissertação apresentada ao Curso de Mestrado do Programa de Pós Graduação em Ciências Odontológicas, Área de Concentração em Odontologia, Ênfase em Patologia Bucal, da Universidade Federal de Santa Maria (UFSM-RS), como requisito parcial para obtenção do título de **Mestre em Ciências Odontológicas.**

Aprovado em 28 de Junho de 2019:

Cristiane Cademartori Danesi, Dr.ª (UFSM-RS)
(Presidente/Orientadora)



Camila Silveira Sfreddo, Dr.ª (UFN-RS)

Raquel Cristine Silva Barcelos, Dr ª. (UFSM-RS)

Santa Maria, RS
2019

DEDICATÓRIA

Dedico este trabalho à minha família, por sua capacidade de acreditar e investir em mim, a presença de vocês significou segurança e certeza de que não estou sozinho nessa caminhada.

AGRADECIMENTOS

A **Deus**, pelo que sou e pelas pessoas que colocou no meu caminho.

À minha família, **Hilton, Marine e Bruna** pelo amor incondicional, oferecendo subsídios para meu crescimento profissional e pessoal. São meus exemplos de vida.

Ao **Marciano** pelo companheirismo, apoio incondicional e incentivo, enfim obrigado por se fazer presente em minha vida.

À minha orientadora, Prof.ª Dr.ª **Cristiane Cademartori Danesi**, pela oportunidade de trabalhar com ela, por ter me direcionado em todos os pensamentos, por se mostrar uma excelente professora e uma ótima pessoa, tens toda a minha admiração.

À minha coorientadora, Prof.ª Dr.ª **Kívia Linhares Ferrazzo** pelos ensinamentos durante minha caminhada, pela ajuda em todos os trabalhos que eu realizei e por ser uma professora acessível e de conhecimento admirável.

À Prof.ª Dr.ª **Karla Kantorski** e ao médico veterinário, Prof. Dr. **Roberto Marinho** pela exaustiva contribuição durante a elaboração e execução deste projeto.

À Dr.ª **Raquel Cristine Silva Barcelos**, por ser esta pessoa iluminada, por estar sempre disponível a me ajudar, pela paciência ao ensinar e me fazer entender tudo o que eu lhe perguntava, pelo carinho e pela amizade sincera. Meu muito obrigado e que nossa amizade nunca se perca.

À Prof.ª Dr.ª **Camila Silveira Sfreddo,** por aceitar fazer parte da banca examinadora desta dissertação dividindo todo o seu conhecimento e por ter sido uma colega de trabalho muito especial e motivadora. Muito Obrigado.

Às minhas colegas e amigas Prof.ª Dr.ª **Luisa Machado Barin** e Me. **Fernanda Maia Pillusky**, pela paciência ao ensinar, pelo carinho e amizade no decorrer dessa minha caminhada. Muito obrigado por tudo.

Às minhas amigas e colegas, **Eva**, **Danielle, Jeanni, Jéssica, Luisa Berlato e Mônica**, obrigado por me aguentarem nos momentos de desabafo e estresse, vocês são incríveis, ter amizade de vocês me faz mais feliz.

Aos **animais utilizados neste estudo**, todo meu respeito.

À coordenação de aperfeiçoamento de pessoal de nível superior CAPES pelo apoio financeiro durante meu estudo.

À **Universidade Federal de Santa Maria**, por disponibilizar cursos gratuitos e de excelência, garantindo acesso universal ao conhecimento.

Enfim, a todos que de alguma forma contribuíram e não foram citados, mas nem por isso esquecidos toda minha gratidão.

“A ciência não pode prever o que vai acontecer. Só pode prever a probabilidade de algo acontecer.”

(César Lattes)

RESUMO

INFLUÊNCIA DA TERAPIA FOTODINÂMICA ANTIMICROBIANA SOBRE O COLÁGENO E O STATUS OXIDATIVO GENGIVAL EM MODELO EXPERIMENTAL DE PERIODONTITE EM RATOS

AUTOR: Victor de Mello Palma
ORIENTADORA: Cristiane Cademartori Danesi
COORIENTADORA: Kívia Linhares Ferrazzo

O objetivo deste estudo foi avaliar a influência da terapia fotodinâmica antimicrobiana (TFDa) com azul de metileno (AM) dissolvido em etanol utilizado como adjuvante à raspagem e alisamento radicular (RAR), para o tratamento da periodontite nas fibras colágenas e dano oxidativo gengival em ratos. Setenta ratos machos Wistar foram distribuídos aleatoriamente em cinco grupos experimentais de acordo com o tratamento periodontal: CN (controle negativo; sem periodontite. n=11); CP (controle positivo; periodontite sem qualquer tratamento. n=11); RAR (periodontite e raspagem e alisamento radicular. n=16), TFDa I (periodontite e RAR + TFDa + AM solubilizados em água. n=16) e TFDa II (periodontite e RAR + TFDa + AM solubilizados em etanol a 20%. n=16). A periodontite foi induzida pela colocação da ligadura ao redor do primeiro molar inferior direito de todos os grupos experimentais, exceto do grupo CN. Os tratamentos periodontais foram realizados após 7 dias de remoção da ligadura. Aos 7 e 15 dias após a remoção da ligadura, os animais foram eutanaseados e as amostras de mandíbula e gengiva foram coletadas para avaliações histológicas e bioquímicas. Os grupos CP e RAR apresentaram maiores níveis de lipoperoxidação gengival aos 7 dias, mas apenas o grupo CP apresentou maior lipoperoxidação gengival no 15º dia comparado ao grupo CN. Além disso, o grupo TFDa II mostrou uma ação protetora na gengiva já aos 7 dias após a remoção da ligadura observada pela manutenção dos níveis de glutationa reduzida (GSH), que foi semelhante ao grupo CN. A TFDa foi capaz de prevenir a degradação do colágeno gengival já no 7º dia. A área de colágeno foi negativamente correlacionada com os níveis de lipoperoxidação na gengiva em todos os grupos. A solubilização do AM em etanol 20% evitou a peroxidação lipídica e a degradação do colágeno a curto prazo na periodontite induzida. A TFDa pode atuar como uma ferramenta adjuvante benéfica para minimizar a degradação do colágeno e os danos oxidativos induzidos pela periodontite. Hipótese, considerando que a periodontite e o consequente estado oxidativo na gengiva foram modificados pela TFDa, é possível que essas alterações fisiológicas estejam relacionadas à hormese.

Palavras-chave: Colágeno tipo I. Colágeno tipo III. Doença Periodontal. Estresse Oxidativo. Gengiva. Hormese. Raspagem e alisamento radicular. Fototerapia.

ABSTRACT

INFLUENCE OF ANTIMICROBIAL PHOTODYNAMIC THERAPY ON COLLAGEN AND GENGIVAL OXIDATIVE STATUS IN EXPERIMENTAL MODEL OF PERIODONTITE IN RATS

AUTHOR: Victor de Mello Palma
ADVISOR: Cristiane Cademartori Danesi
CO-ADVISER: Kívia Linhares Ferrazzo

The objective of this study was to evaluate the influence of antimicrobial photodynamic therapy (PDT) with methylene blue (MB) dissolved in 20% ethanol, used as an adjunct to scaling and root planing (SRP), for the treatment of periodontitis in bone loss, collagen fibers and gingival oxidative damage in rats. Seventy male Wistar rats were randomly assigned to five experimental groups according to periodontal treatment: NC (negative control, no periodontitis. n=11); PC (positive control; periodontitis without any treatment. n=11); SRP (Periodontitis and scaling and root planning. n=16), aPDT I (periodontitis and SRP + aPDT + MB solubilized in water. n=16) and aPDT II (periodontitis and SRP + aPDT + MB solubilized in 20% ethanol. n=16). Periodontitis was induced by placing the ligature around the right first molar of all experimental groups, except the NC group. Periodontal treatments were performed after 7 days of ligation removal. At 7 and 15 days after ligature removal, the animals were euthanized and the mandible and gingival samples were collected for histological and biochemical evaluation. The PC and SRP groups presented higher levels of gingival lipoperoxidation at 7 days, but only the PC group presented higher gingival lipoperoxidation on the 15th day compared to the NC group. In addition, the aPDT II group showed a protective action on the gingiva as early as 7 days after the removal of the ligature observed by the maintenance of reduced glutathione levels (GSH), which was similar to the NC group. aPDT was able to prevent the degradation of gingival collagen as early as day 7. The collagen area was negatively correlated with the levels of lipoperoxidation in the gingiva in all groups. The solubilization of the AM in water/ethanol solution prevented the lipid peroxidation and collagen degradation periodontitis-induced in the evaluated times in a short-term. Hypothesi, considering that the periodontitis and the consequent oxidative status in gingiva were modified by aPDT, it is possible that these physiological changes are related hormesis.

Keywords: Type I collagen. Type III collagen. Periodontal disease. Oxidative stress. Gum. Hormesis. Scaling and root planing. Phototherapy.

.

LISTA DE ILUSTRAÇÕES

ARTIGO

LISTA DE TABELAS

ARTIGO

LISTA DE ABREVEATURAS, SIGLAS E SÍMBOLOS

%	Por cento
°C	Grau Celsius
®	Marca Registrada
AM	Azul de Metileno
CAT	Catalase; “Catalase”
cm2	Centímetro quadrado
DP	Doença Periodontal
dL	Decilitro
DNA	Ácido Desoxirribonucleico; “Deoxyribonucleic Acid”
EO	Estresse Oxidativo
EROs	Espécies Reativas de Oxigênio
EV	Endovenoso
Fs	Fotossensibilizador
g	Grama
GPx	Glutationa Peroxidase; “Glutathione Peroxidase”
GSH	Glutationa Reduzida; “Glutathione”
HE	Hematoxilina/Eosina
H2O	Água
H2O2	Peróxido de Hidrogênio
InGaAlP	Índio, Gálio, Alumínio e Fósforo
IL-1	Interleucina-1
IP	Intraperitoneal
J	Joule
J/cm2	Joule por centímetro quadrado
J/ponto	Joules por ponto
Kg	Quilograma
Laser	Light amplification by stimulated emission of radiation
M	Molar (mol.L-1)
MDA	Malondialdeído; “Malondialdehyde”
mg	Miligrama
mL	Mililitro

mm	Milímetro
mm2	Milímetro quadrado
mol	Quantidade de Substância
mW	MegaWatt
Na	Sódio
nm	Nanômetro
O2	Oxigênio
O2–•	Ânion Superóxido
OH•	Radical Hidroxila
OMS	Organização Mundial da Saúde
OPG	Osteoprotegerina; “Osteoprotegerin”
PMN	Polimorfonucleares
pH	Potencial Hidrogeniônico
RANK	Receptor Ativador do Fator Nuclear kappa B
RANKL	Ligante do Receptor Ativador do Fator Nuclear kappa
RAR	Raspagem e Alisamento Radicular
RL	Radicais Livres
SOD	Superóxido Dismutase; “Superoxide Dismutase”
SSPS	Statistical Package for Social Sciences
STZ	Estreptozotocina; “Streptozotocin”
TBA	Ácido Tiobarbitúrico; “Thiobarbituric Acid”
TBARS	Thiobarbituric Acid Reactive Substances
TFDa	Terapia Fotodinâmica Antimicrobiana
TNF-alfa	Fator de Necrose Tumoral Alfa; “Tumor Necrosis Factor alpha”
UFSM	Universidade Federal de Santa Maria
VIT C	Vitamina C
µm	Micrômetro
µs	Microssegundo

SUMÁRIO

1 INTRODUÇÃO

A periodontite é a infecção oral mais comum em humanos e tem sido a principal causa de perda dentária em adultos (HYVÄRINEN et al., 2016). A periodontite é uma doença imuno-inflamatória crônica (CHANG et al., 2013), multifatorial (LÖE et al., 1965; KIKANE; BARTOLD, 2010) de alta prevalência nas populações, tanto em países desenvolvidos (ALBANDAR et al., 1999), como em países em desenvolvimento (SUSIN et al., 2004). Resulta de um processo de inter-relação entre a placa bacteriana e os tecidos periodontais, culminando em eventos celulares e vasculares. No entanto, existem outros fatores que modificam a instalação e progressão da doença que podem ser relacionados ao hospedeiro ou ao ambiente (BRUNETTI, 2004; ANGELIS, 2011). A resposta do hospedeiro frente ao desafio bacteriano persistente resulta na liberação de mediadores biológicos como as citocinas prostaglandina E2 (PGE2), interleucinas 1 (IL-1) e (IL-6), fator de necrose tumoral alfa (do inglês tumor necrosis factor (TNF-α)) e metaloproteinases de matriz (MMPs). Esses mediadores levam a destruição do periodonto por meio da destruição extracelular da matriz e estimulação da reabsorção óssea (SCHENKEN, 2006).

A reabsorção óssea alveolar é um mecanismo de proteção do organismo. Conforme a reação inflamatória se aproxima do osso alveolar, a reabsorção é iniciada de modo a evitar a invasão bacteriana, mantendo uma distância da periferia do infiltrado inflamatório (COCHRAN,2008). A reabsorção óssea ocorre pela remoção controlada de componentes orgânicos e inorgânicos mediante células específicas, chamadas osteoclastos. Porém, nas doenças periodontais ocorre um desequilíbrio nesse papel regulatório ocasionando a reabsorção óssea que é uma consequência importante, uma vez que o osso degradado não será regenerado (KINANE et al., 2010).

Além do tecido ósseo, a gengiva merece grande atenção por ser a precursora do processo inflamatório e reproduzir os eventos associados ao avanço da DP (BARTOLD; WALSH; NARAYANAN, 2000). O tecido gengival é constituído basicamente por colágeno (EJEIL et al., 2003) dos tipos I e III (LALLIER et al., 2007), que são fundamentais na manutenção da sua estrutura e função (REN et al., 2005) e exercem um papel importante na progressão das doenças periodontais (LORENCINI et al., 2009; SÉGUIER; GODEAU; BROUSSE, 2000). O principal componente estrutural e funcional da matriz colagenosa do tecido conjuntivo gengival é o colágeno tipo I (KESLER et al., 2000), caracterizado pela intensa birrefringência à luz polarizada do amarelo ao vermelho quando corado com Picrosirius red (MONTES; JUNQUEIRA, 1991). O colágeno tipo III é o segundo principal tipo de colágeno do tecido

conjuntivo gengival (NARAYANAN; PAGE; MEYERS, 1980), garante a integridade à distensibilidade tecidual (CHAVRIER et al.,1984; MINOR, 1980) e apresenta fraca birrefringência à luz polarizada, portanto é visualizado na cor verde quando corado com Picrosirius red (MONTES; JUNQUEIRA, 1991). Para quantificar a área ocupada por fibras colágenas no tecido conjuntivo geralmente é utilizada a coloração histológica Tricrômio de Masson que proporciona uma coloração azul a tais fibras. Além disso, essa coloração permite a clara diferenciação entre as fibras colágenas e os demais componentes dos tecidos, como células, queratina, fibras elásticas e musculares (MASSON, 1929).

Na periodontite, contra o biofilme bacteriano os neutrófilos liberam espécies reativas de oxigênio (EROs) como um dos mecanismos de defesa (AKALIN et al., 2008), levando ao aumento dos seus níveis teciduais e sistêmicos (MOSELEY; WADDINGTON; EMBERY,1997). Apesar das EROs serem essenciais para o metabolismo celular normal, em altos níveis elas podem provocar danos aos tecidos (CHAPPLE; MATTHEWS, 2007; WADDINGTON; MOSELEY; EMBERY, 2000). Portanto, as EROs desempenham um duplo papel na patogênese da periodontite: protetora e destrutiva e, além disso, há fortes evidências que correlacionam as EROs à destruição patológica do tecido conjuntivo durante a periodontite (SAKALLIOGLU et al., 2005) e que o estabelecimento e progressão da periodontite envolve a quebra do colágeno (THOMADAKI et al., 2013).

Para combater os efeitos nocivos das EROS, o organismo possui um sistema de defesa antioxidante (AKALIN et al., 2008) que pode ser classificado em enzimático (superóxido dismutase, catalase, glutationa redutase e glutationa peroxidase) e não enzimático (ácido úrico, vitamina C, vitamina E e glutationa reduzida (GSH), entre outros (SCHAFER; BUETTNER, 2001). Quando a produção das EROs ultrapassa a capacidade antioxidante tecidual, instala-se o quadro de estresse oxidativo (EO) (CHAPPLE, 1997; CHAPPLE; MATTHEWS, 2007). A degradação tecidual mediada pelas EROs durante o EO pode ser mensurada pelos níveis do produto final da peroxidação lipídica, como o malondialdeído (MDA) (TSAI et al., 2005; KHALILI; BILOKLYTSKA, 2008). Estudos recentes apontam uma redução na capacidade antioxidante e um aumento de biomarcadores de EO na periodontite (AKALIN et al., 2007; BALTACIOĞLU et al. 2006; MASHAYEKHI et al., 2005; MATTHEWS et al., 2007; TSAI et al., 2005). Além disso, o EO estimula progressivamente a destruição da estrutura periodontal, osso alveolar e tecido conjuntivo (BULLON et al., 2011; LI; GULBINS; ZHANG, 2012; PETTI; SCULLY, 2009), evidenciando uma forte relação dos aspectos bioquímicos com as alterações teciduais (DALAI et al., 2013).

O recurso terapêutico universalmente aceito para o tratamento da periodontite consiste na remoção mecânica de um dos seus fatores etiológicos que é o biofilme bacteriano (BARTOLD; VAN DYKE, 2013). Portanto, o procedimento de raspagem e alisamento radicular (RAR) é considerado o tratamento padrão ouro para a periodontite (COBB, 2002). Tal intervenção se baseia na remoção mecânica de colônias patogênicas e de depósitos mineralizados da superfície dos dentes, culminando na resolução do processo inflamatório e cicatrização tecidual (AXELSSON et al., 2004). Entretanto, a efetividade do tratamento periodontal mecânico pode ser limitada quando a periodontite está correlacionada a fatores de risco que modificam a resposta do hospedeiro como o Diabetes Mellitus (TERVONEN; OLIVER, 1993) e o tabagismo (BERGSTRÖM; ELIASSON; DOCK, 2000) ou por características anatômicas próprias dos dentes, como concavidades, fissuras e áreas de furca que prejudicam a efetiva descontaminação das superfícies (TAKASAKI et al., 2009; ADRIAENS et al., 1988). O reparo insuficiente e o consequente agravo da doença é resultante da carga bacteriana remanescente deste processo (ALWAELI; AL-KHATEEB; AL-SADI, 2013). Para estas situações é proposto o uso de antibióticos como terapia adjuvante (SLOTS, 2004), porém, quando administrados sistemicamente, podem apresentar repercussões também sistêmicas e o seu uso prolongado e indiscriminado leva à resistência bacteriana, sendo assim, a utilização da antibioticoterapia local seria uma alternativa (CASSELL; MEKALANOS, 2001; HAMBLIN; HASAN, 2004).

Estudos experimentais recentes tem demonstrado que a terapia fotodinâmica antimicrobiana (TFDa) vem sendo utilizada como adjuvante ao tratamento periodontal padrão ouro de forma promissora (BARIN et al., 2017; PILLUSKY et al., 2017; GARCIA et al., 2013). A TFDa é definida como uma reação fotoquímica, oxigênio-dependente, na qual a ativação de um corante, denominado de fotossensibilizador, por uma luz de baixa potência e de comprimento de onda específico, promove a geração de EROs, principalmente oxigênio singleto, que atuam danificando e induzindo a morte das células microbianas (ROLIM et al., 2012; GOODSON, 2011). No decorrer da fotoativação, a molécula do fotossensibilizador absorve energia passando do seu estado fundamental para o estado singleto excitado. Dessa forma, o fotossensibilizador pode perder energia como fluorescência ou calor, voltando ao seu estado fundamental ou pode passar ao estado tripleto excitado, menos energético que o estado singleto. O fotossensibilizador no estado tripleto pode sofrer dois tipos de reações: reação do tipo I e do tipo II. Na reação tipo I, o fotossensibilizador reage diretamente com um substrato (bactérias), produzindo EROs. Na reação tipo II, o fotossensibilizador reage com oxigênio molecular formando oxigênio singleto (CASTANO; DEMIDOVA; HAMBLIN, 2004).

Os produtos gerados a partir das reações tipo I e II são citotóxicos e o oxigênio singleto tem sido considerado o principal responsável pelo efeito antimicrobiano da TFDa (GEORGE; KISHEN, 2007). As reações tipo I e tipo II podem ocorrer conjuntamente e a razão entre elas depende tanto da concentração de substrato e de oxigênio, quanto do tipo de fotossensibilizador utilizado (CASTANO; DEMIDOVA; HAMBLIN, 2004) (Figura1).

Figura 1. Mecanismo de ação da TFDa; reações Tipo I e Tipo II. (Adaptado de Soukos; Goodson, 2011).

Alguns estudos vêm sendo realizados visando a avaliação da TFDa como adjuvante a RAR, utilizando como fotossensibilizador o azul de metileno dissolvido em água (CHRISTODOULIDES et al., 2008; MEISEL; KOCHER, 2005). Porém, evidências mostram que essa formulação do fotossensibilizador resulta em limitada produção associada com curta meia vida (4μs) de oxigênio singleto (MEISEL; KOCHER, 2005), além de baixo potencial de difusão tecidual (OCHSNER, 1997). Tais propriedades do fotossensibilizador podem ser influenciadas pelo solvente em que fotossensibilizador é dissolvido. Estudos recentes apontam melhores propriedades fotofísicas, fotoquímicas e fotobiológicas do azul de metileno dissolvido em solvente menos polar que a água, como o etanol, aumentando a penetrabilidade tecidual e meia-vida do oxigênio singleto (GEORGE; KISHEN, 2007; MEISEL; KOCHER, 2005).

A utilização da TFDa como adjuvante ao tratamento periodontal padrão tem demostrado vantagens em modelos animais de periodontite (FILIPINI et al 2019; BARIN et al 2017; PILLUSKY; 2017). Estudos prévios do nosso grupo mostraram que a TFDa como adjuvante a RAR, particularmente quando o AM é dissolvido em etanol, pode induzir a resposta protetora sistêmica contra o estresse oxidativo induzido por periodontite e recuperar o colágeno gengival (PILLUSKY et al., 2017) e maior tecido reparo, além de induzir a recuperação de defesas antioxidantes sistêmicas (BARIN et al., 2017), promovendo assim a cicatrização periodontal.

O presente trabalho tem como objetivo investigar a influência da TFDa com AM dissolvido em etanol a 20% sobre o status oxidativo gengival, bem como no conteúdo de colágeno da gengiva de ratos com periodontite.

2 ARTIGO - COLLAGEN PROTECTION AND IMPROVEMENT OF THE OXIDATIVE STATUS GINGIVAL IN A SHORT-TERM BY ANTIMICROBIAL PHOTODYNAMIC THERAPY WITH PHOTOSENSITIZER IN ETHANOL IN EXPERIMENTAL PERIODONTITIS

Os resultados inseridos nesta dissertação apresentam-se sob a forma de manuscrito, o qual se encontra aqui estruturado da mesma forma a qual foi submetido a Photodiagnosis and Photodynamic Therapy, Qualis A2, Fator de impacto 2.895. Os itens Materiais e Métodos, Resultados, Discussão e Referências encontram-se no próprio manuscrito.

Manuscrito submetido a Photodiagnosis and Photodynamic Therapy e encontra-se sob revisão.

Collagen protection and improvement of the oxidative status gingival in a short-term by antimicrobial photodynamic therapy with photosensitizer in ethanol in experimental periodontitis

Palma VM[a], Barcelos RCS[bd], Pillusky FM[a], Barin LM[a], Pasini MM[a], Vey LT[c], Maciel RM[a], Kantorski KZ[a], Bürger ME[bc], Ferrazzo KL[d], Danesi CC[ad]

[a]Programa de Pós-Graduação em Ciências Odontológicas, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[b]Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[c]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[d]Departamento de Patologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

***Corresponding author**

Victor de Mello Palma

Departamento de Patologia, UFSM

Av. Roraima, 1000.

97105-900 Santa Maria – RS – Brazil

Phone: +55-55-3220-9559

victorpalmaod@hotmail.com

Abstract

Objective To evaluate the influence of antimicrobial photodynamic therapy (aPDT) with methylene blue (MB) dissolved in ethanol, used as an adjunct to scaling and root planing (SRP), in the periodontitis treatment on the collagen fibers and oxidative damage gingival in rats.

Methods Wistar rats were randomly assigned into five experimental groups according to periodontal treatment: NC (negative control; no periodontitis); PC (positive control; periodontitis without any treatment); SRP (periodontitis and scaling and root planing), aPDT I (periodontitis and SRP+aPDT+MB solubilized in water), and aPDT II (periodontitis and SRP+aPDT+MB solubilized in ethanol 20%). The periodontal treatments were performed after 7 days of removal of the ligature. At 7 and 15 days after removal ligature, gingival of the left first mandibular molar was removed for biochemical and histological analysis.

Results The periodontitis was induced by placing of the ligature around of the first right molar in rats. The PC and SRP groups showed higher gingival lipoperoxidation levels at 7 days, but only PC group exhibited higher gingival lipoperoxidation at 15th day. Moreover, it indicated a protective action of aPDT II in gingiva already at 7 days observed by the maintenance of the GSH levels of the gingiva similar to NC group. The aPDT was able to prevent gingival collagen degradation already at 7th day. Collagen area was negatively correlated with lipoperoxidation levels in the gingiva.

Conclusion aPDT can acts a beneficial adjuvant tool to minimize collagen degradation and oxidative damages periodontitis-induced. Hypothesizing, ROS periodontitis-induced generate adaptive responses, which may be related to hormesis, thereby ameliorating gingival oxidative toxicity of the aPDT.

Keywords: periodontal disease; gingiva; oxidative stress; type I collagen, type III collagen; hormesis

Introduction

Periodontal disease is the most common oral infection of humans and has been the major cause of tooth loss in adults [1]. Periodontitis occurs high prevalence in developed [2] and developing countries [3], and presents negative impact on the life quality of the people [4]. The periodontitis is the infect-inflammatory disease that result of the interaction between the bacterial biofilm adhered to dental surfaces and the host response. In the periodontal lesions occur immuno-inflammatory and oxidative responses that cause loss of the periodontal tissue support [5, 6]. Excessive production of reactive oxygen species (ROS) and the oxidative damage consequent are observed [6]. Besides, the inflammation becomes sufficiently intense to degrade the collagen matrix of connective tissue. The increased collagen breakdown causes quantitative and qualitative changes in the collagen matrix, resulting in destruction of up to 70% [7].

The gold standard treatment for periodontitis consist in the mechanical removal of biofilm and of mineralized deposits adhering to tooth surfaces by scaling and root planing (SRP) [8]. However, in some clinical situations, such as different types of periodontitis [9], diabetes mellitus [10], smoke [11], or specific anatomical characteristics of the teeth [12], the success of treatment can be compromise. In these cases, adjuvant therapies to mechanical treatment can be employed, such as antibiotics [13]. However, the consequent bacterial resistance to them prolonged use has become a current issue inevitably growing [14]. In this context, antimicrobial photodynamic therapy (aPDT) has been proposed as an alternative to mitigates the microbes in the subgingival region, once it showed effectiveness in killing periodontal pathogens in mono- and multi-species biofilms [15]. Furthermore, aPDT has been suggested to be non-invasive, have broad spectrum, low risk of developing bacterial resistance and does not have the limit dose level [16, 17].

The aPDT consists of a local application of a photosensitive drug (photosensitizer) and its photo-activation by light with an appropriate wavelength. After absorption of the light energy, the photosensitizer (PS) is activated to an excited state. The excited-PS can perform electronic transfers with the neighboring molecules to form reactive oxygen species (ROS), such as superoxide and hydroxyl radical (type I mechanism) and/or energy transfers with the molecular oxygen, resulting in the generation of singlet oxygen molecules (type II mechanism) [18]. These ROS aPDT-induced are toxic to microbial cells, causing damage to the their cell membrane or DNA [19].

Currently, there are a wide variety of dyes with photosensitizing properties that have been used in aPDT and methylene blue (MB) has been chosen by many authors to perform clinical trials [20]. Usually, the MB is solubilized in water. In this solvent, the singlet oxygen, the main ROS produced, has

low power tissue diffusion (<0.02mm) [21] and half-life of short duration (4μs) [22]. The literature has been reported that the solubilization of the MB in solvents containing ethanol improvements in photophysical and photochemical properties showing that there increase in half-life of singlet oxygen (20μs) and better antimicrobial effect [23].

Our previous studies have shown that aPDT as an adjuvant to the SRP, particularly when the MB is dissolved in ethanol can induce the systemic protective response against oxidative stress periodontitis-induced and recover the gingival collagen [24] and greater tissue repair, besides induce the recovery of systemic antioxidant defenses [25], thus promoting the healing periodontal. Here, we investigate the influence of this aPDT protocol on gingival oxidative status, as well as on the collagen content from gingiva of rats with periodontitis.

Materials and methods

The Figure 1 presents the experimental procedures.

Animals

Seventy male Wistar rats adult (2 months of age) from the breeding facility of Universidade Federal de Santa Maria (UFSM, RS, Brazil) were kept in Plexiglas® cages with water and food *ad libitum* (Supralab®, Alisul Alimentos LTDA, São Leopoldo, RS, Brazil) in a room with controlled temperature (23±1°C) and 12h light/dark cycle. Before starting the procedures, the animals underwent an acclimatization period of 15 days. This study was approved by the Animal Ethical Committee of Universidade Federal de Santa Maria (027132-UFSM), affiliated to the Council for the Control of Animal Experiments (CONCEA), following international norms of animal care and maintenance.

Protocol for experimental periodontal disease

The animals were randomly allocated into five experimental groups: NC (negative control; no periodontitis; n=11); PC (positive control; periodontitis without any treatment; n=11); SRP (periodontitis and scaling and root planning; n=16), aPDT I (periodontitis and SRP+aPDT+MB solubilized in water; n=16), and aPDT II (periodontitis and SRP+aPDT+MB solubilized in ethanol 20%; n=16). In order to induce experimental periodontitis, under general anesthesia (ketamine/xylazine, 70 and 6 mg/kg, intramuscular injection, respectively), one mandibular right first molar of each animal received the cotton ligature in a submarginal

position. The ligature was removed from all animals after 7 days of periodontal disease induction [26].

Scaling and root planning treatment

The SRP was carried out with micro Gracey curettes Mini-Five 1-2 (Hu-Friedy®, Chigaco, IL, USA) through 10 distal-mesial traction movements in the buccal and lingual aspects. The interproximal areas and furcation were instrumented with the same curettes using cervico-occlusal traction movements [26]. One operator blinded for experimental groups performed all the SRP procedures.

Antimicrobial photodynamic therapy protocols

The aPDT was performed with two formulations of MB 0.01% (Sigma-Aldrich, St. Louis, MO, USA) according experimental group: (I) aPDT I employing MB 0.01% solubilized in bidistilled water, and (II) aPDT III employing MB 0.01% solubilized in water/ethanol solution 20% v/v.

The MB formulation was slowly poured into the periodontal pocket around the left mandibular first molar using a syringe (1mL) and an insulin needle (BD® UltrafineTM, U-100, 0.5mL, 8mm x 0.3mm) without a bevel. After 1 minute, low-level laser therapy (LLLT) was applied to three points at each buccal and lingual aspect of the left mandibular first molar. The laser used was an indium-gallium-aluminum-phosphorous (TheraLase®, DMC Equipments, São Carlos, SP, Brazil). The laser parameters were: a wavelength 660nm, continuous emission mode, power output of 30mW transmitter, with spot size of 0.0283cm^2. The laser was activated for 4s in each point (4.94J/cm^2). The tooth received a total energy density of 29.64 J/cm^2 [26].One operator blinded to the experimental groups performed aPDT procedures.

Experimental periods

The periodontal treatments were performed immediately after ligature removal. On days 7 and 15 after periodontal treatments, seven animals from each group were anesthetized with isoflurane (2-3%) (Isothane®, Baxter Healthcare®, Guayama, Puerto Rico) [27] and euthanized by exsanguinations. Their jaws were removed and were fixed with 10% formaldehyde in phosphate buffer (pH 7.2) for 48h.

Histological analysis

Measurements of linear bone loss

For the histomorphometric analyses, histological sections from each specimen, which was hematoxylin and eosin (HE) stained, were selected. Care was taken to obtain histological sections, in which the interproximal alveolar bone crest and the coronal and root pulp chambers of the mandibular right first molar were clearly identified. The linear distance from the cement to the remaining alveolar crest to center parts of the bifurcation and alveolar bone loss measurements in the furcation region of the mandibular right first molar was measured to determine a histometric bone loss (μm) [28]. The linear bone loss in the furcation region of the mandibular right first molar represents the average value obtained from two measurements for each rat of each experimental group (n=6) after 7 days of ligature removal. A blind examiner evaluated the experimental groups with an interval of one week and mean values were calculated, with modifications. Images were obtained through an image analysis system (Axiovision, Carl Zeiss MicroImagnig, Jena, Germany) in a 10x magnification, captured with a digital camera coupled to the light microscope (AxioStar PluSS, Carl Zeiss) and visualized with the aid of a computer with processor (Pentium 4, with 3.00 GHz, 512Mb of RAM - Operating System Microsoft Windows XP - Monitor LG model FLATRONezT710SH, 64M, 17 inches color), associated with a binocular optical microscope (Olympus, model BX51 / BX52), with video camera (Olympus, model OLY-200) attached.

Influence of aPDT on the total collagen area of gingiva - Masson Trichrome staining

The Masson Trichrome histological staining provides blue color to the collagen fibers of the connective tissue and was used to quantify the area occupied by such fibers [29]. The slides were prepared as specific protocol of the product manufacturer (Masson Trichrome with aniline blue, Easy Path®, Erviegas Surgical instruments Ltda, São Paulo, SP, Brazil). The images were captured by digital camera attached to a light microscope (Axiovision, Carl Zeiss MicroImagnig, Jena, Germany), transferred to a computer in JPEG format and with a resolution of 2560x1920 pixels. Quantification of the area percentage (%) occupied by collagen fibers was performed using an image analysis system (FIJI to ImageJ®, version 1.47i, Wayne Rasband, National Institutes of Health, USA) under 400x magnification in five fields random underlying the epithelium of each blade, from left to right, a total of fifteen fields. Collagen area of each blade was given by the average of five determinations and the corresponding area of each animal was given by the mean of three slides area.

Collagen Gingival Deposition Measurement

The collagen deposition by the upper connective tissue (directly under the epithelial membrane) of the gingiva was evaluated. The samples around of the right mandibular first molar of each animal were dissected at the end of the experiments and fixed in 10% formaldehyde in phosphate buffer (pH 7.2) for 24h. The paraffin embedded gingiva specimens were sectioned (5μm), deparaffinized and stained with Picrosirius red commercial kit (Picrosirius Red Staining, Easy Path®, Erviegas Instrumental Cirúrgico Ltda., São Paulo, SP, Brazil) for polarized light microscopic evaluation. The staining with Picrosirius red allows an analysis of collagen fibers in the gingiva by different interference colors, intensity and birefringence of stained tissue. Thus, the staining differentiate collagen fibers type I and type III. Type I collagen (mature) show colors from yellow to red fibers strongly birefringent, and type III collagen (immature) appears green and low birefringence [30]. The stained tissue sections were examined using a microscope (Leica, model DM2000, Germany) with a 20x objective equipped coupled to digital image capture camera (Leica, model DFC295, Germany). The images generated by camera were transferred to a microcomputer and converted into 256 different grey levels [31]. For all the gingival samples, all extension of the histological slide was analyzed. The percentages of type I and III collagen deposition were calculated for each rat, and used to estimate the ratio between the percentages of type I over type III collagen (% collagen I / % collagen III), which is defined as collagen maturation index (CMI). Results >1 were considered as predominance of mature collagen [32], namely, higher percentage of type I collagen on the percentage of collagen type III and demonstrate the state of maturity of healing. For the morphometric determination of the types I and III collagen [33], the examiners underwent training and completed double measurements of 60 specimens, with a 10 days interval between each measurement. The interexaminer and intraexaminer reproducibility revealed a high correlation (Kappa>0.81). The results were expressed as area fraction (AA%) occupied by gingival collagen.

Preparation of gingival sample for biochemical analysis

At week 7 and 15 days of periodontitis experimental induction, animals were euthanized and gingival tissue of the mandibular first molar region were excised from each rat, it was homogenized in Tris HCl buffer (10mM; pH 7.4) (Sigma-Aldrich®, São Paulo, SP, Brazil), and

centrifuged at 3640g for 15min. The supernatants were used for determination of oxidative damage parameters.

Lipid peroxidation estimation of the gingiva

Gingival lipid peroxidation was assessed by quantifying thiobarbituric acid (TBARS) levels as described by Ohkawa et al [34]. The TBARS occurring by excess generation of ROS was determined by the pink chromogen produced by the reaction of the thiobarbituric acid (TBA) to malondialdehyde (MDA) at 100°C and measured spectrophotometrically at 532nm. Results were expressed as nmol MDA/g gingiva.

Estimation of non-enzymatic antioxidant defense GSH gingival

The GSH levels were determined after the reaction of the samples with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, Proquímios®, Rio de Janeiro, RJ, Brazil). Yellow formed was read at 412nm, according Boyne and Ellman [30]. A standard curve was plotted using GSH to calculate GSH levels, which were expressed in GSH µmol/g gingiva.

Statistical analysis

Homogeneity of the data was analyzed by Levene's test. Histological and biochemical evaluations were analyzed by two-way ANOVA followed by Duncan's multiple range test, when appropriate (Software package Statistica 8.0 for Windows was used). Data were expressed as the mean ± SEM, and $p<0.05$ were considered statistically significant for all comparisons made. Linear regression analysis was performed between collagen area and TBARS levels in gingival tissue.

Results

Morphometric Analysis

Measurements of linear bone loss

Was observed that in 7 days, the PC group, which was induced to periodontal disease and was not submitted to treatments, demonstrated more bone loss compared to the NC, SRP, aPDT I and II. The SRP and aPDT I groups showed equivalent and greater bone losses than the NC group. The aPDT II group presented bone loss similar to the aPDT I group, smaller than the PC and SRP groups, and larger than the NC group [$P<0,05$] (Table 1).

Influence of aPDT on the total collagen area of the gingiva

A two-way ANOVA of total collagen area of the gingiva revealed a significant main effect of periodontal treatment, experimental periods and a significant periodontal treatment x experimental periods interaction [F=389.17, *P*<0.0000; 149.32, *P*<0.0000 and 9.81, *P*<0.0000] (Table 1; Fig. 2).

On the 7th and 15th evaluation days, the groups NC, aPDT I and aPDT II presented the highest gingival total collagen area than all the other groups. In the same experimental times, the PC group showed the minor gingival total collagen area. The SRP group had a highest gingival total collagen area than the PC group and minor than the aPDT groups I and II. In the intra-group comparison, after 15 days of periodontal treatment, PC and SRP groups had a higher gingival total collagen area compared to day 7 (Table 1; Fig. 2).

Collagen Maturation Index Measurement

A two-way ANOVA of CMI of the gingiva revealed a significant main effect of periodontal treatment, experimental periods and a significant periodontal treatment x experimental periods interaction [F=3.43, *P*<0.01; 11.41, *P*<0.0000 and 2.44, *P*<0.01].

On the 7th day of evaluation, NC, PC, aPDT I and aPDT II group showed similar gingival CMI, but just NC group showed higher gingival CMI compared to SRP group. In addition, PC, aPDT I and aPDT II groups showed similar gingival CMI.

At 15 days after the end of periodontal treatments, NC, aPDT I and aPDT II groups showed similar CMI, but just NC group showed higher gingival CMI compared to PC group. Additionally, PC and SRP groups showed similar CMI and minor than NC group. (Table 1; Fig. 3).

Biochemical measurements

Influence of aPDT on lipid peroxidation levels in gingiva

A two-way ANOVA of lipid peroxidation revealed a significant main effect of periodontal treatment, experimental periods and a significant periodontal treatment x experimental periods interaction [F=12.04, *P*<0.0000; 3.45, *P*<0.03 and 2.24, *P*<0.03].

The *post-hoc* test showed that PC and SRP groups increased gingival lipid peroxidation levels 7 days after the ligature removal in relation to NC, aPDT I and aPDT II groups, which showed similar values. The evaluations on the 15th days showed that the gingival lipid

peroxidation levels remained higher in PC group compared to other groups, whose values were similar. Additionally, SRP group showed minor gingival lipid peroxidation levels at 15th day than in 7th day of evaluation (Fig. 4).

Influence of aPDT on antioxidant defense levels in gingiva

A two-way ANOVA of gingival GSH levels revealed a significant main effect of periodontal treatment [F=6.82, $P<0.0000$].

The *post-hoc* test showed gingival GSH levels similar in NC group and aPDT II group and higher all other experimental groups 7 days after the ligature removal. In addition, PC, SRP and aPDT I showed gingival GSH levels showed lower levels than NC and aPDT II groups, and the values of both groups were similar. On the 15th day of evaluation, gingival GSH levels was similar among all the experimental groups. Additionally, SRP group showed higher gingival GSH levels at 15th day than in 7th day of evaluation (Fig. 4).

Linear regression analysis of the lipid peroxidation and collagen degradation in the gingiva

Statistical analyses revealed a significant negative correlation between lipid peroxidation with collagen degradation [r=0.25, p=0.000013] (Fig. 5).

Discussion

The experimental model chosen for this study, which employs the ligature placement of mandibular first molar for 7 days, has been used to evaluate the aPDT influence adjuvant to SRP in normal systemically [26], diabetic [35], immunosuppressed [36], and ovariectomized rats [37], in addition to rats submitted nicotine [38]. Previous studies of our group showed that aPDT as an adjunct to the SRP can induce the systemic protective response against oxidative stress periodontitis-induced, recover the gingival collagen [24] and increase the number of blood vessels in the short term thus promoting the healing periodontal [25], particularly when the MB is dissolved in ethanol 20%. In the present study, the periodontitis was induced, demonstrated by the greater bone loss observed in the PC group, which is in agreement with previous studies [40, 41], the ligation was maintained by 7 days and then removed, but removal did not result in bone repair in 7 days, since the PC group presented greater bone loss in relation to all the other groups in the same period, the same was found by Chang et al. [40] in study. The three treatment groups of our study (SRP / aPDT I / aPDT II) presented differences between the control groups (NC and PC), demonstrating that the therapies result in bone repair in the short term. In addition, the periodontitis induced an increase of the MDA levels and decrease

of the antioxidant parameter, represent by GSH levels, in the gingiva of PC group compared to the NC group, suggesting oxidative damage in this oral tissue. The MDA levels are one of the most frequently used indicator of lipid peroxidation, can be a potential biomarker indicating oxidative stress [39] and has been reported higher MDA levels in chronic periodontitis [42]. In addition, recent studies have shown a reduction in the antioxidant capacity and an increase in oxidative stress biomarkers in periodontitis [6], corroborating with our results. The oxidative stress occurs when ROS generation exceeds the tissue antioxidant capacity, that is, it results in an increase production or an endogenous antioxidant defenses decrease, or both [43]. The ROS-mediated tissue damage during the oxidative stress can be measured by the end products levels of lipid peroxidation, such as MDA [44] in the TBARS assay [34]. In this sense, the cellular redox system plays a key role in homeostatic imbalance and damage in the gingiva, which can result from an imbalance between pro-oxidant and antioxidant stimuli.

The SRP, associated with adherence to maintenance schedules, can promotes stability in periodontal levels and promotes the maintenance of the teeth over the years [45]. This procedure employs manual instruments to remove supra and subgingival bacterial deposits and is considered the gold standard in periodontal therapy [46], which new therapies have been compared as well [20]. In this study, the SRP treatment alone was able to decreased lipid peroxidation levels in gingiva the manner time-dependent, confirming the effectiveness of this classic procedure for periodontal treatment [47]. The mechanical instrumentation (SRP) cannot completely remove subgingival biofilm [48], and the aPDT has been shown to be effective as adjunctive therapy to the SRP in the periodontitis treatment in both animal [24, 25, 49] and human studies [50]. In addition, the aPDT as an adjuvant tool associated to SRP may result in decreasing of the ROS generation and of the lipid peroxidation consequent in gingiva, improving periodontal disease treatment when compared to standard treatment (SRP) alone, regardless of the solvent used for solubilization of the photosensitizer, as demonstrated by our results. Furthermore, the solubilization of the MB in water (aPDT I group) or water/ethanol 20% (aPDT II group) solution used as photosensitizer in aPDT did not differ between them as to the lipid peroxidation levels in all analyzed times, but evade its induction by periodontitis.

The glutathione is a oxidized (GSSG) or reduced (GSH) tripeptide considered one of most important endogenous antioxidant defenses, which has nucleophilic and reduced properties [51], and the reduced form can be decreased by oxidative stress [52]. In the present study, the gingival GSH evaluation of groups under periodontitis after periodontal therapy demonstrated that this non-enzymatic antioxidant defense were decreased in SRP and aPDT I

groups compared to levels of the NC group. This result suggests a consumption of gingival GSH due to oxidative stress-induced periodontitis. Our results are according with Azuma et al. [53], which related the gingival GSH/GSSG ratio reduction in the oxidative stress. Nevertheless, the dissolution of MB in ethanol 20% (aPDT II group) showed GSH levels similar to NC group 7 days after the end of periodontal treatment [24, 25]. This finding indicate that when employs the ethanol 20% to solubilize the MB, the aPDT showed more effectiveness and improved therapeutic action as an adjunct therapy to SRP in the periodontal treatment. Furthermore, after 15 days of the completion of periodontal treatments, GSH gingival levels have been completely restored in all groups; while the standard treatment (SRP) alone (SRP group) was able to increase the antioxidant defense compared to the evaluation of 7^{th} day. In this context, the bacterial biofilm reduction from the tooth surface using an association of non-surgical methods, as SPR and aPDT, reduced gingival lipid peroxidation levels, which contributed to the restoration of GSH levels in this tissue. Previous studies have showed that antioxidants can be beneficial as useful tools as adjuncts to improve treatment outcome in periodontitis patients [54, 55]. In addition, the application of the Er:YAG laser (erbium-doped: yttrium, aluminum, and garnet) in chronic periodontitis treatment showed promising experimental and clinical results [56, 57], showing the importance of this type of therapy with light. The oxidative stress has been associated to collagen degradation [58], which is a major component of the soft tissues and bone [59]. Moreover, the periodontitis induces alterations in the gingival connective tissue composition [24, 60]. In the present study, the total area of the gingival collagen was significantly decreased on the 7th day after ligature removal in the PC and SRP groups compared to NC group. The SRP group showed lipid peroxidation levels similar to PC group in 7 days and both higher than the NC group, which can be related to collagen reduction area in this group observed at 7 and 15 days evaluation. In this sense, we may infer that oxidative stress periodontitis-induced degraded the gingival collagen, as demonstrated by area reduction of these fibers in the gingiva. Our finding are according with previous studies, which related the oxidative stress and lower collagen content in tissues [24, 61]. The collagen degradation has been considered the main marker of periodontal disease progression [62]. The collagen area in the gingival tissue in the aPDT I and aPDT II groups were not affected by periodontitis, this was demonstrated by the preservation of the gingival collagen area compared to the NC group. The aPDT could protect the lipid peroxidation in gingiva, and thus reduced oxidative stress-related collagen degradation in these groups, independent of solvent used to solubilized the photosensitizer in all the evaluated times. These

observations suggest that the aPDT helps to prevent gingival collagen breakdown by suppress oxidative stress related to periodontitis.

The collagen deposition has been reported to reflect the healing process [63] and was included in the present study as CMI. The periodontitis affected the gingival collagen maturation in the SRP group, which showed minor CMI than NC group at 7 days of evaluation, indicating the predominance of immature versus mature collagen, but this difference did not show statistical significance. At 15th day, the PC and SRP groups exhibited CMI equivalents and lower than the NC group, indicating collagen deposition, in order to restore the contents of gingival collagen reduced by periodontitis. In contrast, the CMI on both aPDT groups, at 7 and 15th days, was not significantly different from that of NC group, suggesting that both aPDT protocols preserved deposition and consequent collagenous maturation, which was similar to NC group. Based on these results, we can suggest that the repair phase of the periodontitis is related to type III collagen, since that the fibroblasts synthesize high levels of type III collagen and reduced levels of type I collagen [64], which is essential for the reestablishment of functional dynamic conditions, since the type III collagen is essential for the type I collagen formation [65]. Of particular importance, aPDT protocols showed ability to induce collagen maturation in short-term compared standard periodontal treatment (SRP), which may be related to their protective action against oxidative stress-induced periodontitis observed in aPDT groups [24]. According to Mendez et al. [66], Meirelles et al. [67] and Gonçalves et al. [68], enhanced collagen maturation may be seen observed in different protocol employing GaAIAs laser (λ 830nm) at 50J/cm^2, low level laser therapy (LLLT) at an energy density of 20J/cm^2 and laser GaAsAl 30 and 90J/cm^2, respectively.

The photosensitizer excitation results in the ROS generation, which mediate cellular effects such as lipid peroxidation and vascular effects, resulting in direct or indirect cytotoxic effects on the treated cells [69]. In this sense, one hypothesis can be proposed to explain the lower oxidative toxicity of aPDT to periodontal tissue in presence of the periodontitis: development of hormesis due to stressor, as periodontitis and the consequent ROS bacterial biofilm-induced. This hypothesis suggests that organisms exposed to stressors trigger defense mechanisms, which would act much more efficiently than in those not previously subjected to stressors and has been used to explain the increase in the antioxidant defenses in different animal species [70, 71]. Our data also point towards the development of hormesis, since oxidative damage of the gingiva, as lipid peroxidation and collagen degradation were not observed in the groups treated with aPDT, both generators of the ROS, than in group treated

with SRP only (SRP group). Our findings are consistent with development of hormesis during exposure of gingiva to periodontitis following aPDT, allowing physiological changes counteract the damages caused by ROS aPDT-induced. According our findings, low dose of irradiation also showed to be effective on treatment against greater stress, as the gingivitis [72]. In this context, authors conceptualized hormesis, which is a useful way to think about the effect of stressors on the gingiva, i.e., periodontitis and aPDT. The term hormesis has been defined in the literature as the stimulatory effects caused by low levels of potentially toxic agents [73]. In other words, hormesis is a physiological response that helps the organism to react to the continuous presence of a small stimulus such as low ROS levels, which can induce an increase in the antioxidant defenses, promoting compensatory processes following an initial disruption in homeostasis (hormesis hypothesis) [74]. In our study, the lower levels of lipid peroxidation and collagen degradation observed in gingiva of aPDT treated groups may indicate tolerance to a hostile environment and adaptation mechanisms development, even as increment of the defense mechanisms [75], resulting from hormesis. Here, we did not observe an increase in the defense mechanism measured in this study, unlike there was a depletion of GSH gingival levels in all experimental groups at 7 days evaluation. We believe that another antioxidant or defense mechanisms may be involved in the gingival tissue protection, since the GSH is the first line of antioxidant defense that organism have against ROS in favor of homeostase, and therefore the first to be consumed during oxidative stress [76]. In this context, more studies are necessary for better understanding of the hormesis mechanisms at the molecular and cellular level in the different approaches in search of prevention and treatment of periodontal diseases.

The negative correlation between collagen area and lipid peroxidation levels, both observed in gingiva indicate the close relationship between collagen degradation and oxidative damage development in this supporting oral tissue of rats. In accordance to our findings, Gonçalves et al. [68] found a moderately negative correlation between lipid peroxidation levels and CMI in all the groups. Considering that the periodontitis and the consequent oxidative status in gingiva were modified by aPDT, it is possible that these physiological changes are related to adaptation and/or hormesis.

Our results employing aPDT with the MB solubilized in water/ethanol solution, which used as adjuvant therapy to the mechanical treatment of the periodontitis induced in rats was similar on the collagen fibers and oxidative damage gingival to the aPDT using AM solubilized in water. In other words, the solubilization of the AM in water/ethanol solution prevented the lipid peroxidation and collagen degradation periodontitis-induced in the evaluated times in a short-term.

References

[1] K. Hyvärinen, A. Salminen, V. Salomaa, P.J. Pussinen, Systemic exposure to a common periodontal pathogen and missing teeth are associated with metabolic syndrome. Acta Diabetol. 52 (2015) 179-182. doi: 10.1007/s00592-014-0586-y.

[2] J.M. Albandar, J.A. Brunelle, A. Kingman, Destructive Periodontal Disease in Adults 30 Years of Age and Older in the United States, 1988-1994, J Periodol 70 (1999) 13-29. doi: 10.1902/jop.1999.70.1.13.

[3] C. Susin, C.F. Dalla Vecchia, R.V. Oppermann, O. Haugejorden, J.M. Albandar, Periodontal attachment loss in an urban population of brazilian adults: effect of demographic, behavioral, and environmental risk indicators, J Periodontol 75 (2004) 1033-1041. doi: 10.1902/jop.2004.75.7.1033.

[4] L.S. Al-Harthi, M.P. Cullinan, J.W. Leichter, W.M. Thomson, Periodontitis among adult populations in the Arab World, Int Dent J 63 (2013) 7-11. doi: 10.1111/idj.12002.

[5] F.A. Akalin, E. Toklu, N. Renda, Analysis of superoxide dismutase activity levels in gingiva and gingival crevicular fluid in patients with chronic periodontitis and periodontally healthy controls. J. Clin. Periodontol. 32 (2005) 238-243. doi: 10.1111/j.1600-051X.2005.00669.x.

[6] F.A. Akalin, E. Baltacioglu, A. Alver, E. Karabulut, Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. J. Clin. Periodontol. 34:558-565; 2007. doi: 10.1111/j.1600-051X.2007.01091.x.

[7] A. Havemose-Poulsen, P. Holmstrup, K. Stoltze, H. Birkedal-Hansen, Dissolution of type I collagen fibrils by gingival fibroblasts isolated from patients of various periodontitis categories, J Periodontal Res 33 (1998) 280-291. doi: 10.1111/j.1600-0765. 1998. tb02 201.x.

[8] C.M. Cobb, Clinical significance of non-surgical periodontal therapy: an evidence-based perspective of scaling and root planning, J Clin Periodontol 29 (2002) 6-16. doi: 10.1034/j.1600-051X.29.s2.4.x.

[9] N.B. Arweiler, M. Pietruska, J. Pietruski, A. Skurska, E. Dolińska, C. Heumann, T.M. Auschill, A. Sculean, Six-month results following treatment of aggressive periodontitis with antimicrobial photodynamic therapy or amoxicillin and metronidazole. Clin. Oral Invest. 18 (2014) 2129-2135. doi: 10.1007/s00784-014-1193-6.

[10] T. Tervonen, R.C. Oliver, Long-term control of diabetes mellitus and periodontitis, J Clin Periodontol 20 (1993) 431-5. doi: 10.1111/j.1600-051X.1993.tb00384.x.

[11] J. Bergström, S. Eliasson, J. Dock, A 10-year prospective study of tabacco smoking and periodontal health, J Periodontol 71 (2000) 1338-47. doi: 10.1902/jop.2000.71.8.1338.

[12] J.L. Wennström, G. Dahlén, P. Ramberg, Subgingival debridement of periodontal pockets by air polishing in comparison with ultrasonic instrumentation during maintenance therapy. J. Clin. Periodontol. 38 (2011) 820-7. doi: 10.1111/j.1600-051X.2011.01751.x.

[13] J. Slots, Systemic antibiotics in periodontics, J Periodontol 75 (2004) 1553-65. doi: 10.1902/jop.2004.75.11.1553.

[14] T.T. Yoshikawa, Antimicrobial resistance and aging: beginning of the end of the antibiotic era? J Am Geriatr Soc 50 (2002) S226-9. doi: 10.1046/j.1532-5415.50.7s.2.x.

[15] C.R. Fontana, A.D. Abernethy, S. Som, K. Ruggiero, S. Doucette, R.C. Marcantonio, C.I. Boussios, R. Kent, J.M. Goodson, A.C. Tanner, N.S. Soukos, The antibacterial effect of photodynamic therapy in dental plaque-derived biofilms, J Periodontolal Res 44 (2009) 751-9. doi: 10.1111/j.1600-0765.2008.01187.x.

[16] M. R. Hamblin; T. Hasan, Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochemical & Photobiological Sciences, v. 3, p. 436-50. 2004. doi: 10.1039/b311900a.

[17] T. Maisch, Anti-microbial photodynamic therapy: useful in the future? Lasers in Medical Science, v, 22, p. 83-991, 2007. doi:10.1007/s10103-006-0409-7.

[18] N. S. Soukos; J. M. Goodson, Photodynamic therapy in the control of oral biofilms. Periodontol 2000, v. 55, n. 1, p. 143-166, fev. 2011. doi: 10.1111/j.1600-0757.2010.00346.x.

[19] M. Wainwright, Methylene blue derivatives - suitable photoantimicrobials for blood product disinfection? Int J Antimicrobial Agents 16 (2000) 381-394. doi: 10.1016/S0924-8579(00)00207-7.

[20] J. Betsy, C.S. Prasanth, K.V. Baiju J. Prasanthila, N. Subhash, Efficacy of antimicrobial photodynamic therapy in the management of chronic periodontitis: a randomized controlled clinical trial. J. Clin. Periodontol. 41 (2014) 573-581. doi: 10.1111/jcpe.12249.

[21] M. Ochsner, Photophysical and photobiological processes in the photodynamic therapy of tumours, J Photochem Photobiol B Biol 39 (1997) 1-18. doi: 1011-1344/97.

[22] P. Meisel, T. Kocher, Photodynamic therapy for periodontal diseases: State of the art, J Photochem Photobiol B Biol 79 (2005) 159-170. doi: 10.1016/j.jphotobiol.20 04.11.023.

[23] S. George, A. Kishen, Photophysical, photochemical, and photobiological characterization of methylene blue formulations for light-activated root canal disinfection, J Biomed Opt 12 (2007) doi:10.1117/1.2745982.

[24] F.M. Pillusky, R.C.S. Barcelos, L.T. Vey, et al. Antimicrobial photodynamic therapy with photosensitizer in ethanol improves oxidative status and gingival collagen in a short-term in periodontitis. Photodiagn Photodyn 2017; 19:119-127. doi: 10.1016/j.pdpdt.2017. 05.010.

[25] L.M. Barin, R.C.S. Barcelos, L.T. Vey, F.M. Pillusky, V.M. Palma, K.Z. Kantorski, et al. Role of the adjunctive antimicrobial photodynamic therapy to periodontal treatment at plasmatic oxidative stress and vascular behavior. J Photochem Photobiol B. 2017. August;173:538–544. doi: 10.1016/j.jphotobiol. 2017.06.039.

[26] V.G. Garcia, M. Longo, L.A. Fernandes, E.C.G. Junior, C.S. Santinoni, A.F. Bosco, M.J.H Nagata, L.H. Theodoro, Treatment of experimental periodontitis in rats using repeated adjunctive antimicrobial photodynamic therapy, Laser Med Sci 28 (2013) 143 150. doi: 10.1007/s10103-012-1099-y.

[27] N.E. Taylor, J.J. Chemali, E.N. Brown, K. Solt, Activation of D1 dopamine receptors induces emergence from isoflurane general anesthesia, Anesthesiol 118 (2013) 30-39. doi:10.1097/ALN.0b013e318278c896.

[28] P.G.F.P. Oliveira, A.M.M.S. Souza, A.B. Novaes Jr, M. Taba, M.R. Messora, D.B. Palioto, M.F.M. Grisi, A.C. Tedesco, S.L.S De Souza, Adjunctive effect of antimicrobial photodynamic therapy in induced periodontal disease. Animal study with histomorphometrical, immunohistochemical, and cytokine evaluation, Lasers Med Sci 31 (2016) 1275-83. doi: 10.1007/s10103-016-1960-5.

[29] P. Masson, Some histological methods. Trichrome stainings and their preliminary technique, J Tech Methods 12 (1929) 75-XX.

[30] A.F. Boyne, G.L. Ellman, A methodology for analysis of tissue sulfhydryl 535 components, Anal Biochem 46 (1972) 639-653. doi:10.1016/00032697(72)90335-1.

[31] A.L. Ejeil, F. Gaultier, S. Igondjo-Tchen, K. Senni, B. Pellat, G. Godeau, B. Gogly, Are cytokines linked to collagen breakdown during periodontal disease progression?, J Periodontol 74 (2003) 196-201. doi: 10.1902/jop.2003.74.2.196.

[32] I.C.M. Coelho-Lemos, A.C.L. Campos, M. Almeida, S.L. Schüler, J. Gurmini, O. Malafaia, D.F. Andrade, *In Utero* malnutrition influences wound healing of newborn rats as measured by tensile strength and collagen deposition. J. Parenter. Enteral Nutri. 28 (2004) 241-245. doi: 10.1177/0148607104028004241.

[33] L. Rich, P. Whittaker, Collagen and picrosirius red staining: a polarized light assessment of fibrillar hue and spatial distribution, Braz J Morphol Sci 22 (2005) 97-104.

[34] H. Ohkawa, N.Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal Biochem 95 (1979) 351-358. doi: 10.1016/0003-2697(79)90738-3.

[35] J.M. Almeida, L.H. Theodoro, A.F. Bosco, M.J. Nagata, S. Bonfante, V.G. Garcia, Treatment of experimental periodontal disease by photodynamic therapy in rats with diabetes. J. Periodontol. 79 (2008b) 2156-2165. doi: 10.1902/jop.2008.080103.

[36] P.E. Bottura, J. Milanezi, L.A. Fernandes, H.C. Caldas, M. Abbud-Filho, V.G. Garcia, M.A.S.F. Baptista, Nonsurgical periodontal therapy combined with laser and photodynamic therapies for periodontal disease in immunosuppressed rats. Transplant. Proc. 43 (2011) 2009-2016. doi: 10.1016/j.transproceed.2011.03.083.

[37] V.G. Garcia, E.C.G. Júnior, L.A. Fernandes, A.F. Bosco, M.J.H. Nagata, C.A. Casatti, E. Ervolino, L.H. Theodoro, Adjunctive antimicrobial photodynamic treatment of experimentally induced periodontitis in rats with ovariectomy, J Periodontl 84 (2013b) 556-565. doi: 10.1902/jop.2012.120163

[38] V.G. Garcia, L.A. Fernandes, V.C. Macarini, J.M. de Almeida, T.M. Martins, A.F. Bosco, M.J.H. Nagata, J.A. Cirelli, L.H. Theodoro, Treatment of experimental periodontal disease with antimicrobial photodynamic therapy in nicotine-modified rats, J Clin Periodontal 38 (2011) 1106-1114. doi: 10.1111/j.1600 051X.2011.01785.x.

[39] F. Nielsen, B.B. Mikkelson, J.B. Nicholson, H.R. Anderson, P. Grandjean, Plasma MDA as biomarker and oxidative stress: reference /interval and effects of life style pattern. Clin Chem. 43 (1997) 1209-14.

[40] P. C. CHANG, et al., Progression of periodontal destruction and the roles of advanced glycation end products in experimental diabetes. Journal of Periodontology, v. 84, n. 3 (2013) 379-388. doi: 10.1902/jop.2012.120076.

[41] M. S. Rafael, et al., Evaluation of the Host Response in Various Models of Induced Periodontal Disease in Mice, J Periodontol v. 85, n. 3 (2014) 465- 477. doi: 10.190 2/jop.2013.130225.

[42] A.S. Aziz, M.G. Kalekar, A.N. Suryakar, T. Benjamin, M.J. Prakashan, B.M.N. Ahmed, M. Sayyad, Assessment of some biochemical oxidative stress markers in male smokers with chronic periodontitis, Ind J Clin Biochem 28 (2013) 374-380. doi:10.1007/s12291-012-0283-y.

[43] D. Trachootham, J. Alexandre, P. Huang, Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? Nat Rev Drug Discov 2009;8(7):579–591. doi: 10.1038/nrd2803.

[44] C.C. Tsai, H.S. Chen, S.L. Chen, Y.P. Ho, K.Y. Ho, Y.M. Wu, C.C. Hung, Lipid peroxidation: A possible role in the induction and progression of chronic periodontitis, J Periodontal Res 40 (2005) 378-384. doi: 10.1111/j.1600-0765.2005.00818.x.

[45] P. Axelsson, B. Nystro¨m, J. Lindhe, The long-term effect of a plaque control program on tooth mortality, caries and periodontal disease in adults. Results after 30 years of maintenance. J Clin Periodontol 2004; 31: 749–757. doi: 10.1111/j. 1600-051X. 2004.00563.x.

[46] M.L. Balata, L.P. Andrade, D.B.N Santos, A.N. Cavalcanti, U.R. Tunes, E.D.P Ribeiro, S. Bittencourt, Photodynamic therapy associated with full-mouth ultrasonic debridement in the treatment of severe chronic periodontitis: a randomized controlled clinical trial. J. Appl. Oral Sci. 21 (2013) 208-214. doi: 10.1590/1678-7757201302366.

[47] A.M. Mlachkova, C.L. Popova, Efficiency of nonsurgical periodontal therapy in moderate chronic periodontitis, Folia Med 56 (2014) 109-115.

[48] M. Petelin, K. Perkič, K. Seme, B. Gašpirc, Effect of repeated adjunctive antimicrobial photodynamic therapy on subgingival periodontal pathogens in the treatment of chronic periodontitis. Lasers Med. Sci. 30 (2015) 1647-1656. doi: 10.1007/s10103-014-1632-2.

[49] J.M. Almeida, L.H. Theodoro, A.F. Bosco, M.J. Nagata, M. Oshiiwa, V.G. Garcia, In vivo effect of photodynamic therapy on periodontal bone loss in dental furcations. J Periodontol 79 (2008a) 1081-1088. doi:10.1902/jop.2008.070456.

[50] L.H. Theodoro, S.P. Silva, J.R. Pires, G.H. Soares, A.E. Pontes, E.P. Zuza, D.M. Spolidório, B.E. de Toledo, V.G. Garcia VG, Clinical and microbiological effects of photodynamic therapy associated with nonsurgical periodontal treatment. A 6-month follow-up. Lasers Med. Sci. 27 (2012) 687-693. doi: 10.1007/s10103-011-0942-x.

[51] A.P. Arrigo, Gene expression and the thiol redox state, Free Radic Biol Med 27 (1999) 936-944. doi: 10.1016/S0891-5849(99)00175-6.

[52] R.C.S. Barcelos, L.T. Vey, H.J. Segat, D.M. Benvegnú, F. Trevizol, K. Roversi, et al., Influence of trans fat on skin damage in first-generation rats exposed to UV radiation, Photochem Photobiol 91 (2015) 424-30. doi:10.1111/php.12414.

[53] T. Azuma, T. Tomofuji, Y. Endo, N. Tamaki, D. Ekuni, et al., Effects of exercise training on gingival oxidative stress in obese rats. Archives of Oral Biology, 56(8), (2011) 768 774.doi:10.1016/j.archoralbio.2011.01.008

[54] T. Tomofuji, D. Ekuni, T. Sanbe, K. Irie, T. Azuma, T. Maruyama, N. Tamaki, J. Murakami, S. Kokeguchi, T. Yamamoto, Effects of vitamin C intake on gingival oxidative stress in rat periodontitis. Free Radical Bio. Med. 46 (2009) 163-168. doi: 10.1016/j.freeradbiomed.2008.09.040.

[55] P. Kudva, S.T. Tawkhira, N.K. Shekhawat, Effect of green tea catechin, a local drug delivery system as an adjunct to scaling and root planing in chronic periodontitis patients. J. Indian. Soc. Periodontol. 15 (2011) 39-45. doi: 10.4103/0972-124X.82269.

[56] A. Domínguez, C. Gómez, A.I. García-Kass, J.A. García-Nuñez, IL-1b, TNF-α, total antioxidative status and microbiological findings in chronic periodontitis treated with fluorescence-controlled Er:YAG laser radiation, Lasers Surg Med 42 (2010) 24-31. doi: 10.1002/lsm.20873.

[57] I. Ishikawa, A. Aoki, A.A. Takasaki, Potential applications of Erbium:YAG laser in periodontics. J. Periodont. Res. 39 (2004) 275-285. doi: 10.1111/j.1600-0765.2004.00738.x.

[58] C.S. Alge-Priglinger, T. Kreutzer, K. Obholzer, A. Wolf, M. Mempel, M. Kernt, et al., Oxidative stress-mediated induction of MMP-1 and MMP-3 in human RPE cells, Invest Ophthalmol Vis Sci 50 (2009) 5495-503. doi:10.1167/iovs.08-3193.

[59] T. Sorsa, L. Tjaderhane, Y.T. Konttinen, A. Lauhio, T. Salo, H. Lee, L.M. Golub, D.L. Brown, P. Mäntylä, Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. Ann. Med. 38 (2006) 306-321. doi: 10.1080/07853890600800103.

[60] R.C. Page, H.E. Schroeder, Pathogenesis of inflammatory periodontal disease. A summary of current work, Lab Invest 33 (1976) 235-249.

[61] C. Galli, G. Passeri, G.M. Macaluso, Fox Os, Wnts and oxidative stress-induced bone loss: new players in the periodontitis arena? J Periodontal Res 46 (2011) 397-406. doi: 10.1111/j.1600-0765.2011.01354.x.

[62] R.J. Waddington, R. Moseley, G. Embery, Reactive oxygen species: A potential role in the pathogenesis of periodontal diseases. Oral Dis. 6 (2000) 138-151. doi: 1354-523X/00.

[63] H.P. Ehrlich, T.M. Krummel, Regulation of wound healing from a connective tissue perspective, Wound Repair Regen. 4 (1996) 203-206. doi: 10.1046/j.1524-475X.1996.40206.x

[64] H. Larjava, M. Sandberg, R.P. Happonen, E. Vuorio, Differential localisation of type I and type III procollagen messenger ribonucleic acids in inflamed periodontal and periapical connective tissues by in situ hybridization. Lab. Invest. 62 (1990) 96-99.

[65] X. Liu, H. Wu, M. Byrne, S. Krane, R. Jaenisch, Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. Proc. Natl. Acad. Sci. USA. 94 (1997) 1852-1856. doi: 0027-8424y97y941852-5

[66] T.M.T.V. Mendez, A.L.B. Pinheiro, M.T.T. Pacheco, P.M. Nascimento, L.M.P. Ramalho, Dose and wavelength of laser light have influence on the repair of cutaneous wounds. J. Clin. Laser Med. Surg. 22 (2004) 19-25. doi: 10.1089/104454 704773660930.

[67] G.C.S. Meirelles, N.J. Santos, P.O. Chagas, A.P. Moura, A.L.B. Pinheiro, A comparative study of the effects of laser photobiomodulation on the healing of third-degree burns: a histological study in rats. Photomed. Laser Surg. 26 (2008) 159-166. doi: 10.1089/pho.2007.2052.

[68] R.V. Gonçalves, R.D. Novaes, M.C. Cupertino, B. Moraes, J.P.V. Leite, M.C.G. Peluzio, M.V.M. Pinto, S.L.P. Matta, Time-dependent effects of low-level laser therapy on the morphology and oxidative response in the skin wound healing in rats. Lasers Med. Sci. 28 (2013) 383-390. doi: 10.1007/s10103-012-1066-7.

[69] C.M. Brackett, S.O. Gollnick, Photodynamic therapy enhancement of antitumor immunity, Photochem Photobiol Sci 10 (2011) 649-52. doi:10.1039/c0pp00354a.

[70] G.S. Dolci, V.T. Dias, K. Roversi, Kr. Roversi, C.S. Pase, H.J. Segat, A.M. et al., Moderate hypoxia is able to minimize the manganese-induced toxicity in tissues of silver catfish (*Rhamdia quelen*). Ecotoxicology and Environmental Safety 91 (2013) 103-109. doi: 10.1016/j.ecoenv.2013.01.013.

[71] Laughlin Jr, R.B., Ng, J., Guard, H.E., Hormesis: a response to low environmental concentrations of petroleum hydrocarbons. Science 211 (1981), 705–707. doi: 10.1126/science.211.4483.705.

[72] T.D. Luckey. Low dose irradiation as a treatment for gingivitis. Medical Hypotheses (2006) 66, 695–697. doi: 10.1016/j.mehy.2005.10.020.

[73] A.R.D. Stebbing, Hormesis - The stimulation of growth by low levels of inhibitors, Sci Total Environ 22 (1982) 213-234. doi: 10.1016/0048-9697(82)90066-3.

[74] E.J. Calabrese, L.A. Baldwin, L.A., Defining hormesis. Hum. Exp. Toxicol. 21 (2002) 91-97. doi: 10.1191/0960327102ht217oa.

[75] A. Hamdoun, D. Epel, Embryo stability and vulnerability in an always changing world, Proc Nat Acad Sci USA 104 (2007) 1745-1750. doi: 10.1073/ pnas.06101 08104.

[76] C.C. Franklin, Enhanced glutathione: biosynthetic capacity promotes resistence to AS^{3+}-induced apoptosis, Toxicol Lett 193 (2010) 33-40. doi: 10.1016/j.toxlet. 2009 .12.004.

Graphical abstract

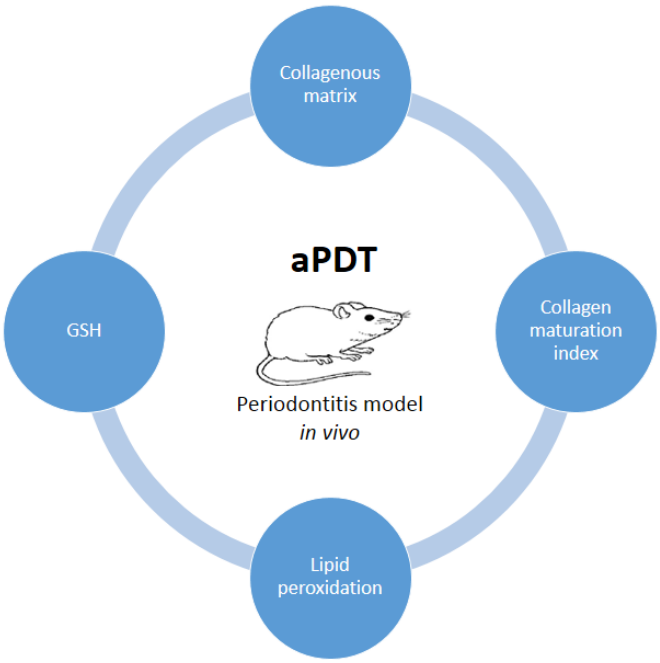


Figure 1. Experimental procedures.

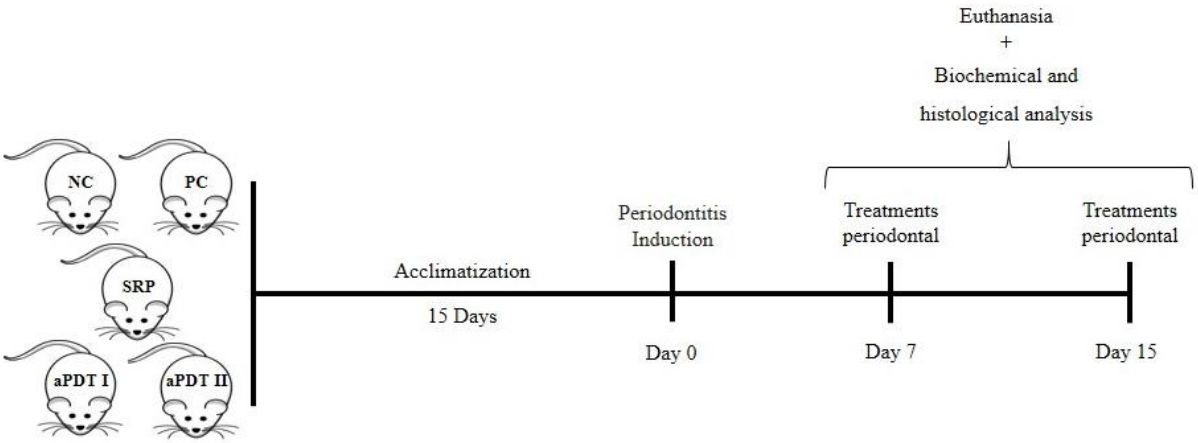
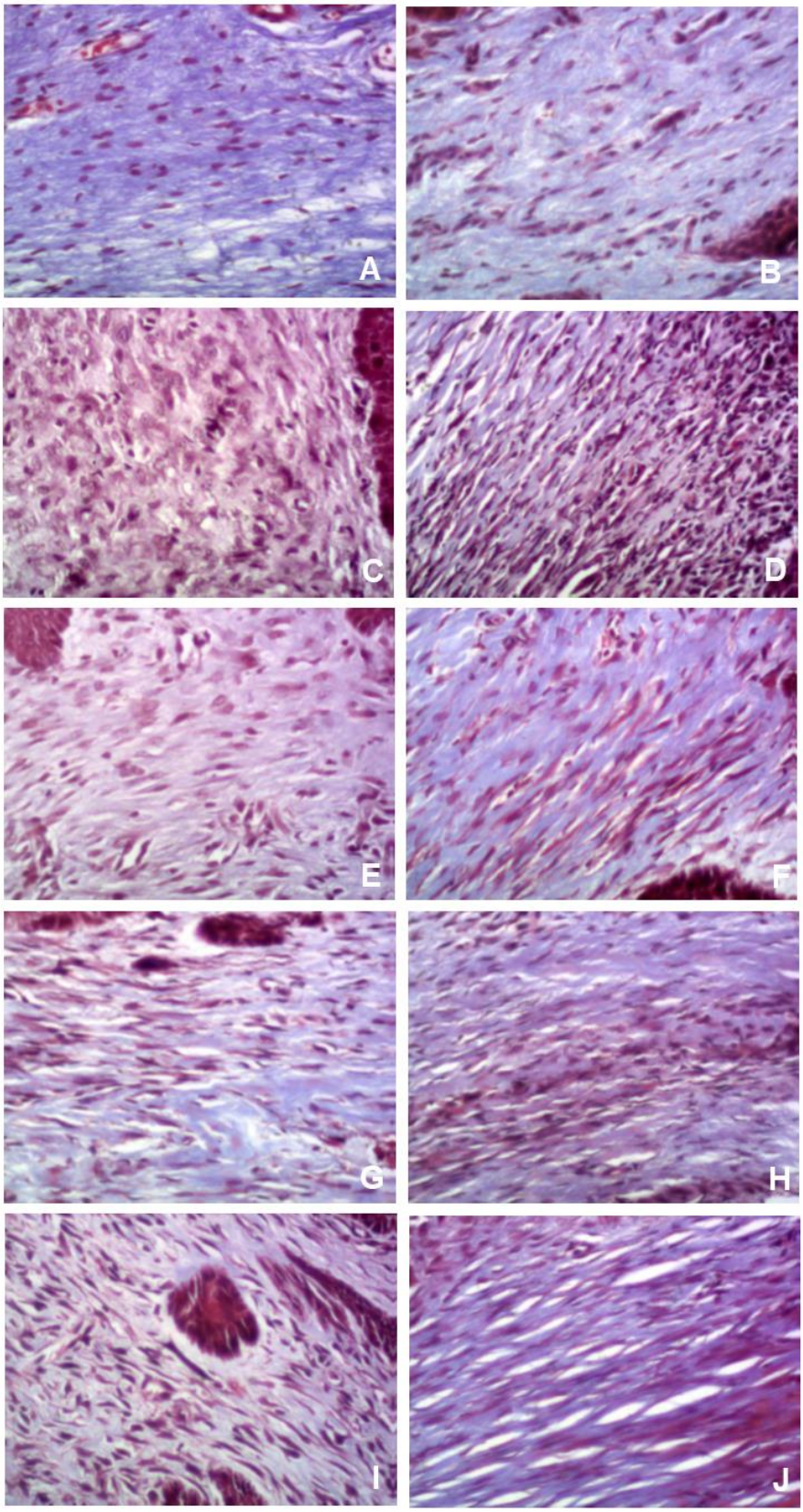
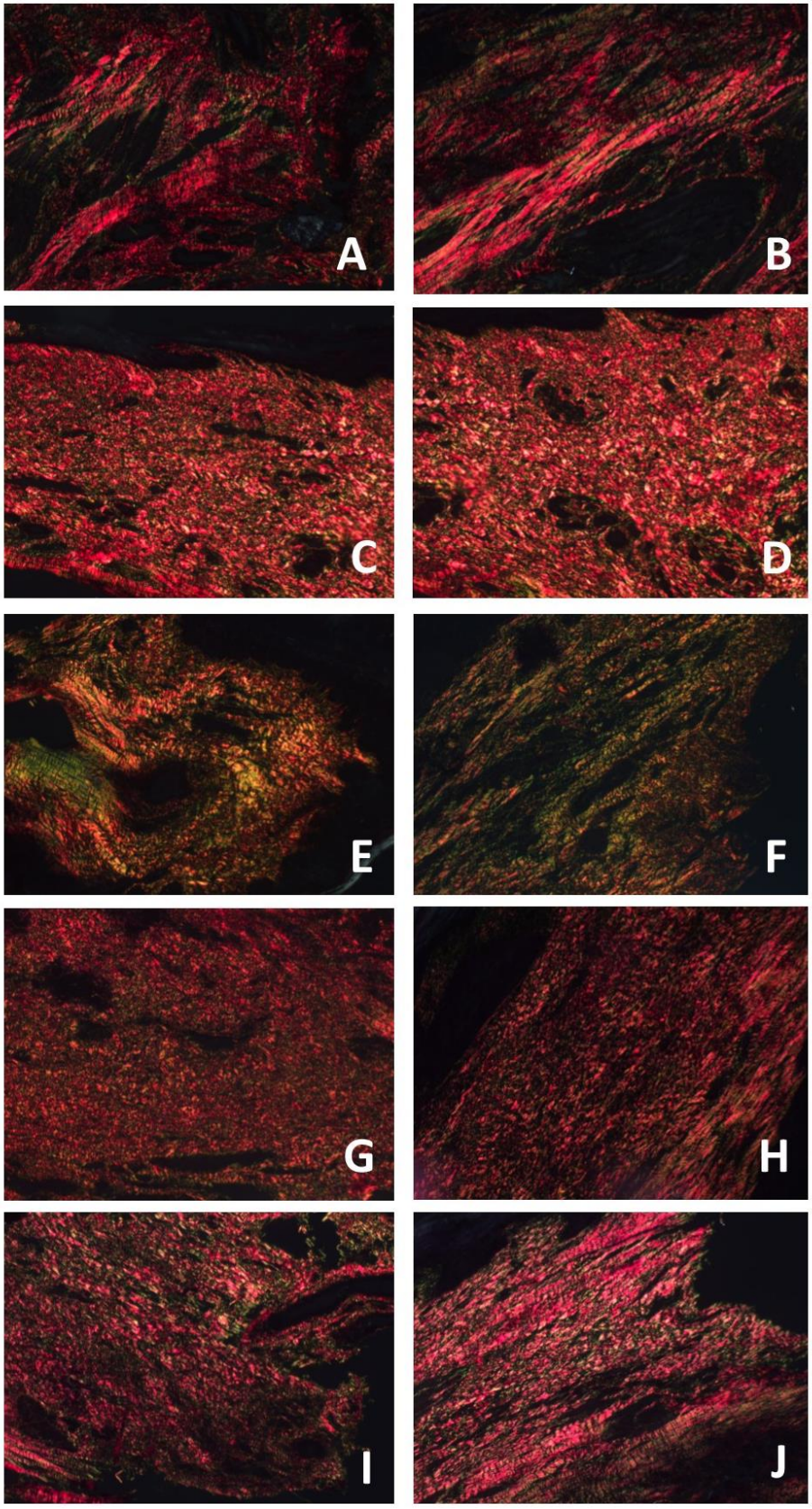


Figure 2. Influence of aPDT on the total collagen area of the gingiva.



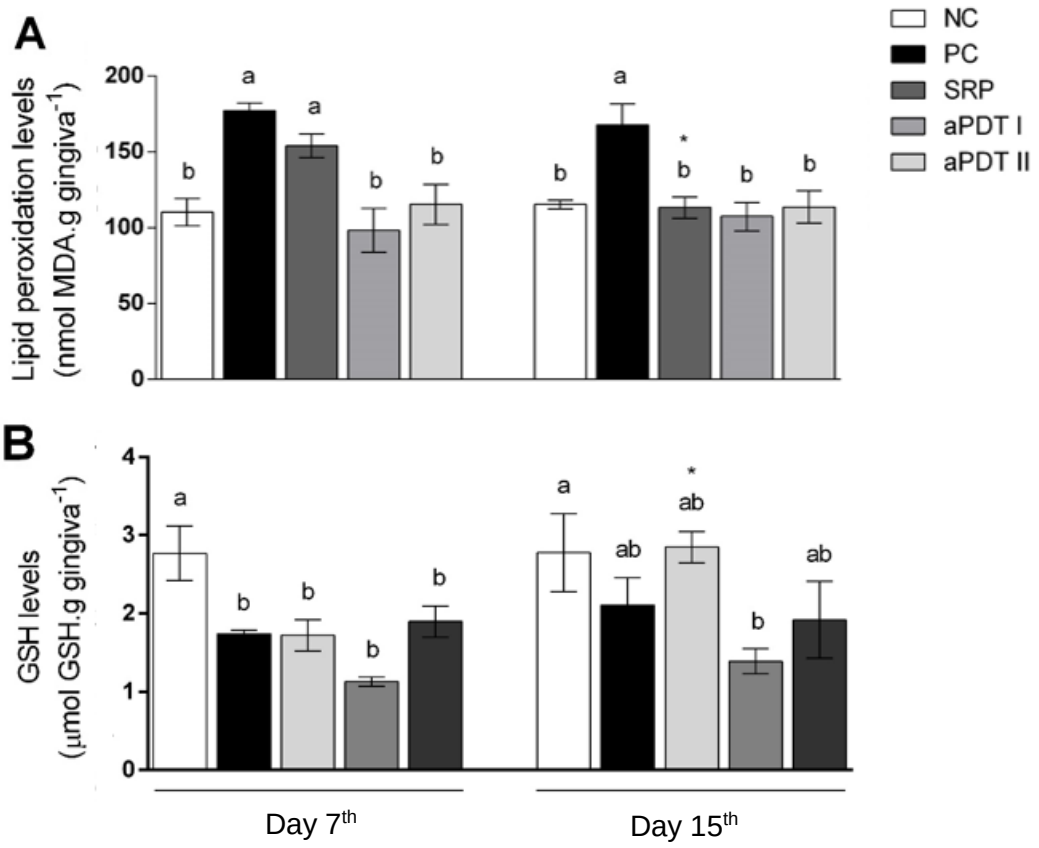
Photomicrograph of the gingival tissue in the region of the first lower molar. NC group: negative control; without disease and without periodontal treatment at 7 days (A) and 15 days (B). Group PC: positive control; disease periodontal and without periodontal treatment at 7 days (C) and 15 days (D). SRP: with disease periodontal and SRP treatment at 7 days (E) and 15 days (F). aPDT I: disease periodontal and treatment aPDT / water at 7 days (G) and 15 days (H). aPDT II: disease periodontal and treatment aPDT / ethanol at 7 days (I) and 15 days (J). (Masson trichrome, original magnification of 400x).

Figure 3. Influence of aPDT on the collagen maturation index.



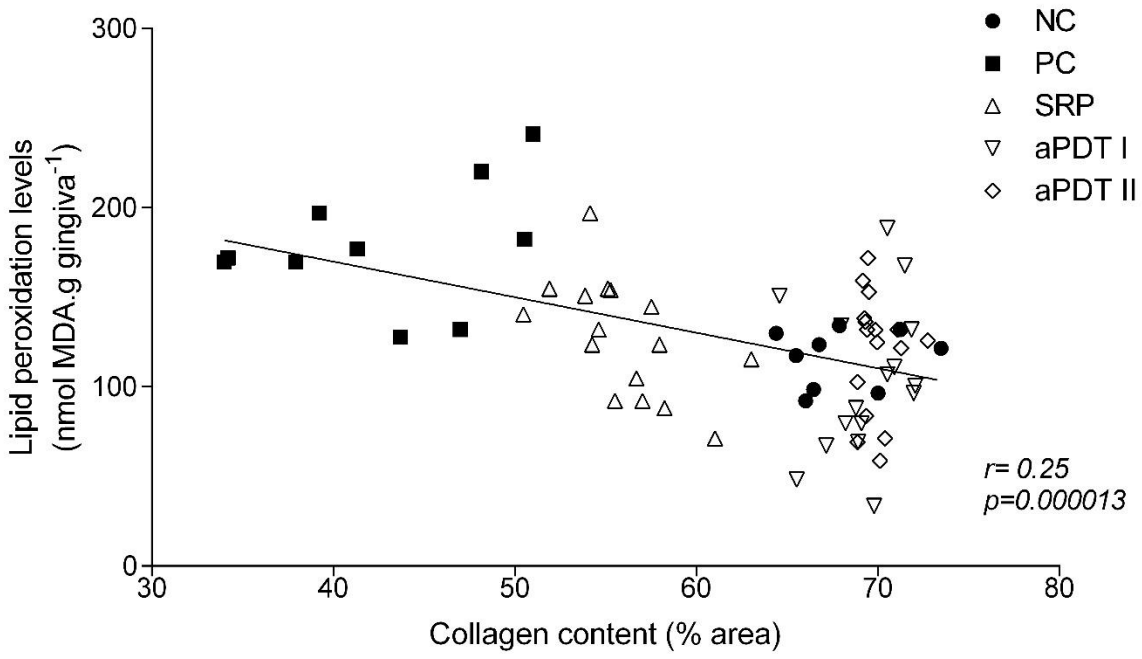
Photomicrograph of the gingival tissue in the region of the first lower molar. NC group: negative control; without disease and without periodontal treatment at 7 days (A) and 15 days (B). Group PC: positive control; disease periodontal and without periodontal treatment at 7 days (C) and 15 days (D). SRP: with disease periodontal and SRP treatment at 7 days (E) and 15 days (F). aPDT I: disease periodontal and treatment aPDT / water at 7 days (G) and 15 days (H). aPDT II: disease periodontal and treatment aPDT / ethanol at 7 days (I) and 15 days (J). (Picrosirius Red, original magnification of 200x).

Figure 4. Influence of aPDT on lipid peroxidation levels and antioxidant defense levels in gingiva.



Influence of TFDa with photosensitizer diluted in different solvents, used as adjuvant to SRP, in the periodontal treatment on gingival levels of lipid peroxidation (A), GSH levels (B). NC (negative control; no periodontitis); PC (positive control; periodontitis without any treatment); SRP (periodontitis and scaling and root planing), aPDT I (periodontitis and SRP+aPDT+MB solubilized in water), and aPDT II (periodontitis and SRP+aPDT+MB solubilized in ethanol 20%). Different lowercase letters (a-b) indicate significant difference between periodontal treatment at the same time of evaluation. * Indicates significant difference of day 7 in the same treatment periodontal disease ($P < 0.05$).

Figure 5. Linear regression analysis of the lipid peroxidation and collagen degradation in the gingiva.



Statistical analysis revealed the following significance levels of P for the values of r -0.25 (P = 0; 00013). NC (negative control; no periodontitis); PC (positive control; periodontitis without any treatment); SRP (periodontitis and scaling and root planing), aPDT I (periodontitis and SRP+aPDT+MB solubilized in water), and aPDT II (periodontitis and SRP+aPDT+MB solubilized in ethanol 20%).

Table 1. Influence of aPDT with methylene blue diluted in water (aPDT I) and in water/ethanol 20% (aPDT II), used as an adjunct to SRP, in the periodontal treatment on the bone loss, collagen degradation and deposition of rats gingiva with periodontitis.

Tissue component	NC group	PC group	SRP group	aPDT I group	aPDT II group
Bone loss					
Day 7th	131.22±2.65^{d}	525.23±30.90^{a}	332.53±8.68^{b}	267.05±5.28^{bc}	257.47±6.14^{c}
Collagenous matrix					
Day 7th	66.97±0.94^{a}	37.34±1.42^{c}	54.16±0.87^{b}	67.95±0.90^{a}	69.65±0.28^{a}
Day 15th	69.25±1.45^{a}	48.23±1.06^{c}*	57.52±1.00^{b}*	70.43±0.49^{a}	70.20±0.42^{a}
CMI					
Day 7th	5.42±0.81^{a}	3.12±1.41^{ab}	0.72±0.10^{b}	2.74±0.70^{ab}	3.48±0.50^{ab}
Day 15th	6.30±1.19^{a}	0.42±0.07^{b}	1.76±0.44^{b}	3.18±0.73^{ab}	2.83±0.40^{ab}

CMI: collagen maturation index. MB: methylene blue. Groups: NC: negative control (no periodontitis); PC: positive control (periodontitis and without any treatment; SRP (periodontitis and scaling and root planning); aPDT I (periodontitis, scaling and root planning and aPDT with MB solubilized in water); aPDT II (periodontitis, scaling and root planning and aPDT with MB solubilized in ethanol 20%). Data are mean ± SEM. Different lower case letters (a-d) indicate significant difference among periodontal treatment in the same evaluation time. *Indicates significant difference of 7th day evaluation in the same periodontal treatment (P<0.05).

Highlights

- aPDT accelerates and facilitates the collagen gingival repair.
- aPDT protected collagen gingival in short-term.
- MB solubilized in ethanol 20% protected gingival lipoperoxidation and the antioxidant defense.

3 CONCLUSÃO

Nossos resultados utilizando o protocolo TFDa II (AM solubilizado em solução de etanol 20% adjuvante a RAR) foi semelhante nas fibras colágenas e no dano oxidativo gengival ao TFDa I (AM solubilizado apenas em água adjuvante a RAR). Em outras palavras, a solubilização do AM em etanol 20% evitou a peroxidação lipídica e a degradação do colágeno a curto prazo induzidos pela periodontite. Considerando que a periodontite e o consequente estado oxidativo na gengiva foram modificados pela TFDa, é possível que essas alterações fisiológicas estejam relacionadas à hormese. Mais estudos são necessários para melhor compreensão dos mecanismos de hormese a nível molecular e celular nas diferentes abordagens em busca de prevenção e tratamento de doenças periodontais.

REFERÊNCIAS

ADRIAENS, P. A. et al. Ultrastructural observations on bacterial invasion in cementum and radicular dentin of periodontally diseased human teeth. **Journal of Periodontology**, v. 59, n. 8, p. 493–503, ago. 1988.

AKALIN, F. A. et al. Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. **Journal Clinical Periodontolology**, v. 34, n. 7, p. 558-565, jul. 2007.

ALBANDAR, J. M.; BRUNELLE, J. A.; KINGMAN, A. Destructive periodontal disease in adults 30 years of age and older in the United States, 1988-1994. **Journal of Periodontology**, v. 70, n. 1, p. 13-29, 1999.

AL-HARTHI, L. S. et al. The impact of periodontitis on oral health-related quality of life: a review of the evidence from observational studies. **Australian Dental Journal**, v. 58, n. 3, p. 274-277, 2013.

ALMEIDA, J. M. et al. In vivo effect of photodynamic therapy on periodontal bone loss in dental furcations. **J Periodontol** n. 79, p. 1081-1088, 2008.

ANGELIS, G. A. M. C. Associação da doença periodontal com Síndrome Coronária Aguda: um estudo caso-controle. 2011. 78 f. Dissertação (Mestrado em Saúde Pública) – Faculdade de Saúde Pública, Universidade de São Paulo, São Paulo. 2011.

AXELSSON, P.; NYSTRÖM, B.; LINDHE, J. The long-term effect of a plaque control program on tooth mortality, caries and periodontal disease in adults. Results after 30 years of maintenance. **Journal of Clinical Periodontology**, v. 31, n. 9, p. 749–757, set. 2004.

BALTACIOĞLU, E. et al. Total antioxidant capacity and superoxide dismutase activity levels in serum and gingival crevicular fluid in post-menopausal women with chronic periodontitis. **Journal Clinical Periodontology**, v. 33, n. 6, p. 385-392, jun. 2006.

BARIN, L.M. et al. Role of the adjunctive antimicrobial photodynamic therapy to periodontal treatment at plasmatic oxidative stress and vascular behavior. **Journal of Photochemistry and Photobiology B: Biology**, v. 173, p. 538–544, 2017.

BARTOLD, P. M.; MCCULLOCH, C. A. Information generation and processing systems that regulate periodontal structure and function. **Periodontology 2000**, v. 63, p. 7-13, 2013.

BARTOLD, P. M.; VAN DYKE, T. E. Periodontitis: a host-mediated disruption of microbial homeostasis. Unlearning learned concepts. **Periodontology 2000**, v. 62, n. 1, p. 203-217, jun. 2013.

BERGSTRÖM, J.; ELIASSON, S.; DOCK, J. A 10-year prospective study of tobacco smoking and periodontal health. **Journal of Periodontology**, v. 71, n. 8, p. 1338-47, ago. 2000.

BRUNETTI MC. Periodontia Médica: Uma Abordagem Integrada, Editora: SENAC – São Paulo, p.23-39, 2004.

BULLON, P. et al. Mitochondrial dysfunction promoted by Porphyromonas gingivalis lipopolysaccharide as a possible link between cardiovascular. **Free Radical Biology and Medicine**, v. 50, p. 1336-1343, 2011.

CARVALHO, A. S. et al. Photodynamic therapy reduces bone resorption and decreases inflammatory response in an experimental rat periodontal disease model. **Photomedicine and Laser Surgery**, v. 29, n. 11, p. 735-740, nov. 2011.

CASSELL, G. H.; MEKALANOS, J. Development of antimicrobial agents in the era of new and reemerging infectious diseases and increasing antibiotic resistance. **Journal of the American Medical Association**, v. 285, n. 5, p. 601-605, 7 fev. 2001.

CASTANO, A. P.; DEMIDOVA, T. N.; HAMBLIN, M. R. Mechanisms in photodynamic therapy: part one—photosensitizers, photochemistry and cellular localization. **Photodiagnosis and Photodynamic Therapy**, v. 1, n. 4, p. 279-293, dez. 2004.

CHANG, P.-C. et al. Progression of periodontal destruction and the roles of advanced glycation end products in experimental diabetes. **Journal of Periodontology**, v. 84, n. 3, p. 379-388, mar. 2013.

CHAPPLE, I. L. C.; MATTHEWS, J. B. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. **Periodontology 2000**, v. 43, p. 160-232, jan. 2007.

CHAPPLE, I. L. Reactive oxygen species and antioxidants in inflammatory diseases. **Journal of Clinical Periodontology**, v. 24, n. 5, p. 287-296, maio. 1997.

CHAVRIER, C. et al. Connective tissue organization of healthy human gingiva. Ultra structural localization of collagen types I-111-IV. **Journal of Periodontal Research**, v. 19, p. 221-229, 1984.

CHRISTODOULIDES, N. et al. Photodynamic Therapy as an Adjunct to Non-Surgical Periodontal Treatment: A Randomized, Controlled Clinical Trial. **Journal of Periodontology**, v. 79, p. 1638-1644, 2008.

COBB, C. M. Clinical significance of non-surgical periodontal therapy: an evidence-based perspective of scaling and root planing. **Journal Clinical Periodontology**, v. 29, n. 2, p. 6-16, 2002.

COCHRAN, D. L. Inflammation and bone loss in periodontal disease. **Journal of Periodontology**, v. 79, n. 8, p. 1569–1576, 1 ago. 2008.

DALAI, C. et al. Correlation between histopathological aspects of periodontitis and biochemical changes of oxidative stress. **Romanian Journal of Morphology Embryology**, v. 54, n. 3, p. 817-822, jan. 2013.

EJEIL, A. L. et al. Are cytokines linked to collagen breakdown during periodontal disease progression? **Journal of Periodontology**, v. 74, p. 196-201, 2003.

GARCIA, V. G. et al. Treatment of experimental periodontitis in rats using repeated adjunctive antimicrobial photodynamic therapy. **Lasers in Medical Science**, v. 28, p. 143-150, abr. 2013.

GEORGE, S.; KISHEN, A. Photophysical, photochemical, and photobiological characterization of methylene blue formulations for light-activated root canal disinfection. **Journal of Biomedical Optics**, v. 12, n. 3, p. 29-34, 2007.

HYVÄRINEN, K; SALMINEN, A; SALOMAA, V; PUSSINEN, PJ. Systemic exposure to a common periodontal pathogen and missing teeth are associated with metabolic syndrome. **Acta Diabetol**. v. 52, p. 179-182, 2016.

KESLER, G.; KOREN, R.; KESLER, A.; KRISTT, D.; GAL, R. Differences in histochemical characteristics of gingival collagen after ER:YAG laser periodontal plastic surgery. **Journal of Clinical Laser Medicine & Surgery**, v. 18, n. 4, p. 203-207, 2000.

KHALILI, J.; BILOKLYTSKA, H. F. Salivary malondialdehyde levels in clinically healthy and periodontal diseased individuals. **Oral Diseases**, v. 14, n. 8, p. 754-60, nov. 2008.

KINANE, D. F.; BERGLUNDH, T; LINDHE, J. Patogênese da periodontite. In: LINDHE, J.; KARRING, T.; LANG, N.P. **Tratado de periodontia clínica e implantologia oral.** 5. ed.Rio de Janeiro: Guanabara, 2010. cap. 11, p. 271–291.

LALLIER, T. E. et al. A simple cell motility assay demonstrates differential motility of human periodontal ligament fibroblasts, gingival fibroblasts, and pre-osteoblasts. **Cell and Tissue Research**, v.328, p. 339-354, 2007.

LOE, H.; THELAIDE, E.; JENSEN, S. B. Experimental gingivitis in man. **The Journal of Periodontology**, v. 36, p. 177–187, 1965.

LORENCINI, M. et al. A new paradigm in the periodontal disease progression: gingival connective tissue remodeling with simultaneous collagen degradation and fibers thickening. **Tissue Cell**, v. 41, n. 1, p.43-50, 2009.

MASSON, P. Some histological methods. Trichrome stainings and their preliminary technique, **J Tech Methods** v. 12. p.75, 1929.

MATTHEWS, J. B. et al. Hyperactivity and reactivity of peripheral blood neutrophils in chronic periodontitis. **Clinical and Experimental Immunology**, v. 147, n. 2, p. 255-264, fev. 2007.

MEISEL, P.; KOCHER, T. Photodynamic therapy for periodontal diseases: State of the art. **Journal.of Photochemistry and Photobiology. B: Biology**, v. 79, n. 2, p. 159-70, 2005.

MINOR, R. R. Collagen Metabolism: A Comparison of Diseases of Collagen and Diseases Affecting Collagen. **American Journal of Pathology**, v. 98, n. 1, Jan. 1980.

MONTES, G. S.; JUNQUEIRA, L. C. U. The use of the pirosirius-polasirius method for the study of the biopathology of collagen. **Memórias do Instituto Oswaldo Cruz**, v. 86, p. 1-11, 1991.

MOSELEY, R.; WADDINGTON, R.J.; EMBERY,G. Degradation of glycosaminoglycans by reactive oxygen species derived from stimulated polymorphonuclear leukocytes. **Biochimica et Biophysica Acta**, v. 1362, p. 221-231, 1997.

NARAYANAN, A. S.; PAGE, R. C.; MEYERS, D. F. Characterization of collagens of diseased human gingiva. **Biochemistry**, v. 19, p. 5037-43, 1980.

OCHSNER, M. Photophysical and photobiological processes in the photodynamic therapy of tumours. **Journal of Photochemistry and Photobiology B: Biology**, v. 39, n. 1, p. 1-18, maio. 1997.

PILLUSKY, F. M. et al. Antimicrobial photodynamic therapy with photosensitizer in ethanol improves oxidative status and gingival collagen in a short-term in periodontitis. **Photodiagnosis and Photodynamic Therapy**, 2017.

REN, L. et al. Lipopolysaccharide-binding protein down-regulates the expression of interleukin-6 by human gingival fibroblast. **Journal of Periodontal Research**, v. 40, p. 407-416, 2005.

ROLIM, J. P. M. L. et al. The antimicrobial activity of photodynamic therapy against Streptococcus mutans using different photosensitizers. **Journal of Photochemistry and Photobiology B: Biology**, v. 106, p. 40-46, 5 jan. 2012.

SAKALLIOGLU, U. et al. Reactive oxygen species scavenging activity during periodontal mucoperiosteal healing: an experimental study in dogs. Arch. **Oral Biol.** 50:1040-1046; 2005.

SCHAFER, F. Q.; BUETTNER, G. R. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. **Free Radical Biology & Medicine**, v. 30, n. 11, p. 1191-1212, 1 jun. 2001.

SÉGUIER, S.; GODEAU, G.; BROUSSE, N. Collagen fibers and inflammatory cells in healthy and diseased human gingival tissues: A comparative and quantitative study by immunohistochemistry and automated image analisis. **Journal of Periodontology**, v. 71, p. 1079-1085,.jul. 2000.

SLOTS, J. Research, Science and Therapy Committee. Systemic antibiotics in periodontics. **Journal of Periodontology**, v. 75, n. 11, p. 1553-65, nov. 2004.

SUSIN, C. et al. Periodontal attachment loss in an urban of Brazilian adults: effect of demographic, behavioral and environmental risk indictors. **Journal of Periodontology**, v.75, n.7, p. 1033-1041, fev. 2004.

TAKASAKI, A.A. et al. Application of antimicrobial photodynamic therapy in periodontal and peri-implant diseases. **Periodontol.** 2000. 51:109-140; 2009.

THOMADAKI, K.; BOSCH, J.A.; OPPENHEIM1, F.G.; HELMERHORST, E.J. The diagnostic potential of salivary protease activities in periodontal health and disease. **Oral Dis**. 19:781-788; 2013.

TSAI, C. C. et al. Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. **Journal Periodontal Research**, v. 40, n. 5, p. 378-384, out. 2005.

WADDINGTON, R. J.; MOSELEY, R.; EMBERY, G. Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. **Oral Diseases**, v. 6, n. 3, p. 138-151, mai. 2000.

ANEXO A - APROVAÇÃO PELA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Influência do solvente fotossensibilizador utilizado na terapia fotodinâmica antimicrobiana no tratamento de periodontite experimental em ratos diabéticos e não diabéticos"

Número do Parecer: 027/2013

Pesquisador Responsável: Prof. Dra. Cristiane Cadermatori Danesi

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO: 05/09/2013

Santa Maria, 05 de setembro de 2013.

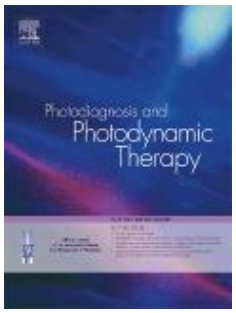
Prof. Dr. Alexandre Krause
Coordenador da Comissão de Ética no Uso de Animais-UFSM

ANEXO B - NORMAS PARA PUBLICAÇÃO NO PERIÓDICO PHOTODIAGNOSIS AND PHOTODYNAMIC THERAPY.

PHOTODIAGNOSIS AND PHOTODYNAMIC THERAPY

AUTHOR INFORMATION PACK

TABLE OF CONTENTS



ISSN: 1572-1000

DESCRIPTION

Official Journal of the European Platform for Photodynamic Medicine Affiliated with the International Photodynamic Association

Also affiliated with the British Medical Laser Association and the Polish Society for Photodynamic Medicine

INDEXED in MEDLINE/PubMed, SciSearch/Science Citation Index Expanded, Current Contents/Clinical Medicine.

Aims and Scope:

Photodiagnosis and Photodynamic Therapy is an international journal for the dissemination of scientific knowledge and clinical developments of **Photodiagnosis** and **Photodynamic Therapy** in all medical specialties. The journal publishes original articles, review articles, case presentations, "how-to-do-it" articles, Letters to the Editor, short communications and relevant images with short descriptions. All submitted material is subject to a strict peer-review process.

AUDIENCE

Professionals in all medical disciplines with an interest in medical and biological applications of lasers and light sources, and photodiagnosis/photodynamic therapy in the treatment of human disease.

IMPACT FACTOR

2017: 2.895 © Clarivate Analytics Journal Citation Reports 2018

ABSTRACTING AND INDEXING

Current Contents / Clinical Medicine

Journal Citation Reports - Science Edition

SciSearch/Science Citation Index Expanded

Scopus

ScienceDirect

National Library of Medicine

Medline/Index Medicus

EDITORIAL BOARD

GUIDE FOR AUTHORS

INTRODUCTION

Scope

Photodiagnosis and Photodynamic Therapy is an international journal for the dissemination of scientific knowledge and clinical developments of Photodiagnosis and Photodynamic Therapy in all medical specialities. The journal publishes original articles, review articles, case presentations, "how-to-do-it" articles, Letters to the Editor, short communications and relevant images with short descriptions. All submitted material is subject to a strict peer review process.

Types of manuscript

Research Papers should report original clinical studies or research not previously published or being considered for publication elsewhere. Work in Progress may also be submitted. See below for the standard layout. Submission of a manuscript to this journal gives the publisher the right to publish that paper if it is accepted. Manuscripts may be edited to improve clarity and expression.

Review articles, including institutional reviews of recent developments are welcome, and will undergo peer review. Reviews should have an abstract of up to 250 words.

Editorials

Although most Editorials in the journal are commissioned, authors may contact the Editor-in-Chief to request submission of their own Editorial.

Correspondence. Readers are encouraged to write about any topic that relates to photodiagnosis or photodynamic therapy, clinical, scientific, educational, social or economic. Letters should be no longer than 500 words and may include discussions on material previously printed in the Journal.

Case report will be considered if formatted as a research letter with 2 figures maximum. Maximum length is up to 1000 words with up to 6 references and 2 tables or figures. There should be no Abstract and no headings.

Short Communications should not exceed 1000 words and should consist of a background section (not to exceed 100 words), aims (not to exceed 50 words), methods (not to exceed 250 words), results (not to exceed 250 words) and conclusion (not to exceed 250 words). An abstract of 150-200 words should also be provided. The editorial team reserves the right to decide which tables/figures submitted are necessary.

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

- E-mail address
- Full postal address

All necessary files have been uploaded:

Manuscript:

- Include keywords
- All figures (include relevant captions)
- All tables (including titles, description, footnotes)
- Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print *Graphical Abstracts / Highlights files* (where applicable) *Supplemental files* (where applicable)

Further considerations

• Manuscript has been 'spell checked' and 'grammar checked'
All references mentioned in the Reference List are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Internet)

• A competing interests statement is provided, even if the authors have no competing interests to declare

• Journal policies detailed in this guide have been reviewed
• Referee suggestions and contact details provided, based on journal requirements

For further information, visit our Support Center.

BEFORE YOU BEGIN

Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

Declaration of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Authors must disclose any interests in two places: 1. A summary declaration of interest statement in the title page file (if double-blind) or the manuscript file (if single-blind). If there are no interests to declare then please state this: 'Declarations of interest: none'. This summary statement will be ultimately published if the article is accepted.

Detailed disclosures as part of a separate Declaration of Interest form, which forms part of the journal's official records. It is important for potential interests to be declared in both places and that the information matches. More information.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic

thesis, see 'Multiple, redundant or concurrent publication' for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service Crossref Similarity Check.

Preprints

Please note that preprints can be shared anywhere at any time, in line with Elsevier's sharing policy. Sharing your preprints e.g. on a preprint server will not count as prior publication (see 'Multiple, redundant or concurrent publication' for more information).

Use of inclusive language

Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Articles should make no assumptions about the beliefs or commitments of any reader, should contain nothing which might imply that one individual is superior to another on the grounds of race, sex, culture or any other characteristic, and should use inclusive language throughout. Authors should ensure that writing is free from bias, for instance by using 'he or she', 'his/her' instead of 'he' or 'his', and by making use of job titles that are free of stereotyping (e.g. 'chairperson' instead of 'chairman' and 'flight attendant' instead of 'stewardess').

Changes to authorship

Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.

Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (see more information on this). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations. If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases.

For gold open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (more information). Permitted third party reuse of gold open access articles is determined by the author's choice of user license.

Author rights

As an author you (or your employer or institution) have certain rights to reuse your work. More information.

Elsevier supports responsible sharing

Find out how you can share your research published in Elsevier journals.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies

Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some funding bodies will reimburse the author for the gold open access publication fee. Details of existing agreements are available online.

After acceptance, open access papers will be published under a noncommercial license. For authors requiring a commercial CC BY license, you can apply after your manuscript is accepted for publication.

Open access

This journal offers authors a choice in publishing their research:

Subscription

- Articles are made available to subscribers as well as developing countries and patient groups through our universal access programs.

- No open access publication fee payable by authors.
- The Author is entitled to post the accepted manuscript in their institution's repository and make this public after an embargo period (known as green Open Access). The published journal article cannot be shared publicly, for example on ResearchGate or Academia.edu, to ensure the sustainability of peer-reviewed research in journal publications. The embargo period for this journal can be found below.

Gold open access

- Articles are freely available to both subscribers and the wider public with permitted reuse.
- A gold open access publication fee is payable by authors or on their behalf, e.g. by their research funder or institution.

Regardless of how you choose to publish your article, the journal will apply the same peer review criteria and acceptance standards.

For gold open access articles, permitted third party (re)use is defined by the following Creative Commons user licenses:

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

For non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article. The gold open access publication fee for this journal is **USD 2500**, excluding taxes. Learn more about Elsevier's pricing policy: https://www.elsevier.com/openaccesspricing.

Green open access

Authors can share their research in a variety of different ways and Elsevier has a number of green open access options available. We recommend authors see our open access page for further information. Authors can also self-archive their manuscripts immediately and enable public access from their institution's repository after an embargo period. This is the version that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and in editor-author communications. Embargo period: For subscription articles, an appropriate amount of time is needed for journals to deliver value to subscribing customers before an article becomes freely available to the public. This is the embargo period and it begins from the date the article is formally published online in its final and fully citable form. Find out more

This journal has an embargo period of 12 months.

Elsevier Researcher Academy

Researcher Academy is a free e-learning platform designed to support early and mid-career researchers throughout their research journey. The "Learn" environment at Researcher Academy offers several interactive modules, webinars, downloadable guides and resources to guide you through the process of writing for research and going through peer review. Feel free to use these free resources to improve your submission and navigate the publication process with ease.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Referees

Referees

Please submit, with the manuscript, the names, addresses and e-mail addresses of three potential referees who are willing to review the article (please obtain confirmation from the referees before submitting your manuscript). Note that the editor retains the sole right to decide whether or not the suggested reviewers are used.

Revised version of the manuscript

On the basis of the comments of the referees and editors, Authors may be asked to revise their manuscript. In order to facilitate the evaluation of the revisions by the referees and editors, upon revision, Authors are asked:

• to indicate all changes to the original manuscript by means of 'track changes'
• to add a letter for the referees, explaining how they dealt with all of the recommendations and questions from the referees. Authors should submit their revised version no later than 9 months after they were informed about the decision that the manuscript needs revision. If no revised manuscript is received 9 months after the decision, the manuscript will be considered as rejected.

PREPARATION

Style

Please use **no abbreviations**. Headlines and Subheadlines should be liberally employed in the Methods, Results, and Discussion sections. Use short paragraphs whenever possible. Clarity of expression, good syntax and the avoidance of medical jargon will be appreciated by the editors, reviewers and readers.

References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/ book title, chapter title/article title, year of publication, volume number/book chapter and the article number or pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Peer review

This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of one independent expert reviewer to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. More information on types of peer review.

Open access

This journal offers authors two choices to publish their research;

1. *Open Access*

o Articles are freely available to both subscribers and the wider public with permitted reuse o An open access publication fee is payable by authors or their research funder

2. *Subscription*

o Articles are made available to subscribers as well as developing countries and patient groups through our access programs (https://www.elsevier.com/access) o No open access publication fee

All articles published open access will be immediately and permanently free for everyone to read and download. Permitted reuse is defined by your choice of one of the following Creative Commons user licenses:

Creative Commons Attribution-NonCommercial-NoDerivs (CC-BY-NC-ND): for non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

Elsevier has established agreements with funding bodies. This ensures authors can comply with funding body open access requirements, including specific user licenses, such as CC-BY. Some authors may also be reimbursed for associated publication fees. https://www.elsevier.com/fundingbodies

If you need to comply with your funding body policy you can apply for the CC-BY license after your manuscript is accepted for publication.

To provide open access, this journal has a publication fee which needs to be met by the authors or their research funders for each article published open access. Your publication choice will have no effect on the peer review process or acceptance of submitted articles.

The open access publication fee for this journal is **$USD 2,500** excluding taxes.
Learn more about Elsevier's pricing policy
https://www.elsevier.com/openaccesspricing

Layout of manuscript

Divide the manuscript into the following sections: Title page, Structured Abstract, Key words (3-6), Introduction, Materials and Methods, Results, Discussion, Acknowledgments, References. The editors will consider the use of other sections if more suitable for certain manuscripts.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**

• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

The **Structured Abstract,** of no more than 250 words, should be written with particular care since this will be the only part of the article studied by some readers. The preferred subheadings are: Background, Methods, Results and Conclusions.

The **Introduction** should be brief and set out the purposes for which the study has been performed along with relevant previous studies only where essential.

The **Materials and Methods** should be sufficiently detailed so that readers and reviewers can understand precisely what has been done without studying the references directly. The description may be abbreviated when well accepted techniques are used.

The **Results** should be presented precisely. Keep discussion of their importance to a minimum in this section of the manuscript.

The **Discussion** should directly relate to the study being reported. Do not include a general review of the topic.

Tables should be typed with double spacing and each should be on a separate sheet. They should be numbered consecutively with Arabic numerals, and contain only horizontal lines. Provide a short descriptive heading above each table with footnotes and/or explanations underneath.

Figures should ideally be submitted in high-resolution TIF format, or alternatively in GIF, JPEG/JPG, or EPS format. The figures should be placed in separate files, named purely with the figure numbers (e.g. "Figure1.tif".) The cost of colour figures will be paid by the author.

Legends for Figures should be typed with double-spacing on a separate sheet.

Gene Accession Numbers

For each and every gene accession number cited in an article, authors should type the accession number in bold, underlined text. Letters in the accession number should always be capitalised. Example: (GenBank accession nos. AI631510 , AI631511 , AI632198, and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117).

Supplementary data

The journal accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, movies, animation sequences, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier web products, including ScienceDirect: http://www.sciencedirect.com

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). You can view example Highlights on our information site.

Graphical abstract

Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a

minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site. Authors can make use of Elsevier's Illustration Services to ensure the best presentation of their images and in accordance with all technical requirements.

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.

• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.
A detailed guide on electronic artwork is available.

You are urged to visit this site; some excerpts from the detailed information are given here. *Formats*

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.

Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings, embed all used fonts.

TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.

TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.

TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;

• Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF) or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) in addition to color reproduction in print. Further information on the preparation of electronic artwork.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is highly encouraged.

A DOI is guaranteed never to change, so you can use it as a permanent link to any electronic article. An example of a citation using DOI for an article not yet in an issue is: VanDecar J.C., Russo R.M., James D.E., Ambeh W.B., Franke M. (2003). Aseismic continuation of the Lesser Antilles slab beneath northeastern Venezuela. Journal of Geophysical Research, https://doi.org/10.1029/2001JB000884. Please note the format of such citations should be in the same style as all other references in the paper.

Data references

This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

Reference management software

Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley. Using citation plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript. More information on how to remove field codes from different reference management software.

Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following

link:

http://open.mendeley.com/use-citation-style/photodiagnosis-and-photodynamic-therapy

When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice.

Reference formatting

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/ book title, chapter title/article title, year of publication, volume number/book chapter and the article number or pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.

Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'

List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.

Examples:

Reference to a journal publication:

[1]J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59. https://doi.org/10.1016/j.Sc.2010.00372.

Reference to a journal publication with an article number:

[2]Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2018. The art of writing a scientific article. Heliyon. 19, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.

Reference to a book:

[3] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000. Reference to a chapter in an edited book:

[4]G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304. Reference to a website:

[5] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/ aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).

Reference to a dataset:

[dataset] [6] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. https://doi.org/10.17632/ xwj98nb39r.1.

Data visualization

Include interactive data visualizations in your publication and let your readers interact and engage more closely with your research. Follow the instructions here to find out about available data visualization options and how to include them with your article.

Research data

This journal encourages and enables you to share data that supports your research publication where appropriate, and enables you to interlink the data with your published articles. Research data refers to the results of observations or experimentation that validate research findings. To facilitate reproducibility and data reuse, this journal also encourages you to share your software, code, models, algorithms, protocols, methods and other useful materials related to the project.

Below are a number of ways in which you can associate data with your article or make a statement about the availability of your data when submitting your manuscript. If you are sharing data in one of these ways, you are encouraged to cite the data in your manuscript and reference list. Please refer to the "References" section for more information about data citation. For more information on depositing, sharing and using research data and other relevant research materials, visit the research data page.

Data linking

If you have made your research data available in a data repository, you can link your article directly to the dataset. Elsevier collaborates with a number of repositories to link articles on ScienceDirect with relevant repositories, giving

readers access to underlying data that gives them a better understanding of the research described.

There are different ways to link your datasets to your article. When available, you can directly link your dataset to your article by providing the relevant information in the submission system. For more information, visit the database linking page.

For supported data repositories a repository banner will automatically appear next to your published article on ScienceDirect.

In addition, you can link to relevant data or entities through identifiers within the text of your manuscript, using the following format: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN).

Mendeley Data

This journal supports Mendeley Data, enabling you to deposit any research data (including raw and processed data, video, code, software, algorithms, protocols, and methods) associated with your manuscript in a free-to-use, open access repository. During the submission process, after uploading your manuscript, you will have the opportunity to upload your relevant datasets directly to *Mendeley Data*. The datasets will be listed and directly accessible to readers next to your published article online.

For more information, visit the Mendeley Data for journals page.

Data statement

To foster transparency, we encourage you to state the availability of your data in your submission. This may be a requirement of your funding body or institution. If your data is unavailable to access or unsuitable to post, you will have the opportunity to indicate why during the submission process, for example by stating that the research data is confidential. The statement will appear with your published article on ScienceDirect. For more information, visit the Data Statement page.

Process of submission

Online submission

Photodiagnosis and Photodynamic Therapy uses an online submission and review system. Authors can upload their article via the Elsevier Editorial System at https://www.evise.com/profile/api/navigate/PDPDT. By accessing the website Authors will be guided stepwise through the uploading of the various files. Editable file formats are necessary. We accept most wordprocessing formats, but Word, WordPerfect or LaTeX is preferred. Figure files (TIFF, EPS, JPEG) should be uploaded separately. Always keep a backup copy of the electronic file for reference and safety. Save your files using the default extension of the program used. The system generates an Adobe Acrobat PDF version of the article which is used for the reviewing process. Authors, Reviewers and Editors send and receive all correspondence by e-mail and no paper correspondence is necessary. For assistance please visit our Support Center.

AFTER ACCEPTANCE

Online proof correction

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.

If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.

We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Offprints

The corresponding author will, at no cost, receive a customized Share Link providing 50 days free access to the final published version of the article on ScienceDirect. The Share Link can be used for sharing the article via any communication channel, including email and social media. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's Webshop. Corresponding authors who have published their article gold open access do not receive a Share Link as their final published version of the article is available open access on ScienceDirect and can be shared through the article DOI link.

AUTHOR INQUIRIES

Visit the Elsevier Support Center to find the answers you need. Here you will find everything from Frequently Asked Questions to ways to get in touch.

You can also check the status of your submitted article or find out when your accepted article will be published.