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INFLUÊNCIA DO FOTOSSENSIBILIZADOR AZUL DE METILENO DISSOLVIDO EM ETANOL NA TERAPIA FOTODINÂMICA ANTIMICROBIANA SOBRE O STATUS OXIDATIVO SISTÊMICO E COLÁGENO GENGIVAL EM MODELO EXPERIMENTAL DE PERIODONTITE

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil
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INFLUÊNCIA DO FOTOSSENSIBILIZADOR AZUL DE METILENO DISSOLVIDO EM ETANOL NA TERAPIA FOTODINÂMICA ANTIMICROBIANA SOBRE O STATUS OXIDATIVO SISTÊMICO E COLÁGENO GENGIVAL EM MODELO EXPERIMENTAL DE PERIODONTITE

Fernanda Maia Pillusky

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 grau de
Mestre em Ciências Odontológicas.

Orientadora: Prof[a] Dr[a] Cristiane Cademartori Danesi
Coorientadora Dr[a] Raquel Cristine Silva Barcelos

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Universidade Federal de Santa Maria
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elaborada por
Fernanda Maia Pillusky

como requisito parcial para obtenção do grau de
Mestre em Ciências Odontológicas

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“Se as coisas são inatingíveis... ora!
Não é motivo para não querê-las...
Que tristes os caminhos, se não fora
A mágica presença das estrelas!”
Mário Quintana

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Odontológicas
Universidade Federal de Santa Maria

INFLUÊNCIA DO FOTOSSENSIBILIZADOR AZUL DE METILENO DISSOLVIDO EM ETANOL NA TERAPIA FOTODINÂMICA ANTIMICROBIANA SOBRE O STATUS OXIDATIVO SISTÊMICO E COLÁGENO GENGIVAL EM MODELO EXPERIMENTAL DE PERIODONTITE

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Data e Local da Defesa: Santa Maria, 28 de agosto de 2015.

O objetivo deste estudo foi avaliar os efeitos da terapia fotodinâmica antimicrobiana (TFDa) usando o fotossensibilizador azul de metileno (AM) dissolvido em etanol sobre o status oxidativo sistêmico, bem como sobre o conteúdo de colágeno gengival de ratos com periodontite. Ratos machos Wistar foram divididos aleatoriamente em dois grupos principais: CN (controle negativo; sem periodontite) e os animais restantes foram o grupo submetido a indução de periodontite. No último grupo, a ligadura de algodão foi colocada no primeiro molar inferior direito de cada animal em uma posição subgengival para induzir a periodontite experimental. Os animais com periodontite foram subdivididos em grupos de acordo com o tratamento periodontal, como segue: grupo RAR (raspagem e alisamento radicular), TFDa I grupo (RAR + TFDa + AM dissolvido em água), e grupo TFDa II (RAR + TFDa + AM dissolvido em etanol). Após 7 dias, a ligadura foi removida e foram realizados os tratamentos periodontais. Aos 7, 15 e 30 dias, os ratos foram submetidos à eutanásia e foi removido o tecido gengival para análise morfométrica. Os eritrócitos foram usados para avaliar o status oxidativo sistêmico. O status oxidativo demostrou maiores níveis de peroxidação lipídica no grupo CP em 7, 15 e 30 dias, e indicou uma influência protetora da TFDa II, nos eritrócitos, já em 15 dias, observada a partir da elevação dos níveis de defesa antioxidante sistêmica. Os achados morfométricos mostraram que o grupo TFDa II restabeleceu o percentual de área total de colágeno também em 15 dias, bem como recuperou a área de colágeno tipo I no mesmo tempo. A partir deste estudo podemos sugerir que TFDa utilizada como um adjuvante ao tratamento padrão periodontal (RAR) aumenta a resposta protetora sistêmica contra o estresse oxidativo induzido pela periodontite, facilitando e acelerando a cicatrização periodontal, particularmente quando o azul de metileno é solubilizado em etanol.

Palavras-chave: Doença Periodontal. Estresse Oxidativo. Gengiva. Colágeno Tipo I. Colágeno Tipo III. Eritrócitos.

ABSTRACT
Master's Dissertation
Graduate Program in Dental Sciences
Federal University of Santa Maria

INFLUENCE OF THE PHOTOSENSITIZER METHYLENE BLUE DISSOLVED IN ETHANOL IN THE ANTIMICROBIAL PHOTHODYNAMIC THERAPY ON SYSTEMIC OXIDATIVE STATUS AND GINGIVAL COLLAGEN IN A MODEL OF EXPERIMENTAL PERIODONTITIS

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CO-ADVISOR: Raquel Cristine Silva Barcelos
Date and Place of Defense: August 28th, 2015, Santa Maria.

The purpose of this study was to evaluate the effects of an antimicrobial photodynamic therapy (aPDT) employing the photosensitizer methylene blue dissolved in ethanol on the systemic oxidative status likewise on the gingival collagen content of rats with periodontitis. Male Wistar rats were randomly divided into two main groups: NC (negative control; no periodontitis) and the remaining animals were submitted to periodontitis induction group. In the last group, the cotton ligature was placed at the first right mandibular molar of each animal in a submarginal position to induce experimental periodontitis. The animals with periodontitis were subdivided into groups according to the periodontal treatment as follow: SRP group (scaling and root planing), aPDT I group (SRP+aPDT+MB dissolved in water), and aPDT II group (SRP+aPDT+MB dissolved in ethanol). After 7 days, the ligature was removed and periodontal treatments were performed. At 7, 15 and 30 days, rats were euthanized and gingival tissue was removed for morphometric analysis. The erythrocytes were used to evaluate systemic oxidative status. Besides that, it indicated a protective influence of aPDT II in erythrocytes already at 15 days observed by the elevation in levels of systemic antioxidant defense. The morphometric findings showed that aPDT II group was the only one that restored the percentage of total collagen area also in 15 days, as well as, aPDT II group recovered the type I collagen area at the same time-point. According to this study we could suggest that aPDT employed as an adjunct to the standard treatment of periodontitis (SRP) increases systemic protective response against oxidative stress periodontitis-induced facilitating and accelerating the periodontal healing particularly when methylene blue is dissolved in ethanol.

Keywords: Periodontal Disease. Oxidative Stress. Gingiva. Type I Collagen. Type III Collagen. Erythrocytes.

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%	Por cento
µs	Microssegundo
DNA	Ácido Desoxirribonucleico
EO	Estresse Oxidativo
EROs	Espécies Reativas de Oxigênio
GSH	Glutationa Reduzida
H_2O_2	Peróxido de Hidrogênio
HOCl	Ácido hipocloroso
MDA	Malondialdeído
NO	Óxido Nítrico
$O2^{\cdot -}$	Superóxido
$^{\cdot}OH$	Hidroxil
RAR	Raspagem e Alisamento Radicular
TBARS	Substâncias Reativas ao Ácido Tiobarbitúrico; Thiobarbituric Acid Reactive Substances
TFDa	Terapia Fotodinâmica Antimicrobiana

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INTRODUÇÃO

A periodontite apresenta alta prevalência nas populações, tanto em países desenvolvidos (ALBANDAR et al., 1999), como em países em desenvolvimento (SUSIN et al., 2004) e tem importante impacto na qualidade de vida dos indivíduos (AL-HARTHI et al., 2013). Essa doença é definida como uma alteração inflamatória crônica que acomete os tecidos de proteção e sustentação dental, como resultado de um desequilíbrio na interação entre o biofilme bacteriano e a resposta de defesa do hospedeiro (CHANG et al., 2013; PAGE; KORNMAN, 1997).

Considerando os tecidos periodontais, a gengiva merece atenção, uma vez que tem função de proteção, além de ser o único elemento diretamente visível ao exame clínico, permitindo a observação dos eventos associados à periodontite (SCHROEDER; LISTGARTEN, 1997). O tecido conjuntivo gengival é composto principalmente por colágeno (EJEIL et al., 2003, SCHROEDER; LISTGARTEN, 1997) dos tipos I e III (LALLIER et al., 2007; REN et al.,2005), os quais são essenciais na manutenção da sua estrutura e função (REN et al., 2005; SÉGUIER; GODEAU; BROUSSE, 2000) desempenhando dessa forma um importante papel na progressão das doenças periodontais (LORENCINI et al., 2009; SÉGUIER; GODEAU; BROUSSE, 2000). A matriz colagenosa do tecido conjuntivo gengival apresenta como principal componente estrutural e funcional 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, por sua vez, constitui 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, sendo visualizado na cor verde quando corado com Picrosirius red (MONTES; JUNQUEIRA, 1991).

O processo inflamatório decorrente da periodontite libera mediadores pró-inflamatórios, os quais acionam enzimas proteolíticas latentes (BARTOLD; MCCULLOCH, 2013; KORNMAN; PAGE; TONETTI, 1997; PAGE et al., 1997), alterando a integridade e estabilidade estrutural do tecido gengival (LORENCINI et al 2009; NANCI; BOSSHARDT, 2006). Esse processo é intenso o bastante para iniciar a degradação da matriz colagenosa do tecido conjuntivo (EJEIL et al., 2003; GOGLY et al., 1997; KESLER et al., 2000; LORENCINI et al., 2009; SILVA et al., 2008), podendo alcançar 70% (HAVEMOSE-POULSEN et al., 1998). Essa degradação pode ocorrer tanto de forma quantitativa, como

qualitativa e é característica proeminente da periodontite (EJEIL et al., 2003; LORENCINI et al., 2009;). Neste contexto, a degradação do colágeno tem sido considerada um marcador da progressão da periodontite (LORENCINI et al., 2009).

Reparação é um estado dinâmico, que compreende diferentes processos simultâneos, entre os quais, a inflamação, a proliferação celular e a síntese ou remodelagem dos elementos que constituem a matriz extracelular, tais como o colágeno tipo I e III, (THOMAS; O'NEILL; HARDING, 1995). Na fase proliferativa ocorre a deposição de colágeno tipo III e na remodelagem há a maturação colagenosa, na qual o colágeno tipo III é substituído principalmente pelo colágeno tipo I (EYDEN; TZAPHLIDOU, 2001; JUNQUEIRA; COSSERMELLI, BRENTANI, 1979; LIU et al., 1997; ROBSON; STEED; FRANZ, 2001). Na cicatrização periodontal, não diferente, esses processos dinâmicos ocorrem para o reestabelecimento funcional tecidual (LARJAVA et al., 1990).

Na periodontite, um dos mecanismos de defesa dos neutrófilos do hospedeiro contra o biofilme bacteriano é a liberação de espécies reativas de oxigênio (EROs) (AKALIN et al., 2008, TATAKIS; KUMAR, 2005), levando ao aumento dos seus níveis teciduais e sistêmicos (MOSELEY; WADDINGTON; EMBERY, 1997). Esses produtos são, principalmente, os radicais superóxido ($O2^{\cdot-}$) e hidroxil ($^{\cdot}OH$), o peróxido de hidrogênio (H_2O_2), óxido nítrico ($NO^{\cdot}$), ácido hipocloroso (HOCl) e oxigênio singleto (CHAPPLE; MATTHEWS, 2007; CHAPPLE, 1997; WADDINGTON; MOSELEY; EMBERY, 2000). Embora várias células possam sintetizar as EROs, a fonte mais significativa são os neutrófilos (CHAPPLE, 1997; CHAPPLE; MATTHEWS, 2007). No entanto, apesar das EROs serem essenciais para o metabolismo celular normal, elas podem provocar danos nos tecidos quando presentes em altos níveis (CHAPPLE, 1997; CHAPPLE; MATTHEWS, 2007; WADDINGTON; MOSELEY; EMBERY, 2000).

Contra os efeitos nocivos das EROS, o organismo possui um sistema de defesa antioxidante (AKALIN et al., 2008). Os antioxidantes podem ser classificados em enzimáticos (superóxido dismutase, catalase, glutationa redutase e glutationa peroxidase) e não enzimáticos (ácido úrico, vitamina C, vitamina E e glutationa reduzida (GSH), entre outros (SCHAFER; BUETTNER, 2001). Quando a produção das EROs excede 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).

A excessiva produção de EROs e o consequente EO tem sido envolvidos na apoptose, inflamação e dano ao DNA (BRIGANTI; PICARDO, 2003). Da mesma forma, têm sido

relacionados à patogênese de muitas doenças, incluindo, mais recentemente, à periodontite (AKALIN et al., 2007; BAUER; BAUER, 1999; BORGES et al., 2007 CHAPPLE, 1997; CHAPPLE; MATTHEWS, 2007; GUSTAFSSON; ASMAN, 1996; MATSUI et al., 2011; MATTHEWS et al., 2007; SEYMOUR; WHYTE; POWELL, 1986; WADDINGTON; MOSELEY; EMBERY, 2000). Esses elevados níveis de EROs gerados por neutrófilos do sangue periférico estão presentes na periodontite durante a resposta inflamatória induzida pelo biofilme bacteriano na cavidade oral e podem atacar o tecido gengival, o ligamento periodontal e os osteoblastos (GUSTAFSSON; ASMAN, 1996; MATTHEWS et al., 2007; SEYMOUR; WHYTE; POWELL, 1986), sendo consideradas um fator inflamatório na doença (ZDARILOVÁ et al., 2010). Estudos recentes revelam uma redução na capacidade antioxidante e um aumento de biomarcadores de EO na periodontite (AKALIN et al., 2007; BALTACIOĞLU et al., 2014; BALTACIOĞLU et al. 2006; MASHAYEKHI et al., 2005; MATTHEWS et al., 2007; TSAI et al., 2005). Além disso, o EO induz progressivamente a destruição da estrutura periodontal, osso alveolar e tecido conjuntivo (BULLON et al., 2011; LI; GULBINS; ZHANG, 2012; PETTI; SCULLY, 2009). Mostrando uma forte relação dos aspectos bioquímicos com as alterações teciduais (DALAI et al., 2013).

O tratamento convencional da periodontite é a raspagem e alisamento radicular (RAR) (COBB, 2002), caracterizada pela remoção mecânica do biofilme em contato com as superfícies dentárias, cujo objetivo primário é desorganizá-lo para restaurar a homeostasia tecidual, uma vez que o biofilme bacteriano desempenha um papel crucial na patogenia da periodontite (BARTOLD; VAN DYKE, 2013; COBB, 2002). Neste contexto, o emprego de tal estratégia tem sido o método universalmente aceito para conter a progressão da doença. Assim, através do controle da infecção bacteriana, as superfícies dentárias tornam-se biocompatíveis, há a liberação de moléculas que modulam a inflamação, estimulando a proliferação celular e a formação do tecido de granulação (BARTOLD; VAN DYKE, 2013). Entretanto, esta modalidade terapêutica pode apresentar efetividade comprometida, tanto quando a periodontite está associada a fatores de risco que modificam a resposta do hospedeiro, como durante o Diabetes Mellitus (TERVONEN; OLIVER, 1993), tabagismo (BERGSTRÖM; ELIASSON; DOCK, 2000), periodontites agressivas (ARWEILER et al., 2014), bem como em algumas situações específicas em que o acesso mecânico é dificultado, como nas regiões de furcas e áreas de concavidades radiculares (WENNSTRÖM; DAHLÉN; RAMBERG, 2011). Nestas situações o uso de antimicrobianos vem sendo proposto (SLOTS, 2004). Contudo, a rápida emergência da resistência bacteriana frente ao uso indiscriminado e prolongado de antibióticos tem se tornado um problema (CASSELL; MEKALANOS, 2001; HAMBLIN; HASAN, 2004; YOSHIKAWA, 2002).

Nesse contexto, a terapia fotodinâmica antimicrobiana (TFDa) associada à RAR tem ganhado força na odontologia moderna (ALVARENGA et al., 2015; BERAKDAR et al., 2012; BOTTURA et al., 2011; DAI; HUANG; HAMBLIN, 2009; GURSOY et al., 2013; HUANG et al., 2012; NOVAES et al., 2012; PRATES et al., 2011; WOOD et al., 1999), como uma alternativa para o uso de antibióticos, uma vez que não é invasiva, possui um amplo espectro de ação, pouco potencial de desenvolver resistência bacteriana e não possui dose limite (BERAKDAR et al., 2012; DAI; HUANG; HAMBLIN, 2009; GURSOY et al., 2013; NOVAES et al., 2012; PRATES et al., 2011).

A TFDa consiste na aplicação de um fotossensibilizador, que ativado por luz de comprimento de onda apropriado, na presença de oxigênio, gera EROs citotóxicas às células-alvo, resultando em morte celular (CASTANO; DEMIDOVA; HAMBLIN, 2004; GE et al., 2011; MAISCH, 2007; ROLIM et al., 2012; SOUKOS; GOODSON, 2011). Durante a fotoativação, a molécula do fotossensibilizador absorve energia passando do seu estado fundamental para o estado singleto excitado. Nesta 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, porém mais estável. O fotossensibilizador no estado tripleto pode sofrer dois tipos de reações (Figura 1). 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; MAISCH, 2007). 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 simultaneamente e a razão entre elas depende tanto da concentração de substrato e de oxigênio, quanto do tipo de fotossensibilizador utilizado (CASTANO; DEMIDOVA; HAMBLIN, 2004).

Estudos vêm sendo realizados visando a avaliação da TFDa como adjuvante ao tratamento periodontal padrão, utilizando como fotossensibilizador o azul de metileno dissolvido em água (CHRISTODOULIDES et al., 2008; MEISEL; KOCHER, 2005). No entanto, evidências mostram que esta formulação do fotossensibilizador resulta em limitada produção de oxigênio singleto associada com curta meia vida (4μs) (MEISEL; KOCHER, 2005) e baixo potencial de difusão tecidual do mesmo (OCHSNER, 1997). Essas propriedades do fotossensibilizador podem ser influenciadas pelo solvente no qual o fotossensibilizador é dissolvido. Recentes pesquisas apontam melhores propriedades fotofísicas, fotoquímicas e fotobiológicas do azul de metileno dissolvido em solvente menos polar que a água, aumentando a penetrabilidade tecidual e meia-vida do oxigênio singleto

(GEORGE; KISHEN, 2007; MEISEL; KOCHER, 2005). Além disso, a inclusão do etanol como solvente do azul de metileno aumentou o efeito antimicrobiano da TFDa frente a biofilmes de *Enterococcus faecalis* e *Aggregatibacter actinomycetemcomitans* (GEORGE; KISHEN, 2007).

Dutra et al. (2013), em um estudo *in vitro*, confirmaram que a produção de EROs oriunda da reação tipo I e tipo II foram maiores quando o azul de metileno foi dissolvido na formulação contendo etanol. Tais resultados podem ser atribuídos à maior produção de oxigênio singleto, aumento da sua meia vida em cinco vezes (20µs) e a menor agregação molecular do azul de metileno. Mediante a desagregação das moléculas do fotossensibilizador, há maior formação da reação tipo II, porém se agregadas em forma de dímeros ocorre maior atividade para transferências eletrônicas com o substrato (mecanismo da reação do tipo I) devido a menor capacidade de capturar energia (PATIL; PAWAR; TALAP, 2000). Deste modo, haverá baixa atividade para transferência de energia ao oxigênio molecular (mecanismo da reação tipo II), resultando em menor produção de oxigênio singleto (GABRIELLI et al., 2004; SEVERINO et al., 2003). Entretanto, ainda que haja um aprimoramento das propriedades físicas e químicas desse fotossensibilizador, a literatura é escassa de estudos experimentais e clínicos utilizando essa formulação e, por isso, seus efeitos teciduais e sistêmicos não estão claramente elucidados.

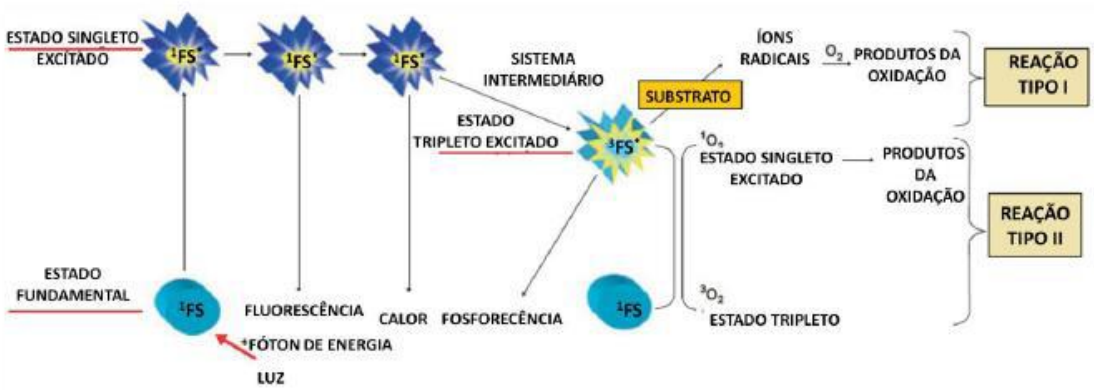

Figura 1. Mecanismo de ação da TFDa; reações Tipo I e Tipo II (Adaptado de Soukos; Goodson, 2011).

A TFDa, como adjuvante ao tratamento periodontal padrão, vem demonstrando vantagens em modelos experimentais de periodontite através de colocação da ligadura no primeiro molar inferior de ratos. Tal modelo vem sendo utilizado para analisar a ação da TFDa na periodontite em ratos sem comprometimento sistêmico (CARVALHO et al., 2011; DE ALMEIDA et al., 2008; GARCIA et al., 2013b), diabéticos (ALMEIDA et al., 2008; AL-ZAHRANI et al., 2009), submetidos à nicotina (GARCIA et al., 2011), imunossuprimidos (BOTTURA et al., 2011) e ovariectomizados (GARCIA et al., 2013a). Nesses estudos foram

avaliados diversos desfechos, por variados métodos como a análise histológica (CARVALHO et al., 2011), morfométrica (ALMEIDA et al., 2008; BOTTURA et al., 2011; GARCIA et al., 2011), imunohistoquímica (GARCIA et al., 2013a) e radiográfica (FERNANDES et al., 2010).

Assim, o objetivo do presente estudo foi avaliar os efeitos da TFDa usando o fotossensibilizador azul de metileno dissolvido em etanol sobre o status oxidativo sistêmico, bem como sobre o conteúdo de colágeno gengival de ratos com periodontite.

OBJETIVOS

Objetivo geral

Avaliar os efeitos da TFDa usando o fotossensibilizador azul de metileno dissolvido em etanol sobre o status oxidativo sistêmico, bem como sobre o conteúdo de colágeno gengival de ratos com periodontite.

Objetivos específicos

- Avaliar os efeitos da TFDa usando o fotossensibilizador azul de metileno dissolvido em etanol sobre o status oxidativo sistêmico de ratos com periodontite;
- Avaliar a influência do fotossensibilizador azul de metileno dissolvido em etanol na TFDa sobre o conteúdo total de colágeno gengival de ratos com periodontite;
- Avaliar a influência do fotossensibilizador azul de metileno dissolvido em etanol na TFDa sobre o conteúdo de colágeno tipo I e tipo III gengival de ratos com periodontite.

MANUSCRITO

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 Free Radical Biology and Medicine, Qualis A1, Fator de impacto 5,736. Os itens Materiais e Métodos, Resultados, Discussão e Referências encontram-se no próprio manuscrito, o qual está disposto da mesma forma que foi submetido.

Manuscrito submetido para Free Radical Biology & Medicine e encontra-se sob revisão.

Antimicrobial photodynamic therapy employing a photosensitizer dissolved in ethanol improves systemic oxidative status and promotes gingival collagen synthesis in a short-term on experimental periodontitis in rats

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Abstract

Objective: The purpose of this study was to evaluate the effects of the antimicrobial photodynamic therapy (aPDT) using the methylene blue (MB) dissolved in ethanol on systemic oxidative status and collagen content from gingiva of rats with periodontitis.

Methods: Male Wistar rats were randomly divided into two main groups: NC (negative control; no periodontitis) and the remaining animals were submitted to periodontitis induction group. In the last group, the cotton ligature was placed at the first right mandibular molar of each animal in a submarginal position to induce experimental periodontitis. The animals with periodontitis were subdivided into groups according to the periodontal treatment as follow: SRP group (scaling and root planing), aPDT I group (SRP+aPDT+MB dissolved in water), and aPDT II group (SRP+aPDT+MB dissolved in ethanol). After 7 days, the ligature was removed and periodontal treatments were performed. At 7, 15 and 30 days, rats were euthanized and gingival tissue was removed for morphometric analysis. The erythrocytes were used to evaluate systemic oxidative status.

Results: The oxidative status showed higher lipid peroxidation levels in PC group at 7, 15 and 30 days. Besides that, it indicated a protective influence of aPDT II in erythrocytes already at 15 days observed by the elevation in levels of systemic antioxidant defense. The morphometric findings showed that aPDT II group was the only one that restored the percentage of total collagen area also in 15 days, as well as, aPDT II group recovered the type I collagen area at the same time point.

Conclusion: In summary, our outcomes suggest that the aPDT as an adjunct to the SRP, a standard periodontal treatment, can induce the systemic protective response against oxidative stress periodontitis-induced and recover the gingival collagen, thus promoting the healing periodontal, particularly when the MB is dissolved in ethanol.

***Keywords*:** periodontal disease, type I collagen, type III collagen, oxidative stress, Picrosirius red, methylene blue

Introduction

Periodontitis is an immune-inflammatory disease initiated and maintained by plaque bacteria and their metabolic products, characterized by persistent inflammation, connective tissue breakdown and alveolar bone destruction [1]. At the present, periodontitis is recognized as one of the most prevalent chronic disease in humans, besides it is the main disease involving human dentition. Thus, affecting a high proportion of the world population [2,3]. Reactive oxygen species (ROS) play a dual role in the pathogenesis of periodontitis: protective and destructive. The ROS released locally during the inflammatory response is one of polymorphonuclear leukocyte defense mechanisms against the pathogenic microflora in periodontitis [4]. Nevertheless, ROS oxidize cellular macromolecules (lipids, proteins and DNA) leading to damages in the periodontal tissue [5]. Besides that, there are strong evidences linking ROS to the pathological destruction of the connective tissue during periodontitis [6]. Additionally, the establishment and progression of periodontitis involves breakdown of collagen [7].

An imbalance between the production of ROS and their detoxification by the antioxidant defense system results in oxidative stress [8], which has been considered a periodontitis biomarker [9]. The antioxidant defense system of periodontal tissue consists in enzymatic and non-enzymatic antioxidants [10]. Of particular significance, lipid peroxides and their metabolic products such as malondialdehyde (MDA) can directly or indirectly affect the homeostasis of the periodontal cells and tissues. Consequently, increased membrane lipid peroxidation can intensify the immune and inflammatory response already installed by periodontitis [11], as the cell proliferation or apoptosis [12]. Therefore, there are close relationships among the ROS production, the loss of antioxidant defenses, the membrane peroxidative damage of cells and inflammatory or degenerative processes of periodontal tissue.

The ROS release is the photodynamic therapy mechanism of action, which involves the combination of visible light (usually by a diode laser) and a photosensitizer [13]. Such medical therapy can destroy selectively bacteria and their by-products [14], thus being called antimicrobial photodynamic therapy (aPDT) [15]. Currently, the aPDT received increased attention as an adjunct tool in periodontitis management for being non-invasive, as well as it has specificity for the target cells, and its activity is initiated only when exposed to the light, besides its inability to develop bacterial resistance [16].

Recent studies evaluated the aPDT in the periodontitis treatment using methylene blue (MB) dissolved in water as a photosensitizer [17,18]. This formulation results in limited singlet oxygen production associated with a short half-life (4μs) [18] and low potential of

diffusion [19]. Thus, the solvent in which the photosensitizer is dissolved can influence the production and half-life of the singlet oxygen. George and Kishen [20] showed that the addition of a lesser polar solvent than water, such as ethanol, increased antimicrobial effect against biofilm. Dutra et al. [21] demonstrated that ROS production was higher in MB formulation containing ethanol as compared to water only. These findings were attributed to an increased production of singlet oxygen along with an increased half-life (20μs) and lower molecular aggregation of MB in the presence of ethanol.

In this context, the present study aimed to evaluate the effects of the aPDT using a photosensitizer dissolved in ethanol on systemic oxidative status likewise on the collagen content from gingiva of rats with periodontitis.

Materials and methods

Animals

Male adult Wistar rats (2 months of age) were used. They were housed at four (±1) animals per Plexiglas cages with free access to food (standard chow; Supralab®, Alisul Alimentos LTDA, São Leopoldo, RS, Brazil) and water in a room with controlled temperature (23±1°C), on a 12h light/dark cycle. Before starting the experimental protocols, animals underwent an acclimatization period of 15 days. The Animal Ethical Committee of Universidade Federal de Santa Maria (027132-UFSM), which is affiliated to the Council of Animal Experiments (CONCEA), following international norms of animal care and maintenance approved all procedures with animals.

Protocol of experimental periodontal disease

The animals were randomly divided into two main groups: NC (negative control; no periodontitis; n=15) and the remaining animals were submitted to periodontitis induction (n=96). In the last group, one mandibular right first molar of each animal received the cotton ligature in a submarginal position to induce experimental periodontitis under general anesthesia (ketamine/xylazine, 70 and 6 mg/kg, intramuscular injection, respectively). The ligature was removed from all animals after 7 days of periodontal disease induction [22], thereafter they were randomly divided into four experimental groups: PC (positive control; without any treatment); SRP (scaling and root planing); aPDT I (SRP plus aPDT with MB dissolved in water); and aPDT II (SRP plus aPDT with MB dissolved in ethanol).

Scaling and root planing procedures

The SRP were performed using the micro Gracey curettes Mini-Five 1-2 (Hu-Friedy®, Chigaco, IL, USA) through 10 distal-mesial traction movements in the buccal and lingual sites. The furcation and interproximal areas were instrumented with the same curettes using cervico-occlusal traction movements [22]. One operator blinded for experimental groups performed all the SRP procedures.

Antimicrobial photodynamic therapy protocols

The aPDT employed two different solvents to dissolve the photosensitizer according to the experimental group: (I) aPDT I used the MB (Sigma-Aldrich, St. Louis, MO, USA) 0.01% solubilized in bidistilled water; and (II) aPDT II used the MB (Sigma-Aldrich, St. Louis, MO, USA) 0.01% solubilized in bidistilled water and ethanol solution. The MB formulation was gently poured into the periodontal pocket around the right mandibular first molar using a syringe (1mL) and an needle (BD® UltrafineTM, U-100, 0.5mL, 8mm x 0.3mm) without a bevel. After 1 minute, low-level laser therapy was applied to three points at each buccal and lingual sides of the right mandibular first molar. The laser employed was an indium-gallium-aluminum-phosphorous (TheraLase®, DMC Equipments, São Carlos, SP, Brazil), 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 29.64J/cm^2. One operator blinded to the experimental groups performed aPDT procedures.

Experimental periods

One third of each experimental group were anesthetized with isoflurane (2-3%) (Isothane®, Baxter Healthcare®, Guayama, Puerto Rico) [23], and euthanized by exsanguination 7, 15, and 30 days after periodontal treatments (Fig. 1).

Biochemical analysis

The blood was collected by cardiac puncture in heparinized tubes and centrifuged at 1300g for 15min to obtain the erythrocytes for the biochemical analysis.

The oxidative damage to lipids, which is estimated by the determination of MDA levels were determined by measuring the accumulation of thiobarbituric acid reactive substances (TBARS) as described by Ohkawa et al. [24], and expressed as nmol MDA/mL erythrocytes.

Reduced glutathione (GSH) levels in erythrocytes, which estimate an antioxidant defense, were determined after reaction with 5,5-dithiobis- (2-nitrobenzoicacid) in accordance with Boyne and Ellman [25] and expressed as nmol GSH/mL erythrocytes.

Morphometric Analysis

Quantitative Determination of Collagen Gingival Content

The area of collagen gingival occupied 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 show colors from yellow to red fibers strongly birefringent, and type III collagen appears green and low birefringence [26].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 [27,28]. For all the gingival samples, all extension of the histological slide was analyzed. For the morphometric determination of the types I and III collagen [29], 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.

Statistical analysis

Levene's test was applied in order to verify the homogeneity of the data. Biochemical and morphometric measurements were analyzed by two-way ANOVA followed by Duncan's multiple range test, when appropriate (Software package Statistica 8.0 for Windows was used). All of the data are expressed as means±SEM. A *P*-value less than 0.05 was considered statistically significant.

Results

Biochemical measurements

Influence of aPDT on lipid peroxidation levels in erythrocytes

A two-way ANOVA of lipid peroxidation revealed a significant main effect of periodontal treatment [F=9.92, *P*<0.0000].

The *post hoc* test showed that PC and SRP groups increased erythrocyte lipid peroxidation levels 7 days after the ligature removal in relation to NC, aPDT I and aPDT II groups, and the values were similar. The evaluations on the 15th and 30th days showed that the erythrocyte lipid peroxidation levels remained higher in PC group compared to other groups, whose values were similar (Fig. 2).

Influence of aPDT on antioxidant defense levels in erythrocytes

A two-way ANOVA of erythrocyte GSH levels revealed a significant main effect of periodontal treatment, experimental periods and a significant periodontal treatment x experimental periods interaction [F=3.33, *P*<0.05; 11.83, *P*<0.0000 and 2.93, *P*<0.01].

The *post hoc* test showed higher erythrocyte GSH levels in PC group compared to all other experimental groups 7 days after the ligature removal. On the 15th day of evaluation, erythrocyte GSH levels in PC, SRP and aPDT II groups showed higher levels than NC and aPDT I groups, and the values of both groups were similar. In addition, aPDT II group presented higher erythrocyte GSH levels 15 days than 7 days after the ligature removal. On the 30th day of evaluation, PC, aPDT I and aPDT II groups showed higher levels than NC and SRP groups, whose values were comparable to each other. In fact, SRP, aPDT I and aPDT II groups showed erythrocyte GSH levels higher at 30th day compared to 7th day of evaluation. Additionally, aPDT I group showed higher erythrocyte GSH levels at 30th day than in 15th day of evaluation (Fig. 3).

Morphometric Analysis

Influence of aPDT on total collagen content of 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=28.72, *P*<0.0000; 40.46, *P*<0.0000 and 4.73, *P*<0.0000] (Table 1; Fig. 4).

On the 7th day of evaluation, NC group showed the highest gingival total collagen area than all other experimental groups. In this same experimental time point, the total collagen

area of the gingiva in aPDT I and aPDT II groups was higher than PC and SRP groups, whose values were comparable.

Additionally, 15 days after the end of periodontal treatment, NC and aPDT II groups showed similar gingival total collagen area, and those values were higher than all other experimental groups. In this same experimental time point of evaluation, the total collagen area of the gingiva in SRP and aPDT I groups was higher than in PC group. In fact, PC and aPDT II groups showed higher total collagen area of the gingiva on the 15th day compared to 7th day of evaluation.

With respect to 30 days after the end of periodontal treatment, PC group showed lower total collagen area of the gingiva than all other experimental groups, whose values were similar. At this same time point of evaluation, PC, SRP, aPDT I and aPDT II showed higher gingival total collagen area compared to 7 days, while SRP and aPDT I groups only showed higher total collagen area of the gingiva compared to 15 days of evaluation.

Influence of aPDT on type I collagen content of gingiva

A two-way ANOVA of type I 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=27.29, P<0.0000; 38.32, P<0.0000 and 4.43, P<0.001] (Table 1; Fig. 4).

On the 7th day of evaluation, NC group showed the highest gingival type I collagen area compared to all other experimental groups. NC and aPDT II groups showed similar type I collagen area of the gingiva, whose values were higher compared to all other experimental groups 15 days after the end of periodontal treatment. In this same experimental time point, gingival type I collagen area of SRP and aPDT I groups were similar and higher compared to PC group. In fact, SRP group showed higher type I collagen area of the gingiva 15 days compared to 7 days after the end of periodontal treatment.

On the 30th day of evaluation, PC group showed lower type I collagen area of the gingiva compared to all other experimental groups and the values were similar. Additionally, PC, SRP, aPDT I and aPDT II groups showed higher type I collagen area of the gingiva 30 days compared to 7 and 15 days after the end of periodontal treatment.

Influence of aPDT on type III collagen content of gingiva

A two-way ANOVA of type III 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=3.57, *P*<0.01; 14.46, *P*<0.0000 and 2.85, *P*<0.001] (Table 1; Fig. 4).

On the 7th day of evaluation, NC and PC groups showed lower gingival type III collagen area compared to all other experimental groups. In this same experimental time point, the gingival type III collagen area of SRP, aPDT I and aPDT II groups was higher than NC and PC groups, whose values were comparable to each other.

PC and SRP groups showed similar type III collagen area of the gingiva, which was higher compared to all other experimental groups 15 days after the end of periodontal treatment. In this same experimental time point, gingival type III collagen area of aPDT I and aPDT II groups were the same. However, those groups showed lower area than PC and SRP groups. PC and SRP groups showed similar type III collagen area of the gingiva, whose values were higher compared to all other experimental groups 15 days after the end of periodontal treatment. The NC group showed lower type III collagen area of the gingiva compared to all other experimental groups at 15th day of evaluation. In fact, PC group showed higher type III collagen area of the gingival at 15 days compared to 7 days after the end of periodontal treatment.

All experimental groups showed similar type III collagen area of the gingival on the 30th day of evaluation. In fact, aPDT I group showed lower type III collagen area of the gingiva at 30th day compared to 7th day of evaluation. Aditionally, PC, SRT, aPDT I and aPDT II groups showed lower gingival type III collagen area on the 30th day of compared to the 15th day of evaluation.

Discussion

The placement of ligatures around teeth is a well-characterized and widely used model of experimental periodontitis in rats [22]. In this study, the periodontitis was successfully induced in Wistar rats by placing a ligature around of the first right molar mandibular in the submarginal position. Moreover, the outcomes of the present investigation affirmed that this experimental model of periodontitis induced an increase in lipid peroxidation levels in erythrocytes of the PC group, observed by MDA levels, throughout the experimental period in comparison to NC group.

The lipid peroxidation is a major outcome of ROS-induced tissue injury [30], since they interact with the polyunsaturated fatty acids in cellular membranes or lipoproteins [11] affecting their structural integrity and function [31]. As lipid peroxidation is an oxidative stress consequence, which has an important role in the pathologic mechanism of periodontitis, MDA levels, as a final product of lipid peroxidation has been used to monitor this process

[32]. In agreement with our findings, studies also demonstrated an increase in lipid peroxidation levels in periodontitis [11,33].

Furthermore, the role of antioxidant defense system in the periodontitis pathology has received increased attention [11]. Among the endogenous non-enzymatic antioxidants, the GSH is considered the main intracellular antioxidant defense due to its nucleophilic properties, which also exists in its oxidized form (GSSG) [34]. Considering that, periodontitis-induced oxidative stress strongly challenges the glutathione system. In the current study, this periodontal disease was related to higher GSH levels in erythrocytes in the PC group in all evaluated time-points compared to NC groups. We believe that these findings may be related with the attempt of this antioxidant defense in counteract high MDA levels observed in PC group. In this regard, GSH together with the blood, represent one of the first systemic defenses against the periodontitis-induced lipid peroxidation [35].

Scaling and root planing procedures are considered the gold standard in periodontal therapy, employing manual instruments to remove supra and subgingival bacterial deposits [36,37], which new therapies have been compared as well [38]. In the present study, SRP group showed lipid peroxidation similar to PC group in 7th day, which can be related to the delayed action of this therapy against to periodontitis-induced lipid peroxidation. In fact, 15 and 30 days after ligature removal, lipid peroxidation in erythrocytes of the SRP group returned to NC group levels, which may have occurred at the expense of increased GSH levels observed at 15th day, and their levels were reduced to NC group levels at 30th day. In accordance to our findings, Mlachkova and Popova [39] demonstrated the effectiveness of SRP as periodontal treatment in moderate and chronic periodontitis. Furthermore, SRP as a periodontal treatment results in a significant decrease of 8-hydroxy-deoxyguanosine, a biomarker of oxidative damage [40].

Recent studies with animals [41] and humans [42] evaluated aPDT in the periodontitis treatment. In this context, the aPDT as tool adjunct to SRP has gained strength in the dentistry field, since it is a non-invasive procedure [43], it has specificity for the target cells, and do not develop bacterial resistance, nor any side effects [16]. Interestingly, in current study, the aPDT I and aPDT II groups exhibited lipid peroxidation levels in erythrocytes similar to NC group in all evaluated time points, affirming its main advantage in localized and specific antimicrobial action on periodontal pockets, which indicates protective influence against systemic oxidative damage periodontitis-induced. Moreover, these groups were also related to similar GSH levels in erythrocytes of NC group 7 days after the ligature removal.

Of particular importance, the dissolution of MB in ethanol (aPDT II group) showed an improvement in the systemic antioxidant defense represented by GSH in erythrocytes 15 days

after the end of periodontal treatment. At 30th day, the aPDT treatments regardless of the vehicle used to dissolve the photosensitizer, were able to increase the GSH levels in erythrocytes compared to their levels exhibit in the evaluations of 7th day. Taken together, these data indicate that when MB is dissolved in ethanol, the aPDT has more effectiveness and improved therapeutic action as an adjunct therapy to SRP in the periodontal treatment. Thereby, this was evidenced by the prevention of periodontitis-induced oxidative damage, besides the increase in the antioxidant defense system in erythrocytes. In this context, promising experimental and clinical results were observed following the application of another laser system, the Er:YAG laser (erbium-doped: yttrium, aluminum, and garnet), in chronic periodontitis [44, 45].

Considering that oxidative stress induces collagen degradation [46] and periodontal tissues are mainly composed by collagen, including soft tissues and bone [47] likewise periodontitis is accompanied by changes in gingival connective tissue composition [48], we also evaluated the influence of aPDT on collagen gingival content of rats with periodontitis. In the present study, the degradation of total gingival collagen reached the maximum level on the 7th day after ligature removal in the PC group. In addition, total collagen of the gingiva was gradually synthesized until the last evaluation in the 30th day in this same group, since showed a gradual increase in its area. Nevertheless, PC group did not completely restore the total collagen area, and did not reach the NC group total collagen area of the gingiva, which can be related to highest MDA levels in erythrocytes following periodontitis induction. As the quantification of the MDA levels is accepted as an oxidative stress biomarker [32], it is feasible that its increase might contribute to collagen degradation observed in PC group. This concept is in agreement with previous reports, which found that oxidative stress is related to lower content of collagen in tissues [49,50]. In addition, degradation of collagen is pointed out as the main marker of periodontal disease progression [51], which is according our data.

The type I and III collagen are the major structural components of the periodontal extracellular matrix. Their synthesis and breakdown are important to maintain the tissue integrity and homeostasis in periodontal connective tissues [52]. Our results showed that the reduction in the total collagen area from gingiva with periodontitis of the PC group is related to the degradation of type I collagen in 7th day of evaluation, which has an important role in the gingival support [53]. As observed in a variety of diseases related to ROS overproduction and to consequent oxidative stress, periodontitis has been described to induce breakdown of type I collagen and collagen degradation in general [54]. Nevertheless, 15 days after ligature removal, the PC group showed higher type III gingival collagen area, which was compared to SRP group. These findings indicate a neoformation of collagen, in which the type III collagen

after maturation became the type I collagen [55]. Interestingly, we observed an increase in the type I collagen area in PC group in the 30th day, which can be related to the maturation of type III collagen and consequent decrease in its area. Thereafter leading to an increase in the total collagen area of gingiva observed in this experimental group. However, 30 days after the ligature removal were not sufficient to restore the total collagen content of the gingiva in PC group.

The aPDT protocols used in this study exerted not only effects on oxidative stress parameters, but also on the collagen content of the gingiva after periodontitis induction. Our results showed that aPDT treatments stimulated the collagen synthesis compared to PC and SRP groups, which have similar amounts at the 7th day. Otherwise, aPDT was not able to recover the total collagen area compared to NC group. Consistent with our results, Pourzarandian et al. [56] reported that the low-laser therapy could promote wound repair through fibroblast proliferation and augmentation of collagen synthesis, thus exhibiting an important role in the extracellular matrix remodeling.

The quantification of gingival collagen type I and type III in rats with ligature-induced periodontitis and submitted to aPDT are still scarce in the literature. In the present study, aPDT groups showed remarkably synthesis of type I collagen, which demonstrated greater area when compared to PC and SRP groups at 7th day after ligature removal. In fact, the aPDT I and II provided neoformation of this macromolecule, however they were still related to a smaller area of gingival type I collagen compared to NC group at this time. These findings may be attributed to the control of periodontitis-induced oxidative stress and consequent partial neoformation of type I collagen by aPDT.

Interestingly, aPDT II group showed capacity to induce the maturation from type III to type I collagen earlier than other treatments (SRP and aPDT I groups). This was demonstrated by higher type I collagen area, and consequently higher total collagen area already in evaluation of the 15th day, which was similar to NC group. In healthy individuals, the type I collagen is found in the highest proportion of the gingival tissue [57]. These findings may be related to the increase of GSH level in erythrocytes observed in aPDT II group after 15th day of evaluation. At the 30th day, all treated groups showed a restoration of gingival collagen content type I and III, which were similar to NC group. To confirm our findings, previous studies have shown that tissue protection against damages to lipids and collagen oxidative stress-mediated can contribute to protect and recover the integrity of gingival tissues [58,59]. From these data, we can suggest that the type III collagen is associated to periodontal disease repair phase (i.e, during periodontal healing the fibroblasts produce high levels of type III collagen and reduced levels of type I collagen) [60] until occurrence of the reestablishment of

functional dynamic conditions, since type III collagen is essential for the formation of type I collagen [55].

Seeking for new potential tools to be used in the treatment of periodontal diseases, the dissolution of MB in ethanol improved its photophysical, photochemical and antimicrobial properties [20]. Here, we demonstrated that such pharmacotechnical manipulation associated to the conventional treatment of periodontitis (SRP) were capable of: 1) prevent systemic lipid peroxidation; 2) increase antioxidant defense; 3) promote collagen synthesis; in addition to 4) promote tissue repair in short-term, which can contribute to the potential therapeutic actions of aPDT in the periodontitis treatment.

Conclusion

So far, no study was developed showing the influence of aPDT, especially with their photosensitizer (MB) dissolved in ethanol on systemic biochemical changes and gingival collagen content, more exactly in the type I and III. As well as the relationship among gingival collagen area and progression or repair stage in periodontitis. From this study, we can propose that aPDT used as an adjunctive to standard periodontal treatment is capable to increase the systemic protective response against periodontitis-induced oxidative stress, thus facilitating and accelerating the periodontal healing, particularly when the MB is dissolved in ethanol.

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Figure 1. Experimental procedures.

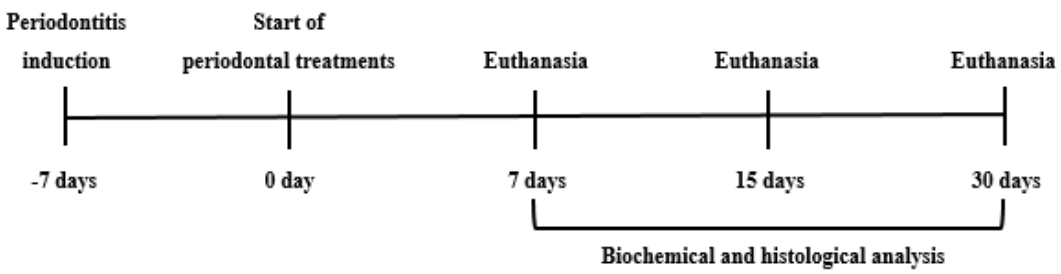


Figure 2. Effects of aPDT with methylene blue dissolved in water (aPDT I) and in ethanol (aPDT II) used as an adjunct to SRP in the periodontal treatment on the lipid peroxidation levels in erythrocytes. Data are expressed as mean ± SEM. Different lowercase letters (a-c) indicate significant differences among periodontal treatment in the same time point evaluated. *Indicates significant differences from 7th day of evaluation in the same periodontal treatment (P<0.05). $^{+}$Indicates significant differences from 15th day of evaluation in the same periodontal treatment (P<0.05).

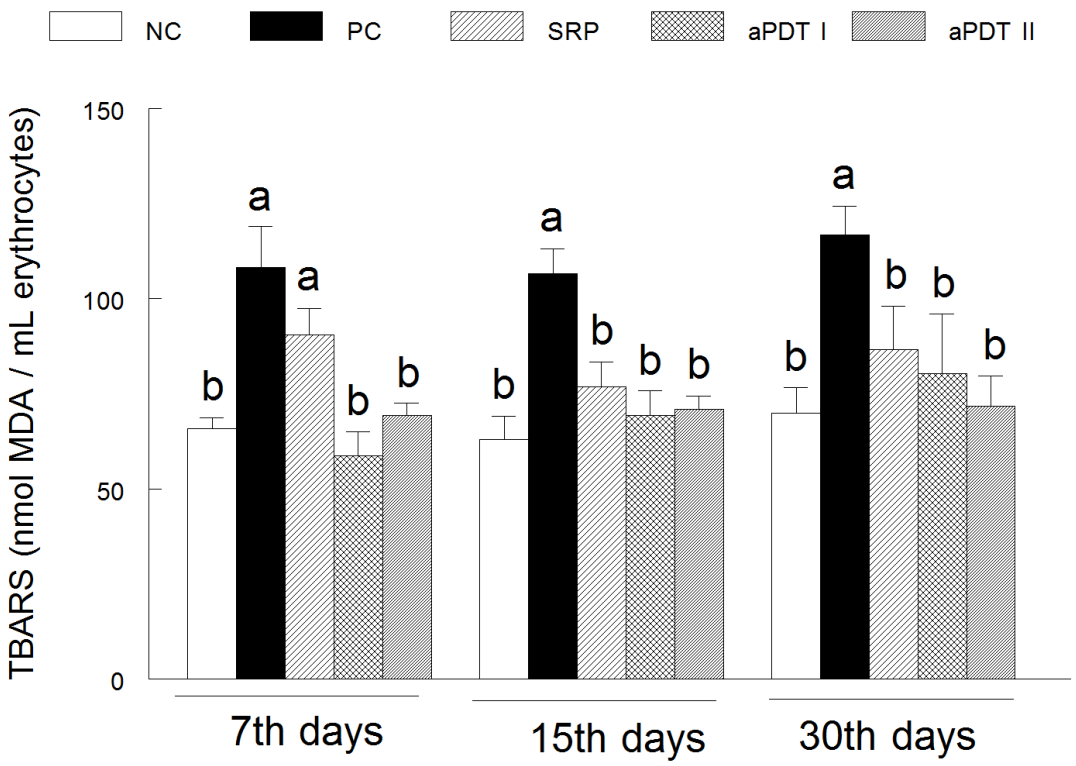


Figure 3. Effects of aPDT with methylene blue dissolved in water (aPDT I) and in ethanol (aPDT II) used as an adjunct to SRP in the periodontal treatment on the GSH levels in erythrocytes. Data are expressed as mean ± SEM. Different lowercase letters (a-c) indicate significant differences among periodontal treatment in the same time point. *Indicates significant differences from 7th day of evaluation in the same periodontal treatment (P<0.05). $^{+}$Indicates significant differences from 15th day of evaluation in the same periodontal treatment (P<0.05).

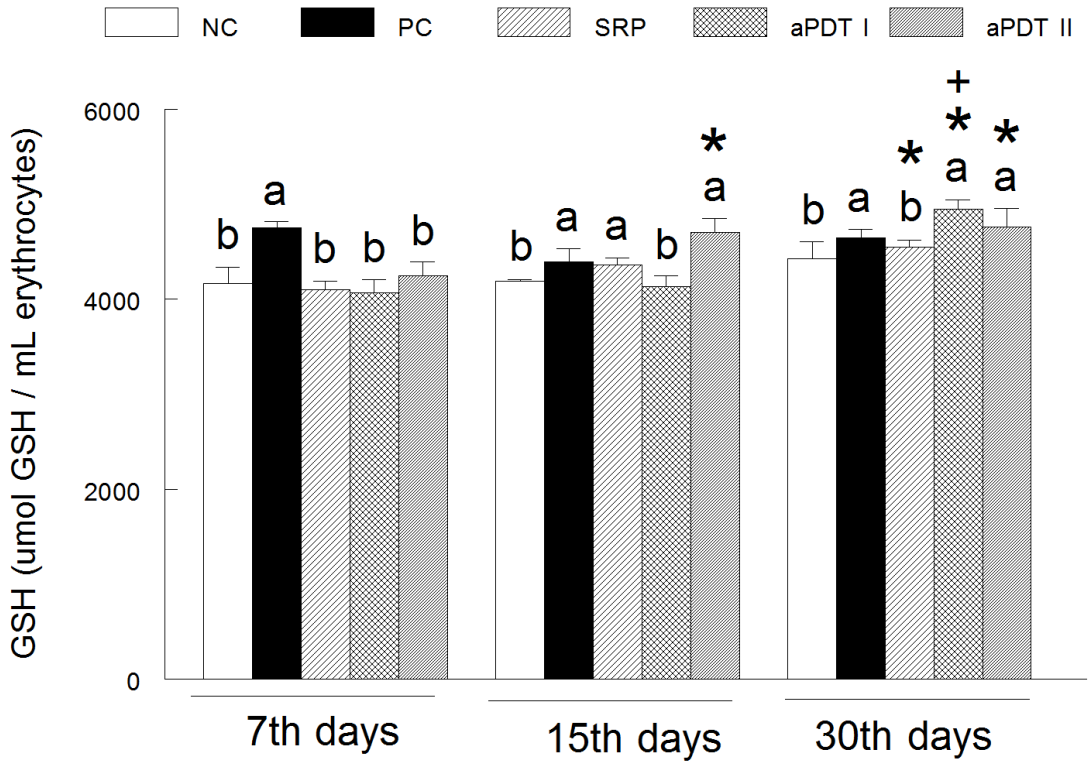
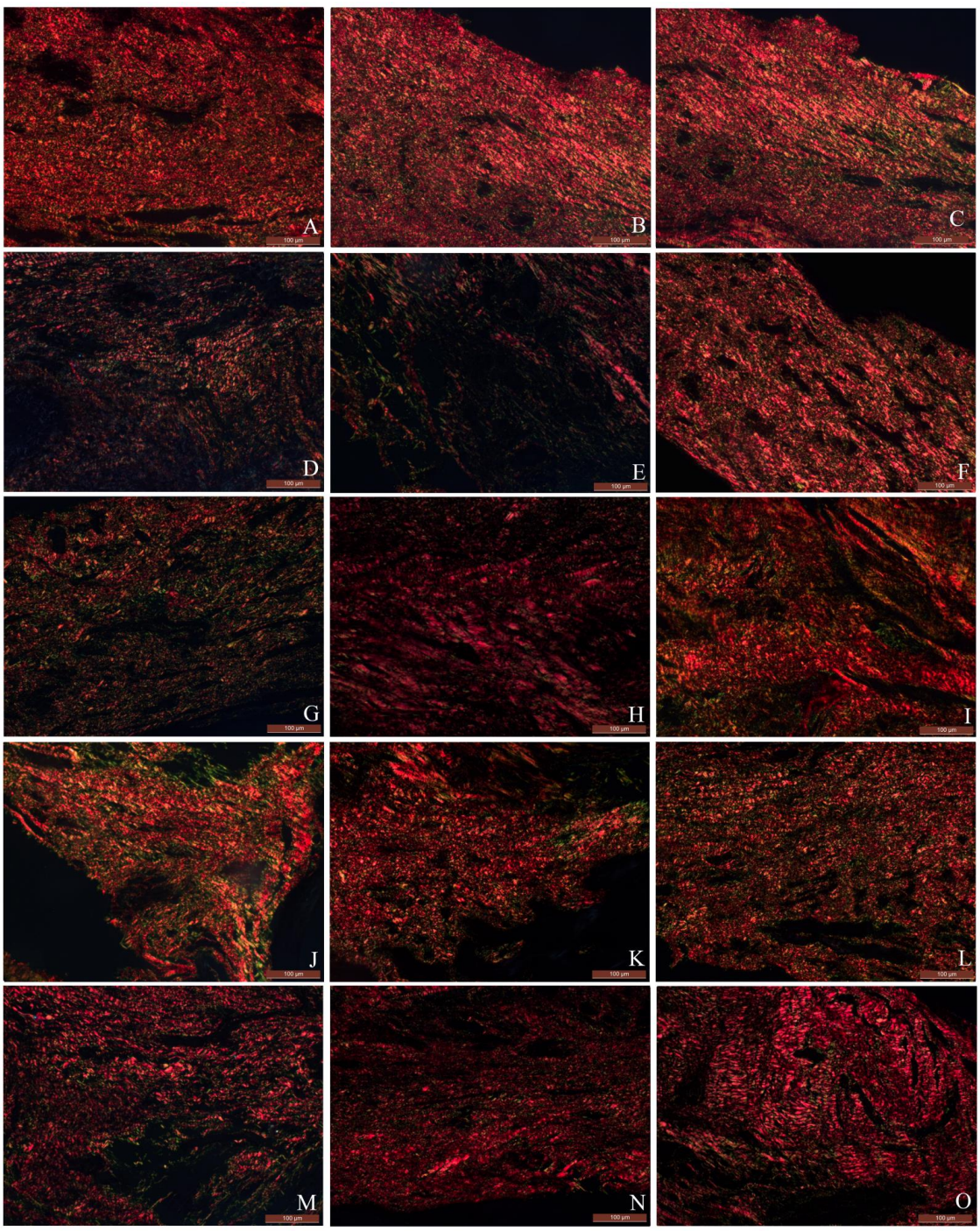


Figure 4. Effects of aPDT with methylene blue dissolved in water (aPDT I) and in ethanol (aPDT II) used as an adjunct to SRP in the periodontal treatment on morphometric changes in the gingival collagen. Five-micrometer histological sections were stained with picrosirius red and examined under a polarizing microscope. The first column corresponds to the 7th day of the evaluation. The second column corresponds to 15th day of the evaluation. The third column corresponds to 30th day of the evaluation. NC group (A, B and C), PC group (D, E and F), SRP group (G, H and I), aPDT I group (J, K and L) and aPDT II group (M, N and O). Magnification 200x. Bars 100µm.



Highlights

- A ligature around of the first right molar mandibular induced periodontitis in rats.
- Periodontitis increased oxidative damages and GSH levels in erythrocytes.
- aPDT facilitates and accelerates the periodontal healing.
- MB in ethanol protected systemic lipoperoxidation and increased antioxidant defense.
- aPDT II promoting collagen synthesis and tissue repair in short-term.

CONSIDERAÇÕES FINAIS

Nos últimos anos, nosso grupo de pesquisa tem estudado a influência da TFDa como terapia adjuvante à RAR no tratamento da periodontite. Primeiramente, estudou-se a alteração do veículo no qual o fotossensibilizador da TFDa é dissolvido (DUTRA et al., 2013). Para tanto, realizou-se a polarização desse veículo com a adição de etanol, a qual mostrou uma maior produção de EROs oriundas das reações tipo I e tipo II em comparação a solubilização em água. Esses achados foram atribuídos a maior produção de oxigênio singleto, aumento da sua meia vida e a menor agregação molecular do azul de metileno na presença de etanol. No prosseguimento dos estudos, Barin et al. (2015) demonstraram que essa alteração do solvente do fotossensibilizador diminuiu a peroxidação lipídica plasmática e aumentou o número de vasos sanguíneos a curto prazo, o que representa menos dano oxidativo sistêmico associado com periodontite e maior reparo tecidual, respectivamente. Além disso, também mostrou que, a longo prazo, a TFDa com o fotossensibilizador azul de metileno dissolvido em etanol induziu a recuperação das defesas antioxidantes sistêmicos.

O presente estudo avaliou os efeitos da TFDa usando o fotossensibilizador azul de metileno dissolvido em etanol sobre o status oxidativo sistêmico, bem como sobre o conteúdo de colágeno gengival de ratos com periodontite. Nesse contexto, analisados em conjunto, os resultados apresentados nesta dissertação demonstram a influência benéfica dessa manipulação farmacotécnica do fotossensibilizador utilizado na TFDa, enquanto terapia adjuvante à RAR, no tratamento da periodontite. Neste contexto, tal estudo demonstrou que a TFDa foi capaz de minimizar o dano tecidual induzido pela periodontite a curto prazo. Tais achados se relacionam diretamente com a prevenção do dano oxidativo sistêmico e aumento de defesas antioxidantes não enzimáticas, além do estímulo à neoformação colagenosa gengival, o que configurou nosso maior objetivo e o maior achado dessa pesquisa.

A partir deste estudo podemos sugerir que TFDa utilizada como um adjuvante ao tratamento padrão periodontal (RAR) é capaz de aumentar a resposta protetora sistêmica contra o EO induzido pela periodontite, facilitando e acelerando a cicatrização periodontal, particularmente quando o azul de metileno é solubilizado em etanol.

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ANEXOS

Anexo A: Parecer de aprovação para experimentação animal da Comissão de Ética no uso de Animais.

Universidade Federal de Santa Maria
1960

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

Comissão de Ética no Uso de Animais - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362

Anexo B: Normas para publicação, segundo o periódico Free Radical Biology & Medicine.