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PAPEL DA TERAPIA FOTODINÂMICA ANTIMICROBIANA ADJUVANTE AO TRATAMENTO PERIODONTAL SOB PARÂMETROS DE ESTRESSE OXIDATIVO PLASMÁTICO E COMPORTAMENTO VASCULAR

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil

2015

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Luisa Machado Barin

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

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por ser minha maior fonte de incentivo e inspiração.
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“Se você quiser alguém em quem confiar
Confie em si mesmo
Quem acredita sempre alcança. ”
Renato Russo

RESUMO

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

PAPEL DA TERAPIA FOTODINÂMICA ANTIMICROBIANA ADJUVANTE AO TRATAMENTO PERIODONTAL SOB PARÂMETROS DE ESTRESSE OXIDATIVO PLASMÁTICO E COMPORTAMENTO VASCULAR

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ORIENTADORA: CRISTIANE CADEMARTORI DANESI
Data e Local de Defesa: Santa Maria, 16 de julho de 2015.

O objetivo deste estudo foi avaliar os efeitos do fotossensibilizador (Fs) azul de metileno (AM) solubilizado em etanol na terapia fotodinâmica antimicrobiana (TFDa) como um tratamento periodontal adjuvante, sob parâmetros de estresse oxidativo (EO) e de comportamento vascular em modelo de rato. Bem como, investigar o maior envolvimento da angiogênese no estágio de avanço ou reparo da doença periodontal (DP). Cento e vinte ratos Wistar adultos machos foram randomizados e divididos em cinco grupos. Os grupos controles eram controle negativo (CN) (sem DP, n = 15) e controle positivo (CP) (com DP, sem qualquer tratamento, n = 15). Os demais grupos apresentavam DP e foram submetidos a diferentes tipos de tratamentos, como raspagem e alisamento radicular (RAR) e irrigação com 1 mL de solução salina (RAR, n = 30); RAR e TFDa com AM solubilizado em água (TFDa I, n = 30); RAR e TFDa com AM contendo etanol (TFDa II, n = 30). A DP foi induzida através da colocação de uma ligadura ao redor do primeiro molar inferior direito. Após 7 dias, a ligadura foi removida e os animais receberam tratamento. Aos 7, 15 e 30 dias, os ratos foram eutanasiados e o tecido gengival circundante à área de indução foi removido para análise histomorfométrica do número e diâmetro dos vasos sanguíneos através da coloração com Hematoxilina e Eosina (HE). O sangue recolhido foi centrifugado e o plasma foi utilizado para determinar os níveis de peroxidação lipídica mensurados pelas substâncias reativas ao ácido tiobarbitúrico (TBARS), vitamina C (VIT C) e glutationa reduzida (GSH). O status oxidativo demostrou maiores níveis de TBARS no grupo CP em 7, 15 e 30 dias, e indicou uma influência protetora da TFDa II no plasma observada a partir de menor peroxidação lipídica. Níveis de GSH foram consumidos nos grupos CP, TFDa I e TFDa II durante o experimento. Ainda, TFDa II também aumentou as defesas antioxidantes no plasma: a) níveis mais elevados de GSH, e b) aumento dos níveis de VIT C. Interessantemente, os níveis plasmáticos de VIT C foram restaurados no grupo TFDa II no trigésimo dia experimental. Os achados histomorfométricos mostraram em 7 dias que os grupos tratados (RAR, TFDa I e TFDa II) apresentaram elevado número de vasos sanguíneos, e o grupo TFDa II apresentou os maiores valores entre eles. A partir destes resultados, fica evidente que TFDa modifica a DP, reduzindo o dano oxidativo sistêmico, e estimula o sistema de defesa antioxidante, protegendo, assim, as zonas afetadas intimamente pela DP em ratos. Além disso, foi observado uma relação entre a maior expressão da angiogênese e o estágio de reparo da DP. Em síntese, sugerimos que a TFDa com AM solubilizado em etanol proporciona melhores respostas terapêuticas no tratamento periodontal.

Palavras-chave: Azul de Metileno. Estresse Oxidativo. Etanol. Neovascularização. Terapia Fotodinâmica Antimicrobiana.

ABSTRACT

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ROLE OF THE ADJUNCTIVE ANTIMICROBIAL PHOTODYNAMIC THERAPY TO PERIODONTAL TREATMENT AT PLASMATIC OXIDATIVE STRESS PARAMETERS AND VASCULAR BEHAVIOR

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Date and Local of Defense: Santa Maria, on July 16th 2015.

The aim of this study was to evaluate the effects of methylene blue (MB) photosensitizer (PS) solubilized in ethanol in antimicrobial photodynamic therapy (aPDT) as an adjuvant periodontal treatment, at oxidative stress (OS) parameters and vascular behavior in rat model. Besides, investigate greater involvement of angiogenesis at the destruction or healing stages of periodontal disease (PD). One hundred and twenty male adult Wistar rats were divided randomly into five groups. The control groups were negative control (NC) (no PD, n=15) and positive control (PC) (with PD, without any treatment, n=15). The other groups had PD and underwent different types of treatment, such as scaling and root planing (SRP) and irrigation with 1mL of saline solution (SRP, n=30); SRP and aPDT with MB solubilized in water (aPDT I, n=30); SRP and aPDT with MB containing ethanol (aPDT II, n=30). The PD was induced via the installation of a ligature around the mandibular right first molar. After 7 days, the ligature was removed and animals received treatment. At 7, 15 and 30 days, rats were euthanized and the gingival tissue surrounding the induction area was removed for histomorphometric analysis of the number and diameter of blood vessels by staining with hematoxylin and eosin (H&E). The collected blood was centrifuged and the plasma was used to determine lipid peroxidation by quantifying thiobarbituric acid reactive substances (TBARS), vitamin C (VIT C) and glutathione reduced (GSH) levels. The oxidative status showed higher TBARS levels in PC group in 7, 15 and 30 days, and indicated a protective influence of aPDT II on plasma observed from lower lipid peroxidation. GSH levels were consumed in PC, aPDT I and aPDT II groups throughout the experiment. Furthermore, aPDT II also increased antioxidant defenses in plasma: i) higher levels of GSH, and ii) increased levels of VIT C. Interestingly, the VIT C plasmatic levels were restored in the aPDT II group in the 30th experimental day. Histomorphometric findings in 7 days showed that treated groups (SRP, aPDT I and aPDT II) showed higher number of blood vessels, and the aPDT II group showed the highest values among them. From these results, aPDT modifies PD course, reducing oxidative systemic damage and stimulating the antioxidant defense system, thus protecting the areas closely affected by PD in rats. Moreover, was observed a relationship between increased expression of angiogenesis and repair stage of the PD. In summary, we suggest that the aPDT with MB solubilized in ethanol provides better therapeutic responses in periodontal treatment.

Keywords: Antimicrobial Photodynamic Therapy. Ethanol. Methylene Blue. Neovascularization. Oxidative stress.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

%	Por cento, Percent
AM	Azul de Metileno
CAT	Catalase
cm^2	centímetro quadrado
DP	Doença Periodontal
DNA	Ácido Desoxirribonucleico; Deoxyribonucleic Acid
EO	Estresse Oxidativo
EROs	Espécies Reativas de Oxigênio
Fs	Fotossensibilizador
GPx	Glutationa Peroxidase
GSH	Glutationa Reduzida; Glutathione Reduced
MDA	Malondialdeído; Malondialdehyde
RAR	Raspagem e Alisamento Radicular
RL	Radicais Livres
SOD	Superóxido dismutase; Superoxide Dismutase
TBARS	Substâncias Reativas ao Ácido Tiobarbitúrico; Thiobarbituric Acid Reactive Substances
TFDa	Terapia Fotodinâmica Antimicrobiana
VIT C	Vitamina C
µs	microssegundo

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1. INTRODUÇÃO

O processo inflamatório é um mecanismo de defesa natural do organismo, não específico e imediato, desencadeado por traumas, agentes químicos, distúrbios imunológicos, genéticos, extremos de temperatura, radiação, hipóxia e microrganismos. A tentativa de eliminar o agente agressor e restaurar a integridade tecidual ativa um conjunto de reações vasculares, celulares e sistêmicas, responsáveis por provocar alterações morfológicas e funcionais nas estruturas acometidas (KUMAR; ABBAS; FAUSTO, 2005; NATHAN, 2002).

A doença periodontal (DP) é uma enfermidade inflamatório-imunológica crônica dos tecidos de proteção e suporte do órgão dental (AL-HARTHI et al., 2013; CHANG et al., 2013; OSORIO et al., 2012), resultante da agressão de patógenos da placa dentobacteriana e a suscetibilidade de defesa do indivíduo (KINANE; BARTOLD, 2007; PAGE; BECK, 1997). A resposta imune do hospedeiro conduz a progressão da doença (COCHRAN, 2008; GENCO, 1992), a qual pode permanecer confinada a área gengival por vários anos ou resultar em destruição do ligamento periodontal e osso alveolar, levando a perda dental (KINANE; BERGLUNDH; LINDHE, 2010; LÖE; THELAIDE; JENSEN, 1965).

Dentre os tecidos periodontais, a gengiva destaca-se por ser precursora do processo inflamatório e repercutir todos os eventos associados ao avanço da DP (BARTOLD; WALSH; NARAYANAN, 2000). Histologicamente, é constituída pelo epitélio oral, epitélio juncional, epitélio sulcular e o tecido conjuntivo denso adjacente. Ambas as estruturas epiteliais e conjuntiva promovem a resposta inflamatória, instigando alterações vasculares e celulares no tecido gengival (KINANE; LINDHE, 2010; SOCRANSKY; HAFFAJEE; LINDHE, 2010).

Devido ao fato do biofilme bacteriano ser o agente etiológico essencial da DP, a remoção mecânica destas colônias patogênicas e de depósitos mineralizados por meio de procedimentos de raspagem e alisamento radiculares (RAR) caracteriza o tratamento convencional. Esta terapia possibilita que as superfícies dentárias tornem-se biocompatíveis, ocorrendo resolução do processo inflamatório, cicatrização e homeostasia tecidual (BARTOLD; VAN DYKE, 2013; COBB, 1996; OSORIO et al., 2012). Estudos longitudinais têm demonstrado que esta modalidade de tratamento associada com um programa periódico de controle do biofilme permitem a estabilidade dos níveis de inserção periodontal e a manutenção

dos dentes ao longo dos anos (AXELSSON; NYSTRÖM; LINDHE, 2004; CHECCHI et al., 2002; FARDAL; JOHANNESSEN; LINDEN, 2004).

No entanto, a efetividade do tratamento periodontal mecânico pode ser limitada por características anatômicas, como fissuras, concavidades e áreas de furca que dificultam a adequada descontaminação das superfícies (ADRIAENS et al., 1988; MATIA et al., 1986). A carga bacteriana remanescente deste processo é responsável pelo inadequado reparo e consequente agravo da doença (ALWAELI; AL-KHATEEB; AL-SADI, 2013).

Como terapia adjuvante para estas situações é proposto o uso de antibióticos (SLOTS, 2004), porém deve-se considerar que antibióticos administrados sistemicamente podem apresentar repercussões também sistêmicas, reações de hipersensibilidade, infecções oportunistas e resistência bacteriana (CASSELL; MEKALANOS, 2001). Além disso, as prescrições indiscriminadas e a falta de adesão dos pacientes em seguir integralmente o regime de tratamento favorecem a mutação bacteriana e o predomínio de espécies resistentes (ARDILA; GRANADA; GUZMÁN, 2010; GENCO, 1981; HEITZ-MAYFIELD, 2009). Bem como, a modificação, heterogeneidade e disposição em biofilme desses microrganismos podem levar esta terapia ao fracasso (PRESHAW, 2004a, 2004b).

Recentemente, a literatura tem apontado a terapia fotodinâmica antimicrobiana (TFDa) como uma modalidade terapêutica adjuvante promissora (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), apresentando como principais vantagens a baixa probabilidade de desenvolver resistência bacteriana, possuir ação local, não ser invasiva, ter amplo espectro de atuação e não possuir dose-limite (BERAKDAR et al., 2012; DAI; HUANG; HAMBLIN, 2009; GURSOY et al., 2013; NOVAES et al., 2012; PRATES et al., 2011).

Tal terapia é definida como uma reação fotoquímica, oxigênio-dependente, em que a ativação de um corante, conhecido como fotossensibilizador (Fs), por uma luz visível e de comprimento de onda apropriado, leva a geração de espécies reativas de oxigênio (EROs), principalmente oxigênio singleto e outros radicais livres (RL), produtos extremamente tóxicos e letais às células microbianas (CASTANO; DEMIDOVA; HAMBLIN, 2004; GE et al., 2011; MAISCH, 2007; ROLIM et al., 2012; SOUKOS; GOODSON, 2011).

Após a fotoativação, a molécula do Fs absorve energia passando do seu estado fundamental para o estado singleto excitado. Nesta forma, o Fs pode perder energia por

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 Fs no estado tripleto pode sofrer dois tipos de reações (Figura 1). Na reação tipo I, o Fs reage diretamente com um substrato, como as bactérias ou moléculas do meio, produzindo RL, como superóxido, peróxido de hidrogênio e radicais hidroxila. Na reação tipo II, o Fs 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, porém o oxigênio singleto parece ser o principal responsável pelo efeito antimicrobiano da TFDa (GEORGE; KISHEN, 2007). As reações tipo I e tipo II podem ocorrer simultaneamente, a razão entre elas depende da concentração de substrato e de oxigênio, e do tipo de Fs utilizado (CASTANO; DEMIDOVA; HAMBLIN, 2004).

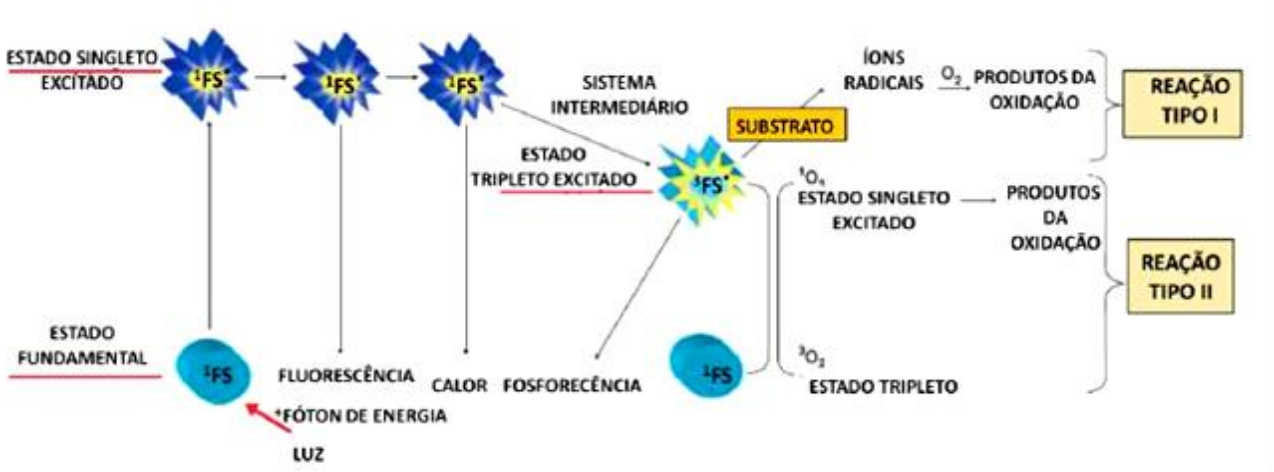

Figura 1. Mecanismo de ação da TFDa: reações Tipo I e Tipo II. (Adaptado de Soukos; Goodson, 2011).

A interação do Fs com as células alvo do tecido torna-o o fator mais influente na ação da TFDa. Atualmente, uma gama de corantes com propriedades fotossensibilizantes são utilizados, porém o azul de metileno (AM) tem sido eleito por apresentar alto potencial de formação de RL, alta solubilidade em água, mínima coloração da mucosa, longa história de segurança no seu uso, baixíssima toxicidade, penetração imediata no biofilme dental e

eliminação com sucesso de vários tipos de bactérias patogênicas (ALVARENGA et al., 2015; GURSOY et al., 2013; NUÑEZ et al., 2015).

Os estudos clínicos realizados até o presente momento avaliando a TFDa no tratamento da DP utilizam como Fs o AM diluído em água. Há evidências de que esta formulação resulta em limitada produção de oxigênio singleto associada com curta meia vida (4 μs) (MEISEL; KOCHER, 2005) e baixo potencial de difusão do mesmo (OCHSNER, 1997). A produção e meia vida do oxigênio singleto podem ser influenciadas pelo solvente no qual o Fs é diluído. Estudos recentes demostraram melhores propriedades fotofísicas, fotoquímicas e fotobiológicas em fotossensibilizantes dissolvidos em solvente menos polar que a água, aumentando a penetrabilidade tecidual e meia-vida do oxigênio singleto (GEORGE; KISHEN, 2007; MEISEL; KOCHER, 2005).

George e Kishen (2007) demonstraram que a inclusão do etanol no Fs AM, aumentou o efeito antimicrobiano da TFDa frente a biofilmes de *Enterococcus faecalis* e *Aggregatibacter actinomycetemcomitans*. Dutra et al. (2013) demonstraram *in vitro* que a produção de EROs oriunda da reação tipo I e tipo II foram maiores em formulação de AM contendo etanol em comparação a água somente. Esses achados foram atribuídos a maior produção de oxigênio singleto, aumento da sua meia vida (20 μs) e a menor agregação molecular do AM. Quando as moléculas do Fs estão desagregadas, 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). Assim, há 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).

O uso adjuvante da TFDa ao tratamento periodontal convencional tem demonstrado benefícios adicionais em modelos animais de DP. A colocação da ligadura no primeiro molar inferior de ratos suscita a presença da doença. Este modelo vem sendo utilizado para estimar a ação da TFDa em ratos sistemicamente normais (CARVALHO et al., 2011; DE ALMEIDA et al., 2008; GARCIA et al., 2013), com diabetes (ALMEIDA et al., 2008), imunossuprimidos por tracolimus (BOTTURA et al., 2011), ratos submetidos à nicotina (GARCIA et al., 2011) e em animais ovariectomizados (GARCIA et al., 2013). Inúmeros desfechos foram avaliados nestes estudos e, dentre os métodos utilizados para mensurá-los, destacam-se a análise histológica (CARVALHO et al., 2011), histométrica (ALMEIDA et al., 2008; BOTTURA et al., 2011;

GARCIA et al., 2011), imuno-histoquímica (GARCIA et al., 2013) e radiográfica (FERNANDES et al., 2010).

Com o mesmo intuito de avaliar os efeitos gerados pela aplicação de terapias e consequente atividade da DP, novos paramêtros de mensuração estão sendo propostos. Dentre eles, biomarcadores do estresse oxidativo (EO) (BALTACIOĞLU et al., 2014; BORGES et al., 2007). O quadro de EO é definido pela produção excessiva de EROs frente a incapacidade do sistema de defesa antioxidante em combatê-las. Tal processo pode causar danos celulares as macromoléculas, como lipídios, proteínas e DNA (HALLIWELL; GUTTERIDGE, 1985). Estima-se que de 1 a 3 bilhões de EROs são geradas diariamente por célula, para evitar a lesão tecidual oxidativa e manter a integridade da membrana celular a presença das defesas antioxidantes são imprescindíveis (AMES; SHIGENAGA; HAGEN, 1993; BARBOSA et al., 2010; BRIGANTI; PICARDO, 2003; KATIYAR et al., 2001).

O sistema de defesa antioxidante compreende antioxidantes enzimáticos e não enzimáticos (BRIGANTI; PICARDO, 2003; LOPEZ-TORRES et al., 1998). Entre os enzimáticos, a catalase (CAT), a superóxido dismutase (SOD) e a glutationa peroxidase (GPx) desempenham um papel central. Os antioxidantes não enzimáticos são constituídos pelo α-tocoferol, ubiquinona, β-caroteno, ácido ascórbico/vitamina C (VIT C) e a glutationa reduzida (GSH) (BRIGANTI; PICARDO, 2003; BURTON; INGOLD, 1984; DI MASCIO et al., 1990; MEISTER, 1988; SCHAFER; BUETTNER, 2001; SIES, 1999).

Por outro lado, os produtos finais de peroxidação lipídica, como o malondialdeído (MDA) afetam direta ou indiretamente a homeostase das células e dos tecidos. O aumento da peroxidação lipídica da membrana celular pode provocar uma resposta imune e inflamatória, ativar a expressão gênica, a proliferação celular ou iniciar a apoptose (BRIGANTI; PICARDO, 2003). O método utilizado para mensurar o dano oxidativo é denominado substâncias reativas ao ácido tiobarbitúrico (TBARS), baseado na reação do MDA com o ácido tiobarbitúrico (OHKAWA; OHISHI; YAGI, 1979). Recentes estudos relatam a presença de níveis elevados de MDA na DP, considerando que este pode ser um biomarcador de EO para avaliar atividade da DP (BALTACIOĞLU et al., 2014; DALAI et al., 2013; KHALILI; BILOKLYTSKA, 2008).

Durante o processo inflamatório da DP, as EROs são geradas pela ativação de leucócitos polimorfonucleares, uma vez que são um dos mecanismos de defesa frente às bactérias patogênicas. Quando presentes em excesso geram efeitos nocivos ao tecido gengival, ao ligamento periodontal e aos osteoblastos (CHAPPLE; MATTHEWS, 2007; CHAPPLE, 1997;

GUSTAFSSON; ASMAN, 1996; SEYMOUR; WHYTE; POWELL, 1986; WADDINGTON; MOSELEY; EMBERY, 2000). Ohnishi et al. (2009), em um modelo animal de camundongos, confirmam que as EROs são responsáveis pela perda óssea alveolar.

Nos últimos anos, uma grande atenção tem sido dada a esta associação entre EROs, produtos de peroxidação lipídica e sistema de defesa antioxidante sistêmicos na DP (DALAI et al., 2013), uma vez que, parece haver mais EROs circulando sistemicamente durante a DP quando comparado aos indivíduos periodontalmente saudáveis (GUSTAFSSON; ASMAN, 1996; MATTHEWS et al., 2007; SEYMOUR; WHYTE; POWELL, 1986). Outra associação, revela a redução da capacidade antioxidante frente ao aumento de biomarcadores do EO (AKALIN et al., 2007; BALTACIOĞLU et al., 2006; BAUER; BAUER, 1999; CHAPPLE; MATTHEWS, 2007; MASHAYEKHI et al., 2005; MATTHEWS et al., 2007; TSAI et al., 2005).

Como repercussão local das alterações geradas pela atividade da DP ocorrem modificações de cunho vascular. No intuito de investigar tais transformações, é necessário conceituar o processo angiogênico e suas implicações. Assim, a angiogênese designa a produção de novos capilares a partir de vasos preexistentes e faz-se presente em todos eventos fisiológicos e patológicos que demandem maior aporte sanguíneo (BUSCHMANN; SCHAPER, 1999, EILKEN; ADAMS, 2010; FILHO, 2009; KARAMYSHEVA, 2008; SURI, 1998).

A neovascularização é primordial em processos fisiológicos como a embriogênese (AUERBACH; AUERBACH, 1997), o ciclo reprodutivo feminino (FREDERICK; SHIMANUKI; DIZEREGA, 1984) e a cicatrização (NISSEN et al., 1998). Autores apontam que cerca de 60% do tecido de granulação é composto por vasos sanguíneos (ARNOLD; WEST, 1991), bem como os neovasos são necessários transportar células fagocitárias e remover produtos de excreção, permitindo o reparo em sua íntegra (WHALEN; ZETTER, 1992).

Entretanto, o crescimento excessivo e persistente de neovasos, é característico de processos patológicos (FOLKMAN; KLAGSBRUN, 1987), que estão associados à cronicidade de enfermidades. Dentre estas, destaca-se a artrite reumatoide (COLVILLE-NASH; SCOTT, 1992), tumores sólidos (CARMELIET; JAIN, 2000), tumores hematológicos (PEREZ-ATAYDE et al., 1997), psoríase (CREAMER et al., 1997), retinopatia diabética (RUDERMAN; WILLIAMSON; BROWNLEE, 1992) e processos inflamatórios (BAGLI et al., 2004), como a DP (JACKSON et al., 1997).

A angiogênese e a inflamação crônica são eventos co-dependentes (BAGLI et al., 2004; JACKSON et al., 1997; SZEKANECZ; KOCH, 2004), a angiogênese expande a microvasculatura do tecido aumentando o influxo de células inflamatórias, que necessitam de maior aporte sanguineo para suprir o processo imune metabolicamente ativo (BAGLI et al., 2004; CHUNG; FERRARA, 2011; MAJNO, 1998; SZEKANECZ; KOCH, 2004).

Diante disto, a angiogênese atua diretamente no curso da DP, sendo responsável tanto pela manutenção dos tecidos periodontais, como pelo avanço e reparo da doença (ASPRIELLO et al., 2009; BOOTH et al., 1998; CETINKAYA et al., 2007; JOHNSON; SERIO; DAI, 1999; YOSHIDA et al., 2011; ZOELLNER; CHAPPLE; HUNTER, 2002). Contudo, estudos são conflitantes, no que tange a principal influência do processo de angiogênese entre os diferentes estágios da DP (ARTESE et al., 2010; ASPRIELLO et al., 2009; BOOTH et al., 1998; CETINKAYA et al., 2007; JOHNSON; SERIO; DAI, 1999), demostrando-se variável a respeito do número e diâmetro dos vasos, podendo estarem aumentados (BOOTH et al., 1998), diminuídos (CHAPPLE; KUMAR; HUNTER, 2000), ou não afetados (UNLÜ et al., 2003).

Cetinkaya et al. (2007), elaboraram o primeiro estudo que associou a expressão do número e diâmetros dos vasos sanguíneos no tecido gengival de ratos, durante estágios de destruição e reparo da DP. Tais autores encontraram os maiores números de vasos sanguíneos presente no grupo de cicatrização e os menores resultados foram verificados no grupo com doença. Além disso, os vasos oriundos do processo inflamatório crônico apresentavam diâmetro aumentado, já os neovasos que provenientes do processo de reparo eram de menor diâmetro. Essas observações associadas concluíram uma maior relação do processo angiogênico com o processo de reparo da DP.

A avaliação histomorfométrica do número e calibre dos vasos (CETINKAYA et al., 2006, 2007) pode elucidar a maior da angiogênese na atividade da DP (ASPRIELLO et al., 2009; BASSIOUNY; ALAYED; ALSALMAN, 2014; CETINKAYA et al., 2007; KASPRZAK et al., 2012). Ainda, a morfogênese vascular envolve uma complexa interação entre as células endoteliais e o ambiente extracelular (UCUZIAN et al., 2010). As células endoteliais proliferam, passam por ativação, migração, alinhamento, formam tubos, ramificações e anastomoses. Todo esse processo tem início com a dilatação de vênulas preexistentes, as quais podem sofrer brotamento ou intussuscepção, em que os capilares se dividem originando dois ou mais vasos que serão maturados e remodelados ao longo do tempo (CARMELIET, 2003; CLAPP et al., 2009).

Ao considerar que nenhum estudo animal ou humano foi realizado a fim de verificar as potencialidade da inclusão do etanol na formulação do Fs AM, a presente pesquisa objetiva avaliar os efeitos gerados pelo uso deste protocolo para o Fs (Fig. 2) na TFDa (Fig. 3), em ratos com DP induzida experimentalmente, através de parâmetros de EO plasmático e análise histomorfométrica vascular do tecido conjuntivo gengival. Bem como, estima-se investigar a maior participação do processo angiogênico na atividade da doença periodontal. A hipótese conceitual do estudo considera que a inclusão do etanol potencialize a produção de oxigênio singleto, minimize os níveis de peroxidação lipídica e aumente o número de vasos sanguíneos, demostrando um maior efeito antimicrobiano e melhor ação terapêutica. Assim como, espera-se uma maior associação entre a angiogênese e o estágio de reparo periodontal.

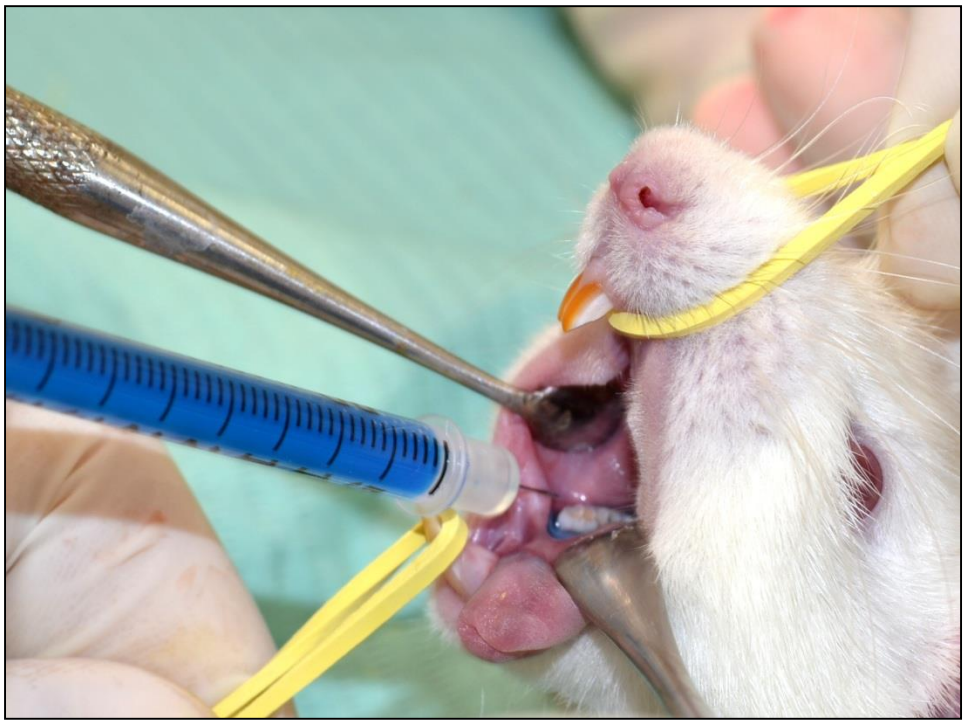

Figura 2. Aplicação do fotossensibilizador em modelo animal.

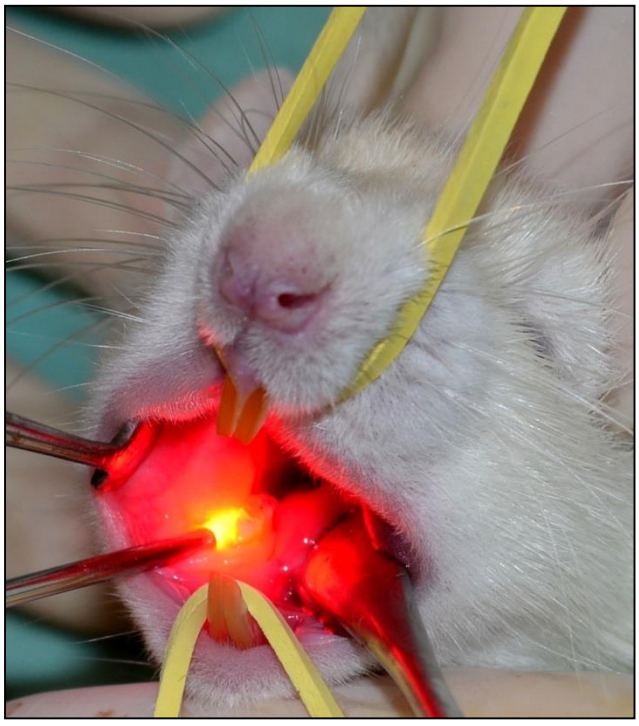

Figura 3. Fotoativação do fotossensibilizador em modelo animal.

1.1. Artigo

ROLE OF THE ADJUNCTIVE ANTIMICROBIAL PHOTODYNAMIC THERAPY TO PERIODONTAL TREATMENT AT PLASMATIC OXIDATIVE STRESS AND VASCULAR BEHAVIOR

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ABSTRACT

Background: The aim of this study was to evaluate the effects of a new formulation of methylene blue (MB) photosensitizer solubilized in ethanol in antimicrobial photodynamic therapy (aPDT) as an adjuvant periodontal treatment, at plasmatic oxidative stress and vascular behavior in rat model. Besides, respond to the current question about the greater involvement of angiogenesis at the destruction or healing stages of periodontitis.

Methods: One hundred and twenty male adult Wistar rats were divided into five groups. The control groups were negative control (NC, no periodontitis, n=15) and positive control (PC, with periodontitis, without any treatment, n=15). The other groups had periodontitis and were treated with scaling and root planing (SRP, n=30), SRP and aPDT with MB solubilized in water (aPDT I, n=30), SRP and aPDT with MB solubilized in ethanol (aPDT II, n=30). The periodontitis was induced at the mandibular right first molar. After 7 days, the ligature was removed and the animals received treatment. At 7, 15 and 30 days, rats were euthanized, and gingival tissue surrounding the induction area was removed for histomorphometric analysis of the number and diameter of blood vessel by hematoxylin and eosin (H&E) staining techniques. The collected blood was centrifuged and the plasma was used to determine lipid peroxidation by quantifying thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) and vitamin C (VIT C) levels.

Results: The oxidative status showed higher TBARS levels in PC group in 7, 15 and 30 days, and indicated a protective influence of aPDT II on plasma observed from lower lipid peroxidation. GSH levels were consumed in PC, aPDT I and II groups throughout the experiment. Furthermore, aPDT II also increased antioxidant defenses, interestingly, the VIT C levels were restored in this group in the 30th day. Histomorphometric findings in 7 days showed, in treated groups (SRP, aPDT I and II), a higher number of blood vessels, and the aPDT II group showed the highest values among them.

Conclusion: From these results, aPDT modifies periodontitis course, reducing oxidative systemic damage, and stimulating the antioxidant defense system, thus protecting the areas closely affected by periodontitis in rats. Moreover, there is a relationship between the angiogenesis and healing stage of the periodontitis. In summary, we suggest that the aPDT with MB solubilized in ethanol provides better therapeutic responses in periodontitis treatment.

Keywords: Oxidative stress; photosensitizing agents; ethanol; gingiva.

1. Introduction

Periodontitis is a chronic inflammation that affects the protective and support tissue of dental organ [1], presents negative impact in the quality of life of individuals [2], besides an elevated worldwide prevalence [3]. The progression of this pathology is caused by the interaction between pathogenic microorganisms adhered in the dental surfaces and susceptible host [1]. The mechanical removal of dental plaque through scaling and root planning (SRP) is the key of the periodontal treatment [4]. However, mechanical removal of plaque and reduction of the infectious cell numbers can be impaired in sites with difficult access, as furcations, deep invaginations, and concavities [5]. Some therapeutic alternatives, such as systemic and local antibiotics, have been used in unresponsive cases to conventional treatment, although this therapy brings undesirable side effects and bacterial drug resistance development [6].

Recently, studies showed satisfactory results employing the antimicrobial photodynamic therapy (aPDT) in experimental [7,8] and clinical periodontal disease treatment [9]. The major advantages of this therapy are its specificity for the target cells, the fact it doesn't induce any side effects, initiation of activity only when exposed to light, and its inability to develop bacterial resistance [10]. The aPDT is a localized treatment that requires two other components in addition to oxygen: a light source and a photosensitizer (PS), both capable of binding to target cells in the oxygen presence. The PS absorbs energy directly from a light source and transfers it to an oxygen molecule, resulting in the reactive oxygen species (ROS) formation (type 1 reaction) and singlet oxygen (type II reaction) [11]. The ROS and singlet oxygen are toxic to microbial cells, causing irreversible damage to the cell cytoplasmic membrane or to the DNA, which culminates in bacterial cell death [12].

The most important factor that influences the outcome of the aPDT is the interaction between the PS and the target cells. A range of photosensitizing dyes are available for use in the aPDT [11]. The methylene blue (MB) have been successfully used as a PS that eliminates several types of bacteria [13], but its solubility in water generates a limited production of singlet oxygen, associated with short half-life (4μs) [14], and low potential for diffusion [15]. The literature has demonstrated an improvement in the photochemical and photophysical properties of the formulations containing MB solubilized in ethanol, which reaches a better antimicrobial effect [16].

The ROS release is one of the defense mechanisms against bacterial biofilm. The ROS have a dual role: they are essential for maintenance of normal cellular metabolism, and can also induce significant oxidative damage as a result of the free radical chain reactions they trigger [12,17]. The body has developed an antioxidant system against the harmful effects of ROS consisting of enzymatic and non-enzymatic antioxidants. Among the enzymatic antioxidants, glutathione peroxidase, catalase and superoxide dismutase play a central role. The non-enzymatic antioxidants are α-tocopherol, ubiquinone, ascorbic acid (vitamin C) and glutathione reduced (GSH) [18]. When the oxidant-antioxidant balance is disturbed in favor of ROS, the oxidative stress (OS) occurs, which culminates in tissue damage [17,18].

Currently, new parameters have been proposed to evaluate the periodontitis activity. OS biomarkers have been suggested to assess disease progression [19,20]. The plasmatic malondialdehyde (MDA) may be a potential biomarker of the OS frequently used indicators of lipid peroxidation [21], which has been implicated with several disease processes and can be measured by the TBARS method [22]. Many researchers have reported higher MDA levels in chronic periodontitis [19,23,24]. A large amount of ROS is released in peripheral blood neutrophils in periodontitis compared to the healthy subjects [25]. Furthermore, the recent research showed an increase of the OS biomarkers and a reduced antioxidant capacity in periodontitis [19,20,26].

Likewise, the angiogenesis, defined as the process by which new blood vessels are produced by sprouting from established vessels [27], also have influence on the periodontitis development. Studies about the blood vessel numbers in the pathogenesis of periodontitis have had conflicting results, suggesting that are increased [28], decreased [29], or unaffected [30]. However, the major role of angiogenesis in promoting the progression or the healing of periodontitis is still unclear [31,32].

In this sense, this study had two aims: to evaluate the aPDT effect employing the MB PS solubilized in ethanol as adjunct to the mechanical treatment of the experimental periodontitis at plasmatic OS and vascularization with regard to the number and diameters of blood vessels. Besides, respond to the current question about the greater involvement of angiogenesis at the destruction or healing stages of periodontitis.

2. Methods

2.1.Animals

One hundred and twenty adult male Wistar rats (250–300 g), from the breeding facility of Federal University of Santa Maria (RS, Brazil), were placed in cages (five animals per cage) with free access to food and water in a room with controlled temperature (23°C±1°C), relative humidity of the air close to 60% and exhaust air. The environment possessed noise control (maximum 85 decibels) and standard light–dark illumination cycle (12 hours each). Animal care and study protocols were approved by the Animal Ethical Committee (027/2013), affiliated to the Council for the Control of Animal Experiments, following international norms of animal care and maintenance.

2.2.Protocol of Experimental Periodontitis

Rats were randomly assigned into the five experimental groups: negative control (NC; no periodontitis; n=15); positive control (PC; with periodontitis and without any treatments; n=15); SRP (with periodontitis, treated with SRP and irrigation with 1mL of saline solution; n=30); aPDT I (with periodontitis and treated with SRP and aPDT with MB solubilized in water; n=30) and aPDT II (with periodontitis and treated with SRP and aPDT with MB solubilized in ethanol; n=30). Each animal of the PC and treatment groups received a cotton ligature, under anesthesia (ketamine/xylazine, 70 and 6 mg/kg, intramuscular injection, respectively), in the mandibular right first molar in the submarginal position in order to induce experimental periodontitis. After 7 days, the cotton ligatures were removed from all animals [8] and the animals of the SRP, aPDT I and aPDT II groups were treated.

2.3.Scaling and Root Planing Procedures

The SRP was carried out with micro Gracey curettes Mini-Five 1-2 (Hu-Friedy®, Chicago, IL, USA) through 10 distal-mesial traction movements in the buccal and lingual sides. The furcation and interproximal areas were instrumented with the same curettes using cervico-

occlusal traction movements [8]. One operator blinded for experimental groups performed all the SRP procedures.

2.4.Antimicrobial Photodynamic Therapy Protocols

The aPDT employed the MB PS (Sigma Aldrich®, St. Louis, MO, USA) dissolved in different solutions according to the experimental groups: aPDT I (MB 0.01% solubilized in bidistilled water) and aPDT II (MB 0.01% solubilized in bidistilled water, carboxymethyl cellulose and ethanol solution (77:3:20)). The MB formulations or its vehicle were slowly applied into the periodontal pocket around the mandibular right first molar using a syringe (1mL) and insulin needle (BD® Ultrafine™, U-100, 0.5mL, 8mmX0.3mm). After one minute, low-level laser therapy Indium–Gallium–Aluminum–Phosphorus (InGaAlP) (TheraLase®, DMC Equipments, São Carlos, SP, Brazil) with wavelength of 660 nm, continuous emission mode, power output of 30mW emitter, was applied to three equidistant points on the mandibular right first molar on the buccal and lingual sides. In each spot the laser was applied by 4s (0.14J/point), the tooth received 0.84J of energy and a total energy density of 29.64J/cm^2 [8]. A blind operator for the experimental groups performed all the aPDT procedures.

2.5.Experimental Periods

On days 7, 15 and 30 after ligature removal of the mandibular right first molar, all animals were anesthetized with isoflurane (2-3%, inhaled) (Isothane®, Baxter Healthcare® of Puerto Rico, Guayama, Porto Rico, USA) and euthanized by exsanguinations (Fig. 1).

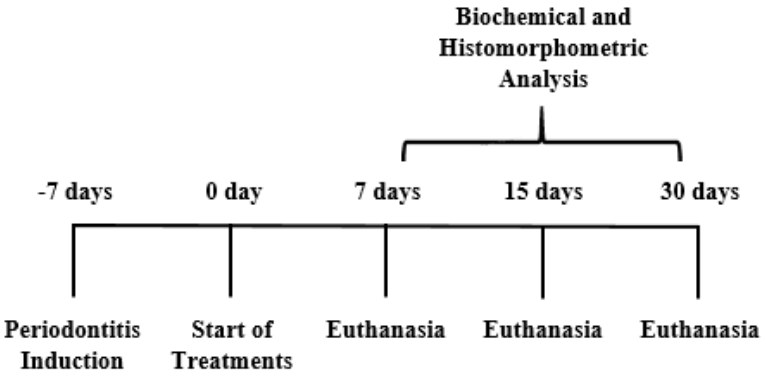

Fig. 1. Presents the experimental procedures.

2.6.Biochemical Analysis

The collected blood (collected by cardiac puncture in heparinized tubes) was centrifuged at 1300g for 15min for plasma and used for the determination of MDA levels, which estimate oxidative damage to lipids, and for GSH and vitamin C (VIT C) levels, which estimate the antioxidant defenses.

2.6.1.Lipid Peroxidation Estimation of Plasma

The plasma lipid peroxidation is assessed by quantifying thiobarbituric acid reactive substances (TBARS) through the pink chromogen produced by the reaction of thiobarbituric acid to MDA at 100°C, measured spectrophotometrically at 532nm [22]. Results were expressed as nmol MDA/mL plasma.

2.6.2.Reduced Glutathione of Plasma Levels

The plasma levels of GSH were determined after plasma reaction with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, Proquímios®, Rio de Janeiro, RJ, Brazil), according to Ellman (1959) [33] with modifications [33,34]. The protein fraction plasma was precipitated with 20% trichloroacetic acid, followed by centrifugation. A standard curve was constructed using GSH order to estimate the GSH content expressed in GSH nmol/mL plasma.

2.6.3.Vitamin C Estimation of Plasma Levels

The VIT C of plasma was estimated as described by Galley et al. (1996) [35] in some modifications [34,35]. The plasma was precipitated with 5% trichloroacetic acid solution and, then centrifuged. The supernatants were mixed with 2,4-dinitrophenylhydrazine (4.5 mg/ml) and 13.3% trichloroacetic acid and incubated for 3h at 37°C. A sulfuric acid solution (65%) was added and the samples measured spectrophotometrically at 520nm. A standard curve using ascorbic acid was used to calculate the VIT C content expressed as μg VIT C/mL plasma.

2.7.Histomorphometric Analysis

The surrounding buccal gingival tissue of the mandibular right first molar was removed from all the animals, each gingival biopsy was soaked in 10% neutral buffered formalin for 24h and then embedded into paraffin. Serial sections were cut with a thickness of 6µm in a mesio-distal direction and stained with hematoxylin and eosin (H&E).

In the H&E-stained sections, histomorphometric analysis was performed by light microscope (Binocular Optical Microscope ZEISS, Axio Lab.A1, Germany), and the images were transferred to a monitor with a camera apparatus (AxioCam, ERc 5S, Germany). In the gingival connective tissue, the number of sectioned blood vessel profiles was counted in three individual fields of 60x60µm^2 area per section at an objective magnification of x400 [31]. The diameter of two randomly selected sectioned blood vessel profiles per section was measured at an objective magnification of x100 [31]. The mean of measurements was used for data analysis. For the histometric analysis the examiners underwent training and completed double measurements of 30 specimens, with a 1-week interval between each measurement. The interexaminer and intraexaminer reproducibility revealed a high correlation (Kappa>0.85).

2.8.Statistical Analysis

Levene's test was applied in order to verify the homogeneity of the data. Biochemical and histomorphometric 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 of less than 0.05 was considered as statistically significant.

3. Results

3.1.Biochemical Assessments

Results were expressed as mean values and standard error in evaluations of TBARS, GSH and VIT C plasmatic in the experimental groups. In Fig. 2 a comparison between the NC and PC groups showed significant lipid peroxidation promoted by periodontitis at all times (P<0.05). The effect of treatment with aPDT II on lipid peroxidation is significantly visible

until the 7th day after treatment (P<0.05), from day 7, all treatments (SRP, aPDT I and II) were able to block the lipid peroxidation, especially between 15th and 30th days.

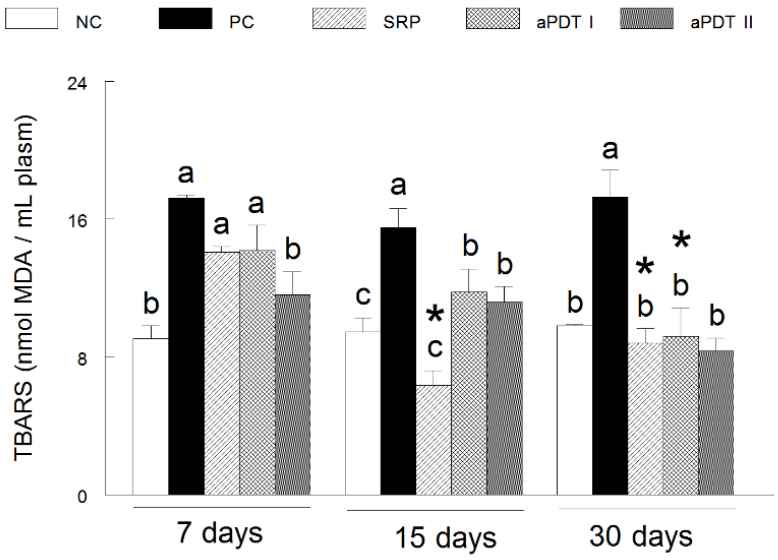

Fig. 2. Effect of aPDT with PS diluted in differents solvents, used as an adjunct to SRP, in the periodontal treatment on the lipid peroxidation levels in plasma. Different lower case letters (a-c) indicate significant difference among periodontal treatment in the same evaluation time. *Indicates significant difference of 7 day evaluation in the same periodontal treatment (P<0.05).

Fig. 3 and 4 (GSH and VIT C) showed the status of the antioxidant defense. Until the 7th day, the presence of periodontitis did not affect the GSH levels, on the contrary, when the periodontitis was treated the GSH levels were increased significantly (P<0.05). However, GSH levels were consumed in PC, SRP, aPDT I and II groups throughout the experiment (P<0.05). On the other hand, on the 30th day there was a significant increase of VIT C and GSH levels in the aPDT II group compared to SRP and aPDT I groups (P<0.05) and no significant difference in VIT C levels between the NC and aPDT II. The SRP and aPDT I groups also were able to inhibit depletion of VIT C levels compared to PC group (P<0.05).

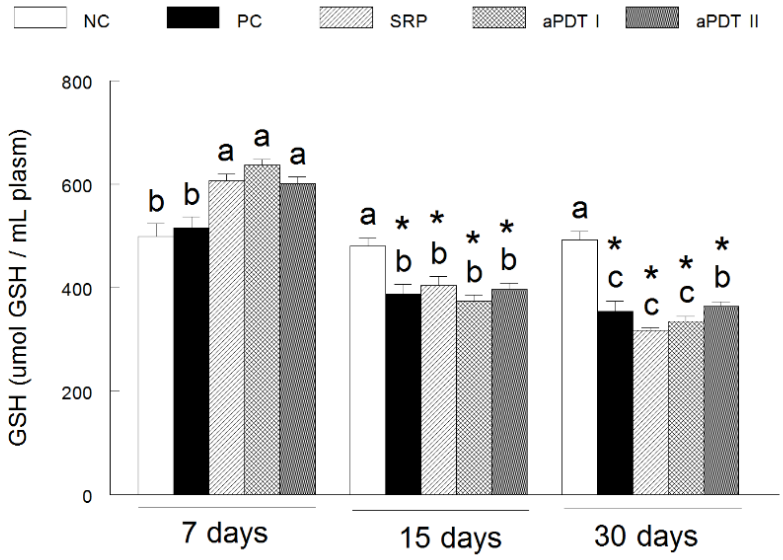

Fig. 3. Effect of aPDT with PS diluted in differents solvents, used as an adjunct to SRP, in the periodontal treatment on the GSH levels in plasma. Different lower case letters (a-c) indicate significant difference among periodontal treatment in the same evaluation time. *Indicates significant difference of 7 day evaluation in the same periodontal treatment (P<0.05).

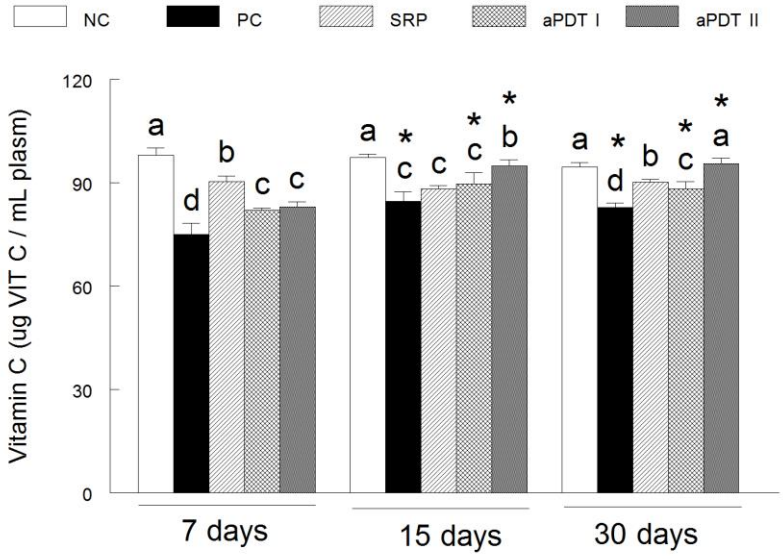

Fig. 4. Effect of aPDT with PS diluted in differents solvents, used as an adjunct to SRP, in the periodontal treatment on VIT C levels in plasma. Different lower case letters (a-c) indicate significant difference among periodontal treatment in the same evaluation time. *Indicates significant difference of 7 day evaluation in the same periodontal treatment (P<0.05).

3.2. Histomorphometric Analysis

Table 1. Effect of aPDT with PS diluted in differents solvents, used as an adjunct to SRP, in the periodontal treatment on the number and diameter of blood vessels of the gingiva.

	Blood vessels					
	Number (unit)			**Diameter (µm)**		
	7th day	**15th day**	**30th day**	**7th day**	**15th day**	**30th day**
NC	2.53±0.29^{c}	3.33±0.13	3.20±0.37^{a}	15.75±3.33	15.52±2.46^{a*}	24,89±1,13^{a*}
PC	1.87±0.13^{c}	2.80±0.16*	2.92±0.22^{a*}	13.05±1.37	13.74±0.90^{a*}	27.20±3.14^{b*}
SRP	3.54±0.22^{b}	2.78±0.19	2.29±0.18^{b*}	11.96±1.58	18.81±1.46^{a*}	13.16±0.92^{c}
aPDTI	3.04±0.25^{b}	2.81±0.16	2.50±0.25^{a}	12.78±2.04	11.74±0.76^{a}	11.97±0.86^{c}
aPDTII	4.83±0.42^{a}	3.00±0.38*	2.18±0.14^{b*}	12.39±1.38	9.79±0.77^{b}	12.60±1.16^{c}

Data are mean ± SEM. Different lower case letters (a-c) indicate significant difference among periodontal treatment in the same evaluation time. *Indicates significant difference of 7 day evaluation in the same periodontal treatment (P<0.05).

Regarding the histomorphometric evaluation of blood vessels shown in Table 1, it was observed until the 7th day, in relationship to NC and PC groups, that all treatments significantly influenced the increase in number of vessels, however, the increase was significantly higher in the aPDT II group. Between the 7th and the 30th days, there was no statistically significant difference in the number of blood vessels of the NC group (Fig. 5A and B), on the other hand, in the same period, there was a significant increase in these numbers in PC group (P<0.05) (Fig. 5C and D) and a decrease in the number of vessels in the treated groups, especially in aPDT II group (P<0.05) (Fig. 5E and F). Regarding the blood vessels diameter, until the 7th day, there was no statistically significant difference among the groups. From the 7th day, diameter differences reached statistical significance, between the PC and the aPDT II groups from the 7th to the 15th day, and between the PC and the other groups from the 15th to the 30th days (P<0.05).

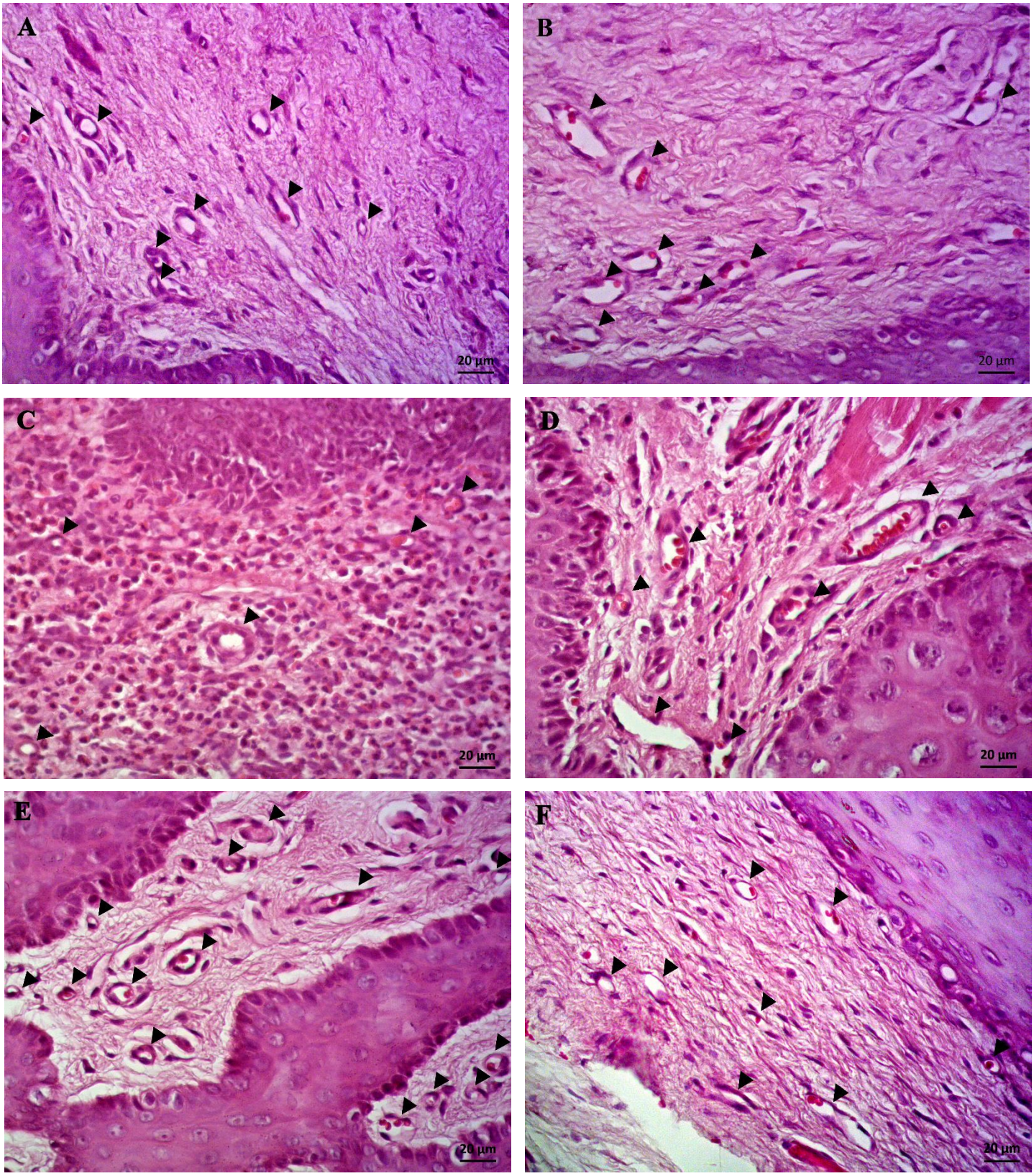

Figure 5. Gingival connective tissue. Arrowheads indicate sectioned blood vessels. **A, B)** Similar number of blood vessels of the NC group between 7 and 30 days, respectively. **C, D)** Increase in the number of blood vessels indicating increased vascularization by spontaneous attempt to repair in the PC group between 7 and 30 days. **E, F)** Decrease in the number of blood vessels indicating advanced healing and structural stability of the tissue in the aPDT II between 7 and 30 days. (H&E; bar:20µm).

4. Discussion

The experimental model of periodontitis used in the current study was able to increase the lipid peroxidation plasmatic levels in PC group, represented by the MDA levels, in all analyzed times, e.g., 7, 15 and 30 days compared to NC group. Moreover, periodontitis was related to the decrease in the GSH and VIT C plasmatic levels, suggesting systemic OS in PC group. These findings confirm previous studies that showed reduction in the antioxidant capacity and increase in OS biomarkers in periodontitis [19,20,26]. Considering the local and systemic inflammatory response during the periodontitis [25,36], this pathology can deplete GSH and VIT C plasmatic levels [37]. Our findings also showed that proinflammatory and pro-oxidant properties of the periodontitis were reflected on the non-enzymatic antioxidant defense system of the plasma, especially on GSH and VIT C, which levels especially decreased in the PC group.

In the present study, the scaling and root planing (SRP group) decreased the plasmatic lipid peroxidation levels just after 15 days of completion of this periodontal treatment, showing a delayed effectiveness of this traditional method. Moreover, GSH plasmatic levels increased after 7 days of SRP treatment, which can be related to a systemic attempt to combat the high MDA levels shown by this group in this moment. However, in 15 and 30 days after SRP procedure, the GSH plasmatic levels decreased, indicating an overloaded antioxidant defense system. These findings indicate that this reduced oxidative damage may be related to the decreased levels of antioxidant defense observed in plasma of SRP group, once the antioxidant defense was consumed to combat the periodontitis-induced lipid peroxidation. Confirming our findings, SRP has been related to the successful management of periodontitis [38].

Nevertheless, the SRP procedure has limitations presumably due to the persistence of periodontal pathogens and subsequent recolonization [39]. In this context, the search for new approaches to improve the effectiveness of periodontal therapy is needed. Moreover, the use of systemic antibiotics and the consequent increase in bacterial resistance can also justify the use of other adjuvant tools for periodontal treatment [14]. The aPDT emerges as an alternative for microbial reduction, with minimal side effects [10]. Therefore, advantages of this therapy make it a promising adjuvant method to conventional periodontal treatments. The aPDT, as an adjuvant tool associated to SRP and employing its PS soluble in water, was able to decrease lipid peroxidation plasmatic after 30 days of this treatment, as demonstrated by aPDT I group.

This finding may have been at the expense of GSH and VIT C plasmatic consumption, which can be decreased by OS [40].

The most important and recognized factor to regulate aPDT action is the result of the interaction between the PS and the target tissue cells [11]. In this context, the solubilization of MB in ethanol has been related to its improvement in the photochemical and photophysical properties, besides improving the antimicrobial effect [16]. Here, we tested such pharmacotechnical alteration for the first time *in vivo* in order to evaluate its effect on plasmatic OS, as well as on number and diameter of gingival blood vessels. aPDT II was more beneficial than aPDT I, while aPDT II showed protective influences against periodontitis-induced systemic damage. This hypothesis is supported by our findings after induction of periodontitis in a PDT II group: (i) prevention of damage to plasmatic lipids in the short and long term, e.g, 7 and 30 days after periodontitis induction; (ii) total recovery of VIT C plasma levels 30 days after treatment; and (iii) increased in the gingival blood vessels number in the 7th days after aPDT. Thereby, the aPDT II group showed the same lipid peroxidation plasmatic levels compared to NC group in the 30th day, reflecting a lower periodontitis-induced systemic OS.

In addition, GSH plasmatic levels increased in 7 days after aPDT II procedure and decreased in the 15th, which demonstrates the antioxidant defense system responsiveness against the periodontitis. Moreover, the later consumption of this non-enzymatic antioxidant observed in 15th day targets to combat the disease deleterious effects, which is consistent with the results about lipid peroxidation observed in this group. Furthermore, it is plausible that consumption of GSH might occur in order to regenerate VIT C, which had its increased levels at a time-dependent manner. Interestingly, 30 days after periodontitis induction, in the aPDT II group was observed a total recovery of the VIT C plasmatic levels. Taken together, we can infer that aPDT II has the best performance and therapeutic action as adjuvant therapy to SRP, demonstrated by minor oxidative damage and recovery of antioxidant system in the plasma. Here, oxidative insult to lipids were minimized in the plasma of rats submitted to aPDT II, indicating that this therapy can excite antioxidant defense mechanisms, thus attenuating the oxidative damage induced by periodontitis.

VIT C is the main hydrophilic antioxidant present in blood, which acts primarily by scavenging ROS [41], and can completely protect against peroxidative damage and OS [41,42]. Our results demonstrated that periodontitis decreased VIT C plasmatic levels in PC group at all times observed compared to NC group. These findings indicate that the consumption of this

antioxidant restores the balance between pro-oxidant and antioxidant mechanisms, once PC group demonstrated the highest lipid peroxidation plasmatic levels. Tomofuji et al. (2009) [43] showed VIT C systemic decrease by periodontitis, which is according to our findings. Furthermore, the severity of periodontitis and VIT C plasmatic levels have been negatively related [44]. In addition, our results showed that SRP, aPDT I and II periodontal treatments showed a reduction in VIT C levels to a lesser extent than PC group in plasma, which may be related to the decrease of inflammatory and oxidative damage-induced periodontitis. As previously discussed, interestingly, there was a full recovery of VIT C plasmatic levels only in the aPDT II group in the 30th day. Thus, we can infer that GSH consumption showed by this group was due to its role in converting VIT C back to its active form [45].

The periodontal vasculature is affected profoundly during the periodontitis, but characteristics of vascular behavior are still scarce and conflicting in the literature [31,46]. Recent research showed a positive relationship between blood vessels number increase and periodontitis repair stage, while the disease progress is characterized by a smaller vascular neoformation [31]. Accordingly, in the current study, in 7 days, the periodontitis decreased blood vessel numbers in PC group, while a high neovascularization was observed in the treated groups. Of particular importance, the largest number of blood vessels was observed in the aPDT II group, which can be related to the lowest lipid peroxidation plasmatic levels indicating the best therapeutic action. These findings demonstrated a positive relationship between increased angiogenesis and the healing stage of periodontitis. The low level laser therapy had been demonstrated to modulate biological mechanisms in wound healing [47], angiogenesis [48], and inflammation [49]. Moreover, recent studies reported that the angiogenesis occurrence in several experimental models is critical to therapy success [48], and may be closely related to our findings. Thus, our findings confirm the hypothesis of the present study, claiming that the best effect was reached with aPDT with MB FS dissolved in ethanol, represented by lower lipid peroxidation levels and total recovery of antioxidant defense of VIT C. Furthermore, over time, it was observed an increase in angiogenesis in PC group, probably due to its spontaneous attempt to repair the periodontitis-induced damage. Whereas there was a blood vessel numbers decrease in the treated groups, due to advanced or complete repair, resorption vessels and structural stability of tissues.

Vessels coming from a healing exhibit small diameters compared to blood vessels arising from a chronic inflammatory process that, in turn, present large diameters [31]. This

vascular behavior was also found in this study, once all periodontal treatments showed smaller blood vessels diameters compared to both NC and PC groups. In untreated chronic inflammatory periodontal disease, there is an extensive vasculature remodeling and selective increase in larger diameter vessels [31]. Furthermore, Chapple et al. 2000 [29] demonstrated that density of blood vessels increased in periodontal disease during remodeling phase, conforming our findings. Other hypothesis for the increase in diameter of blood vessels observed in NC and PC groups during the experimental period may be related to an increase in its wall, which may be consequence of the angiogenesis [50] or due to increased length and/or tortuousness of existing vessels [51]. In addition, SRP group showed an unexpected increase in diameter of blood vessels in the 15th day which can be justified by previous argumentations or yet by increase of the capillary permeability [52].

Taken together, our results demonstrated that the aPDT II used as adjuvant to periodontitis conventional treatment was able to decrease plasmatic lipid peroxidation and increase blood vessel numbers to short-term, representing less systemic oxidative damage associated with periodontitis and greater tissue repair, respectively. Furthermore, in the long term, this therapy induced the recovery of systemic antioxidant defenses. Thus, confirming the existent biological plausibility in aPDT, the findings of the present study showed for the first time *in vivo* that aPDT with MB PS solubilized in ethanol provides additional therapeutic benefit compared to aPDT with MB solubilized in water or SRP periodontal treatment only. In addition, angiogenesis can be more related to the healing stage of periodontitis. Based on these preliminary findings, additional studies are necessary to confirm the relations described here, in order to increase knowledge about the aPDT, as well as on the relationship between periodontitis and angiogenesis.

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2. CONSIDERAÇÕES FINAIS

Este estudo foi conduzido em animais sob condições experimentais de indução da doença periodontal e todos os procedimentos e etapas foram cuidadosamente planejados e executados (Anexo B). Esforços foram feitos para controlar ao máximo possíveis vieses que pudessem comprometer a validade interna deste estudo. Nossos achados devem ser extrapolados com cautela e devem ser inferidos para populações com as mesmas características, mediante as mesmas condições de tratamento da doença periodontal, a fim de evitar conclusões errôneas que possam comprometer a validade externa.

Em suma, este estudo é original, com potencial promissor devido a inclusão do etanol na formulação do fotossensibilizador azul de metileno, aplicado na terapia fotodinâmica antimicrobiana como tratamento adjuvante para doença periodontal, ter apresentado a curto prazo os menores níveis de peroxidação lipídica plasmática, representando um menor dano oxidativo. Do mesmo modo, que simultaneamente, expressou o maior número de vasos sanguíneos, demonstrando uma maior resposta reparacional. Além disso, a longo prazo, esta terapia induz a recuperação das defesas antioxidantes sistêmicas, recuperarando totalmente os níveis plasmáticos de vitamina C, evidenciando a eliminação do processo de doença.

Com base em todos resultados apresentados, podemos inferir pela primeira vez que este protocolo demonstrou melhor efeito terapêutico dentre as demais terapias testadas neste modelo experimental animal. Novas pesquisas devem ser realizadas nesta esfera, com o intuito de fortalecer nossos achados e posteriormente ser executados estudos clínicos com humanos. À medida que, nossos esforços são voltados para uma melhoria no tratamento de indivíduos com doença periodontal, visando sempre o melhor prognóstico dos mesmos.

Em relação aos parâmetros de aferição deste estudo, foram eleitos biomarcadores de estresse oxidativo e a avaliação histomorfométrica vascular, ambos métodos diferenciados e pouco utilizados para avaliações deste cunho. Houve uma relação positiva entre o aumento do número de vasos e depleção do dano oxidativo sistêmico, indicando o maior papel da angiogênese no reparo da doença periodontal. Esta premissa contribui com a literatura vigente que busca revelar a maior associação do processo angiogênico nos estágios da doença periodontal. O uso da técnica de imunoistoquímica com anticorpos específicos para marcação vascular, poderia ser acrescentado fidelizando ainda mais tais achados.

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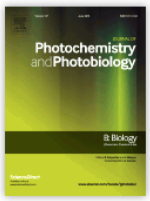

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[1] B.K. Armstrong, A. Kricker, The epidemiology of UV induced skin cancer, J. Photochem. Photobiol. B 63 (2001) 8-18.
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[2] W. Strunk Jr., E.B. White, The Elements of Style, third ed., Macmillan, New York, 1979.
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[3] G.R. Mettam, L.B. Adams, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing, Inc., New York, 1994, pp. 281-304.

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Anexo B – Aprovação da Comissão de Ética no Uso de Animais-UFSM

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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 C – Comprovante de submissão do artigo de pesquisa no periódico Journal of Photochemistry and Photobiology B: Biology

Author's Decision

Thank you for approving "ROLE OF THE ADJUNCTIVE ANTIMICROBIAL PHOTODYNAMIC THERAPY TO PERIODONTAL TREATMENT AT PLASMATIC OXIDATIVE STRESS AND VASCULAR BEHAVIOR". An email has been sent to you confirming that the journal has received this submission. Your Co-Author(s) may also receive this email, depending on the journal policy.

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Dear Dr. Luisa Barin,

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

Journal: Journal of Photochemistry and Photobiology B: Biology
Corresponding Author: Raquel Barcelos
Co-Authors: Luisa M Barin, M.D.; Luciana T Vey, M.D.; Fernanda M Pillusky, M.D.; Karla Z Kantorski, Ph.D; Marilise E Bürger, Ph.D; Roberto M Maciel, Ph.D; Cristiane C Danesi, Ph.D
Title: ROLE OF THE ADJUNCTIVE ANTIMICROBIAL PHOTODYNAMIC THERAPY TO PERIODONTAL TREATMENT AT PLASMATIC OXIDATIVE STRESS AND VASCULAR BEHAVIOR

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