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Luisa Machado Barin

RESPOSTAS HISTOLÓGICAS, BIOQUÍMICAS E IMUNO-HISTOQUÍMICA DE RATOS DIABÉTICOS COM PERIODONTITE TRATADOS COM TERAPIA FOTODINÂMICA ANTIMICROBIANA

Santa Maria, RS
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Orientadora: Profa. Dra. Cristiane Cademartori Danesi

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“O impossível existe
até que alguém duvide dele
e prove o contrário”

Albert Eistein

RESUMO

RESPOSTAS HISTOLÓGICAS, BIOQUÍMICAS E IMUNO-HISTOQUÍMICA DE RATOS DIABÉTICOS COM PERIODONTITE TRATADOS COM TERAPIA FOTODINÂMICA ANTIMICROBIANA

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A presente tese foi estruturada em dois estudos experimentais animais, apresentados em forma de artigos, que investigaram os efeitos da alteração do fotossensibilizador (Fs) azul de metileno (AM), solubilizado em etanol, utilizado na terapia fotodinâmica antimicrobiana (TFDa), como tratamento adjuvante à raspagem e alisamento radicular (RAR), na doença periodontal (DP) com ou sem o envolvimento da diabetes melittus (DM). O primeiro artigo avaliou as respostas teciduais sistêmica e local geradas por este novo Fs na presença da DM. Além disso, verificou-se a relação do fator de crescimento endotelial vascular (VEGF) nos processos de avanço e reparo da DP. Oitenta ratos Wistar receberam injeção intraperitoneal de estreptozotocina e após 14 dias alguns ratos receberam a ligadura no primeiro molar inferior direito para indução experimental da DP. Os grupos experimentais foram: CN (controle negativo, sem DP); CP (controle positivo, com DP e sem tratamento); RAR (com DP e RAR); TFDa I (com DP e RAR + TFDa + AM solubilizado em água) e TFDa II (com DP e RAR + TFDa + AM solubilizado em etanol). Após 7 dias, as ligaduras foram removidas e os animais foram tratados, aos 7 e 15 dias, foram eutanasiados, e as amostras coletadas para avaliação bioquímica, histológica e imuno-histoquímica. Em 7 e 15 dias, o grupo da TFDa II apresentou menor intensidade inflamatória, menor intensidade do VEGF, menor quantidade de vasos sanguíneos (apenas nos 7 dias) e menor dano oxidativo (igual ao grupo CN ao atingir 15 dias), quando comparado ao grupo RAR. A TFDa II não mostrou diferenças estatísticas significativas em relação à TFDa I. Conclui-se que a TFDa II foi capaz de facilitar e acelerar o reparo tecidual periodontal a curto prazo e possui potencial para o tratamento periodontal em pacientes com diabetes possibilitando respostas reparadoras mais rápidas. A TFDa apresenta benefícios adicionais quando comparada apenas à RAR. A intensidade do VEGF foi maior em ratos diabéticos com DP e pode ter maior influência durante o processo de avanço da DP. O segundo estudo avaliou as repostas nos tecidos periodontais geradas por este mesmo protocolo, porém em ratos sistemicamente saudáveis. Cento e vinte ratos Wistar foram randomizados e divididos nos mesmo grupos experimentais do estudo acima. A DP também foi induzida por ligadura, que permaneceu por 7 dias, após, os animais foram tratados e eutanasiados em 7, 15 e 30 dias. As amostras foram coletadas para avaliação histológica e imuno-histoquímica. Os grupos tratados demonstraram menor intensidade inflamatória e do VEGF, quando comparados ao grupo CP. As respostas apresentadas entre os grupos tratados foram similares, porém os casos de ausência inflamatória foram observados apenas com a TFDa em 7 dias. Interessantemente, apenas a TFDa II, aos 30 dias apresentou ausência quase total de infiltração inflamatória. Conclui-se que, a TFDa proporcionou melhores respostas ao tecido periodontal, percebidas pelo menor grau inflamatório quando comparados ao RAR em curto prazo de tempo e a TFDa II demostrou maior capacidade de reparo a longo prazo. A maior expressão do VEGF foi observada no CP, sendo relacionada com o avanço da DP.

Palavras-chave: Angiogênese. Azul de Metileno. Diabetes Mellitus. Doença Periodontal. Estresse Oxidativo. Imuno-histoquímica.

ABSTRACT

HISTOLOGICAL, BIOCHEMICAL AND IMMUNO-HISTOCHEMICAL RESPONSES OF DIABETIC RATS WITH PERIODONTITIS TREATED BY ANTIMICROBIAL PHOTODYNAMIC THERAPY

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ADVISOR: CRISTIANE CADEMARTORI DANESI

The present thesis is structured in two animal experimental studies, presented as article that investigated the effects of methylene blue (MB) photosensitizer (Fs) solubilized in ethanol on antimicrobial photodynamic therapy (aPDT) as adjuvant treatment in scaling and root planing (SRP) in the periodontal disease (PD), with or without the involvement of diabetes mellitus (DM). The first article evaluated the systemic and local tissue responses generated by this new Fs in the presence of DM. In addition, it was verified the relationship of vascular endothelial growth factor (VEGF) in the PD advance and repair processes. Eighty Wistar rats received intraperitoneal injection of streptozotocin and after 14 days some rats received ligadure in the mandibular right first molar for experimental induction of PD. The experimental groups were NC (negative control, no PD); PC (positive control, with PD and without treatment); SRP (with PD and SRP); aPDT I (with PD and SRP + aPDT + MB solubilized in water) and aPDT II (with PD and SRP + aPDT + MB solubilized in ethanol). After 7 days, ligadures were removed and animals were treated, then euthanized at 7 and 15 days, and samples collected for biochemical, histological and immunohistochemical evaluation. At 7 and 15 days, the aPDT II group showed lower inflammatory and VEGF intensities, lower blood vessel numbers (only 7 days) and lower oxidative damage (as well as the NC group when reaching 15 days) when compared to the SRP group. The aPDT II demonstrated no statistically significant differences in relation to the aPDT I. It was concluded that the aPDT II was able to facilitate and accelerate the periodontal tissue repair in the short-term and has the potential for periodontal treatment in patients with diabetes allowing faster repairing responses. The aPDT presented additional benefits when compared to SRP alone. The intensity of VEGF was higher in diabetic rats with PD and may have greater influence during advance process of PD. The second study evaluated the responses in the periodontal tissues generated by the same protocol, but in systemically healthy rats. One hundred and twenty Wistar rats were randomized and divided into the same experimental groups as the previous study. The PD was also ligature-induced, remaining for 7 days, then the animals were treated and euthanized at 7, 15 and 30 days. Samples were collected for histological and immunohistochemical evaluation. Treated groups showed lower inflammatory and VEGF intensities when compared to the CP group. Treated groups presented similar responses, however cases of inflammatory absence were observed only in aPDT at 7 days. Interestingly, only aPDT II at 30 days revealed almost total absence of inflammatory infiltration.It was concluded that aPDT provided better responses to periodontal tissue, found by the lower inflammatory degree when compared to SRP in the short-term and the aPDT II demonstrated a greater long-term repair capacity. The highest expression of VEGF was observed in PC, being related to the PD progression.

Keywords: Angiogenesis. Diabetes Mellitus. Immunohistochemistry. Methylene Blue. Oxidative Stress. Periodontal Disease.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

%	Por cento
°C	Grau Celsius
®	Marca Registrada
™	Marca Comercial
ADA	Associação Americana de Diabetes; “American Diabetes Association”
AGEs	Produtos Finais de Glicação Avançada; “Advanced Glycation End-products”
AIDS	Síndrome da Imunodeficiência Adquirida; “Acquired Immune Deficiency Syndrome”
AM	Azul de Metileno
CAT	Catalase; “Catalase”
cm^2	Centímetro quadrado
DBF	Formato de Banco de Dados; “Database Format”
DP	Doença Periodontal
dL	Decilitro
DM	Diabetes Mellitus
DM1	Diabetes Mellitus tipo 1
DM2	Diabetes Mellitus tipo 2
DNA	Ácido Desoxirribonucleico; “Deoxyribonucleic Acid”
EO	Estresse Oxidativo
EROs	Espécies Reativas de Oxigênio
EV	Endovenoso
Fs	Fotossensibilizador
g	Grama
GPx	Glutationa Peroxidase; “Glutathione Peroxidase”
GSH	Glutationa Reduzida; “Glutathione”
HbA1c	Hemoglobina Glicada
HE	Hematoxilina/Eosina
H_2O	Água
H_2O_2	Peróxido de Hidrogênio
IDF	Federação Internacional de Diabetes; “International Diabetes Federation”

InGaAlP	Índio, Gálio, Alumínio e Fósforo
IL-1	Interleucina-1
IP	Intraperitoneal
J	Joule
J/cm^2	Joule por centímetro quadrado
J/ponto	Joules por ponto
Kg	Quilograma
Laser	Light amplification by stimulated emission of radiation
M	Molar (mol.L-1)
MDA	Malondialdeído; “Malondialdehyde”
mg	Miligrama
mL	Mililitro
mm	Milímetro
mm^2	Milímetro quadrado
mol	Quantidade de Substância
mW	MegaWatt
Na	Sódio
nm	Nanômetro
O_2	Oxigênio
$O_2^{-\bullet}$	Ânion Superóxido
$OH^{\bullet}$	Radical Hidroxila
OMS	Organização Mundial da Saúde
OPG	Osteoprotegerina; “Osteoprotegerin”
PMN	Polimorfonucleares
pH	Potencial Hidrogeniônico
RANK	Receptor Ativador do Fator Nuclear kappa B; “Receptor Activator of Nuclear factor Kappa-B”
RANKL	Ligante do Receptor Ativador do Fator Nuclear kappa B; “Receptor Activator of Nuclear factor Kappa-B Ligand”
RAR	Raspagem e Alisamento Radicular
RL	Radicais Livres
SOD	Superóxido Dismutase; “Superoxide Dismutase”
SSPS	Statistical Package for Social Sciences
STZ	Estreptozotocina; “Streptozotocin”

TBA	Ácido Tiobarbitúrico; “Thiobarbituric Acid”
TBARS	Substâncias Reativas ao Ácido Tiobarbitúrico; “Thiobarbituric Acid Reactive Substances”
TFDa	Terapia Fotodinâmica Antimicrobiana
TNF-alfa	Fator de Necrose Tumoral Alfa; “Tumor Necrosis Factor alpha”
UFSM	Universidade Federal de Santa Maria
VEGF	Fator de Crescimento Vascular Endotelial; “Vascular Endothelial Growth Factor”
VIT C	Vitamina C
µm	Micrômetro
µs	Microssegundo

SUMÁRIO

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).

Nesse contexto, a doença periodontal (DP) trata-se de uma enfermidade infecto-inflamatória 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 dento bacteriana 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, podendo levar à perda dental (KINANE; BERGLUNDH; LINDHE, 2010; LÖE; THELAIDE; JENSEN, 1965).

Dentre os tecidos periodontais, o tecido conjuntivo gengival destaca-se por atuar como barreira mecânica frente ao ataque dos periodontopatógenos. Por sua vez, sua proximidade com a superfície dental e o biofilme presente, permite que este seja precursor do processo inflamatório e repercuta nos eventos associados ao avanço da doença (BARTOLD; WALSH; NARAYANAN, 2000). Sendo que, tais eventos são responsáveis pela liberação de mediadores químicos que modulam a intensidade da resposta celular e vascular (KINANE; LINDHE, 2010; SOCRANSKY; HAFFAJEE; LINDHE, 2010).

Verifica-se que o principal mediador responsável pela expansão vascular é o fator de crescimento endotelial vascular (VEGF), o qual recruta e ativa as células endoteliais possibilitando a origem de novos capilares (DE LA PAZ et al., 2012; DVORAK, 2000; SZABO et al., 2000). Esse processo denomina-se angiogênese (BUSCHMANN; SCHAPER, 1999, EILKEN; ADAMS, 2010; KARAMYSHEVA, 2008), tendo o VEGF como importante mediador na DP, imprescindível para a manutenção, reparo e progressão da doença (R et al., 2014; ZOELLNER; CHAPPLE; HUNTER, 2002; YOSHINO et al., 2003). A intensidade do VEGF exerce notável influência na

etiopatogenia dessa doença (BASSIOUNY; ALAYED; ALSAMAN, 2014; CHAPPLE; KUMAR; HUNTER, 2000; JOHNSON; SERIO; DAI, 1999), podendo ser potencializado na presença de hipóxia (BROGI et al., 1994; VASCONCELOS et al., 2016), óxido nítrico (EL-AZAB; HAZEM; MOUSTAFA, 2012; JOZKOWICZ et al., 2001) e estresse oxidativo (EO) (CHUA; HAMDY; CHUA, 1998). O EO tem um papel significativamente importante durante o processo inflamatório, sendo apontado como um biomarcador da atividade periodontal, através da mensuração das espécies reativas de oxigênio (EROs), produtos de peroxidação lipídica e sistemas de defesa antioxidantes (BALTACIOĞLU et al., 2014; DALAI et al., 2013).

Ademais, vale ressaltar, que a presença de comprometimento sistêmico, como o diabetes mellitus (DM) e, consequentemente a hiperglicemia, permite alterações diretas e indiretas de tais mediadores biológicos (BORRELL; PAPAPANOU, 2005). Assim, a associação entre DM e DP promove uma resposta inflamatória local e sistêmica exacerbada (LALLA et al., 2000; SCHALLHORN, 2016), alterando a integridade e estabilidade estrutural dos tecidos conjuntivo e ósseo (LORENCINI et al., 2009). Sendo que, tal fato promove maior severidade e prevalência da DP em indivíduos diabéticos (CINTRA et al., 2016; PUCHER; OTOMO-CORGEL, 2002).

Evidências relatam que pacientes diabéticos não respondem ao tratamento periodontal convencional, de raspagem e alisamento radicular (RAR), da mesma forma que pacientes não diabéticos (ALTAMASH; KLINGE; ENGSTRÖM, 2016; TERVONEN; KARJALAINEN, 1997). Nestas situações, o uso de antimicrobiano pode ser sugerido como terapia coadjuvante (SLOTS, 2004), porém, sua administração sistêmica pode resultar em infecções oportunistas, reações de hipersensibilidade e desenvolvimento de resistência bacteriana (CASSELL; MEKALANOS, 2001). A heterogeneidade e a disposição dos microrganismos em camadas no biofilme possibilitam a proteção dos mesmos frente aos agentes antibacterianos, contestando a indicação desta terapia (PRESHAW, 2004a; 2004b). Recentes revisões sistemáticas têm apresentado benefícios clínicos limitados em diabéticos submetidos à RAR associada a antibióticos sistêmicos (GRELLMANN et al., 2016; SANTOS et al., 2015).

Mediante a esse argumento, a terapia fotodinâmica antimicrobiana (TFDa) é apresentada como uma alternativa terapêutica coadjuvante promissora, tendo como vantagem a baixa probabilidade de desenvolver patógenos multirresistentes e possuir ação local, ideal para infecções localizadas e associadas a complicações sistêmicas

(GOULART et al., 2010; HAMBLIN; HASAN, 2004; MAISCH, 2007; WAINWRIGHT et al., 1997). Ensaios clínicos randomizados demonstraram efeitos adicionais da TFDa associada à RAR, em comparação com RAR apenas (ALWAELI; AL-KHATEEB; AL-SADI, 2013; BETSY et al., 2014; BRAUN et al., 2008; GIANNELLI et al., 2012). Revisões sistemáticas apontam para resultados estatisticamente significantes a favor da terapia combinada, porém com benefício clínico questionável em pacientes diabéticos (ABDULJABBAR et al., 2016; SGOLASTRA et al., 2013a).

Possibilidades para melhorar o efeito da TFDa estão sendo investigadas e o estudo do solvente no qual o fotossensibilizador (Fs) é dissolvido torna-se uma questão oportuna. Um estudo apenas sobre TFDa realizadas até o presente momento utilizam como Fs o azul de metileno (AM) dissolvido em água (ALWAELI; AL-KHATEEB; AL-SADI, 2013). No entanto, estudos recentes, *in vitro*, apontam melhores propriedades fotofísicas e fotoquímicas quando o Fs é dissolvido em solvente com polaridade menor que da água, como o etanol, culminando em melhor efeito antimicrobiano (GEORGE; KISHEN, 2007; Prochnow et al., 2016).

O DM apresenta-se como um grande problema de saúde pública mundial (GUARIGUATA et al., 2014; WILD et al., 2004). A DP acomete cerca de um terço desses indivíduos (KIDAMBI; PATEL, 2008), manifestando-se de forma mais severa (BASSIM; WARD; DENUCCI, 2007; LÖE, 1993; MARTÍNEZ; FEBLES; ESPORRÍN, 2014). Diante desse panorama e com o intuito de proporcionar benefícios adicionais para o tratamento da DP, com ou sem envolvimento da DM, o presente estudo objetivou testar um protocolo pioneiro da TFDa, adjuvante à RAR, com inclusão do etanol na solubilização do Fs AM em dois experimentos animais. O primeiro estudo avaliou as respostas teciduais sistêmica e local do hospedeiro após aplicação da TFDa no tratamento da DP associada com comprometido sistêmico (DM) e aferidas por análises histológicas, bioquímicas e imuno-histoquímica. Bem como, objetivou relacionar o fator de crescimento endotelial vascular (VEGF) com o processo de avanço e reparo durante o curso da DP. Já o segundo experimento visou testar o mesmo protocolo acima descrito, em ratos com periodontite sem DM, sob parâmetros histológicos e imuno-histoquímico do VEGF, avaliados localmente nos tecidos periodontais.

2 REVISÃO DA LITERATURA

2.1 DOENÇA PERIODONTAL: ETIOPATOGENIA

A DP é reconhecida como 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). É resultado da agressão de patógenos da placa dentobacteriana e a suscetibilidade inata de defesa do indivíduo (KINANE; BARTOLD, 2007; PAGE; BECK, 1997). A resposta imune do hospedeiro conduzirá a progressão da doença (COCHRAN, 2008; GENCO, 1992), a qual pode permanecer confinada a área gengival por vários anos ou resultar em perda do ligamento periodontal e osso alveolar (KINANE; BERGLUNDH; LINDHE, 2010; LÖE; THELAIDE; JENSEN, 1965; SCHENKEIN, 2006).

O curso inflamatório da doença caracteriza-se por alterações vasculares, celulares e enzimáticas. Os fenômenos vasculares são caracterizados pela proliferação vascular, aumento do fluxo sanguíneo, aumento da permeabilidade vascular e presença de exsudato. As células predominantes neste processo são os neutrófilos, macrófagos, linfócitos e plasmócitos. A ativação leucocitária resulta em fagocitose das substâncias nocivas, produção de mediadores químicos que modulam a intensidade da reação e produção de substâncias que incluem enzimas lisossômicas e EROs (COCHRAN, 2008).

Os mediadores químicos liberados localmente podem ser divididos em categorias, como: citocinas, hormônios e fatores de crescimento. Hodiernamente, há um alerta para as interações de alguns fatores de crescimento, devido estes participarem ativamente da patogênese da DP, modificando o quadro clínico da doença (COCHRAN; WOZNEY, 1999; OZMERIC 2004). A produção de mediadores inflamatórios sinaliza as atividades celulares associadas com a reabsorção óssea e a degradação da matriz extracelular do tecido conjuntivo gengival e do ligamento periodontal (SOCRANSKY; HAFFAJEE, 1992). Com o desenvolvimento de uma inflamação crônica, mudanças tornam-se sobrepostas e tanto os elementos agudos como crônicos coexistem nas lesões periodontais (KINANE; BERGLUNDH; LINDHE, 2010; PAGE; SCHROEDER, 1976).

A DP é referida por sua etiologia multifatorial, modulada por diversos fatores intrínsecos e extrínsecos. Os fatores intrínsecos abrangem a genética, as variações

na estrutura do tecido (imunidade inata), as respostas de anticorpos (imunidade adaptativa) e os mediadores inflamatórios (KINANE; BARTOLD, 2010). Dentre os fatores extrínsecos, os destaques são: sexo, idade, condição social, tabagismo, etilismo e diabetes (TATAKIS; KUMAR, 2005). Segundo Susin et al. (2007), fatores demográficos, socioeconômicos e genéticos, são considerados indicadores de risco, enquanto o fumo e, principalmente, o diabetes, são as exposições que apresentam as maiores evidências de fator de risco à DP.

2.2 DOENÇA PERIODONTAL: FATORES INTRÍNSECOS

2.2.1 Metabolismo Ósseo Alveolar

A reabsorção óssea é um mecanismo de proteção do organismo, pois conforme a reação inflamatória se aproxima do osso alveolar, a reabsorção é iniciada, de modo a evitar a invasão bacteriana no osso, mantendo uma distância da periferia do infiltrado inflamatório (COCHRAN, 2008). A reabsorção ocorre pela remoção controlada de componentes orgânicos e inorgânicos, mediante a ação de células específicas, os osteoclastos. Esses são células multinucleadas derivadas de células estaminais hematopoiéticas, pertencentes à linhagem dos monócitos/macrófagos (BAR-SHAVIT, 2007).

A proteína ligante do receptor ativador do fator nuclear kappa B (RANKL), os seus receptores ativadores do fator nuclear kappa B (RANK) e a osteoprotegerina (OPG) apresentam papéis fundamentais na biologia dos osteoclastos, sendo que a interação RANKL/RANK é essencial para a estimulação da osteoclastogênese e promoção da reabsorção óssea. Por outro lado, a interação RANKL/OPG inibe a estimulação da osteoclastogênese, impedindo assim a reabsorção (YASUDA et al., 1998).

A inflamação mobiliza os clastos principalmente via fator de necrose tumoral alfa (TNF-alfa), interleucina-1 (IL-1) e prostaglandina E2, ativando ligações como RANKL/RANK. O curso da reabsorção depende de uma complexa interação entre as células ósseas, dentárias e inflamatórias dos tecidos adjacentes. Ainda a ativação de células B, que se diferenciariam em plasmócitos secretores de imunoglobulinas, além de produtores de RANKL, também ativam o desenvolvimento de osteoclastos e promovem a destruição óssea (YASUDA et al., 1998).

O sistema RANK/RANKL/OPG, as citocinas, fatores de crescimento, hormônios e outros componentes extracelulares, possuem efeitos reguladores no metabolismo dos tecidos duros (COCHRAN, 2008; NAIR et al., 1996; PHAN et al., 2004; YASUDA et al., 1998).

2.2.2 Angiogênese

A manutenção das funções orgânicas dos tecidos depende do adequado suprimento de oxigênio, nutrientes, moléculas e células do sistema imune, as quais são carreadas via sanguínea. Com isso, pode-se afirmar que a vitalidade do organismo é proporcionada pela rede vascular (KARAMYSHEVA, 2008; POTENTE; GERHARDT; CARMELIET, 2011).

A formação dos vasos sanguíneos envolve dois processos fundamentais: a vasculogênese e angiogênese. A vasculogênese forma um plexo vascular primitivo, originada por intermédio de células endoteliais precursoras, os angioblastos, restrita a fase embrionária. Já a angiogênese designa a produção de novos capilares a partir de vasos preexistentes, através do crescimento de brotos endoteliais (ASPRIELLO et al., 2011; BUSCHMANN; SCHAPER, 1999, EILKEN; ADAMS, 2010; FILHO, 2009; KARAMYSHEVA, 2008; SURI, 1998).

A morfogênese vascular obedece a uma sequência de eventos altamente organizada, os quais possuem coordenado aparecimento espacial e temporal de células e de moléculas específicas (LIEKENS; DE CLERCQ; NEYTS, 2001). Mediante ao estímulo químico, as células endoteliais de vasos pré-existentes são ativadas. Essas células liberam enzimas proteolíticas, que degradam a membrana basal adjacente (MIGNATTI; RIFKIN, 1996). As células endoteliais iniciam a migração em direção à matriz extracelular degradada e proliferam, formando um broto capilar que será maturado e remodelado (CLAPP et al., 2009).

A neovascularização é essencial nos processos biológicos que demandam maior aporte sanguíneo, como em processos fisiológicos de: embriogênese (AUERBACH; AUERBACH, 1997), ciclo reprodutivo feminino (FREDERICK; SHIMANUKI; DIZEREGA, 1984) e 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 neovasos são necessários para transportar células fagocitárias e remover produtos de excreção, permitindo o reparo tecidual em

sua íntegra (WHALEN; ZETTER, 1992). Entretanto, o crescimento excessivo e persistente de neovasos é característico de processos patológicos (FOLKMAN; KLAGSBRUN, 1987) e está associado 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), diabetes e 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, as quais necessitam de maior aporte sanguíneo para suprir o processo imune metabolicamente ativo (BAGLI et al., 2004; CHUNG; FERRARA, 2011; MAJNO, 1998; SZEKANECZ; KOCH, 2004). A formação de vasos é imprescindível para a manutenção e reparo dos tecidos periodontais, porém em excesso e de forma permanente pode modificar a tragetória da DP, contribuindo para a maior progressão 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).

Em relação aos mecanismos de homeostasia em tecidos saudáveis, esses mantêm os níveis de oxigênio entre 2,5% a 9%. Porém, em situações inflamatórias de aumento de metabolismo celular e multiplicação de patógenos, os níveis de oxigênio podem ser considerados críticos, com concentrações inferiores a 1% (CLAPP et al., 2009; ELTZSCHIG; CARMELIET, 2011). Verifica-se que o gatilho proliferativo para ativação das células endoteliais provém principalmente desta reposta à hipóxia tecidual e conseguinte liberação de fatores de crescimento e citocinas pró-angiogênicas (KNIGHTON; SILVER; HUNT, 1981; RANDI; LAFFAN; STARKE, 2013).

2.2.3 Fator de Crescimento Endotelial Vascular (VEGF)

O VEGF, que apresenta atividade exclusivamente angiogênica, é considerado um potente regulador, devido sua atuação específica sobre o endotélio (HOEBEN et al., 2004; OLSSON et al., 2006; ROY; BHARDWAJ; YLÄ-HERTTUALA, 2006), sendo estimado como principal indutor da angiogênese em eventos fisiológicos e patológicos do organismo (FERRARA, 2009; MOENS et al., 2014; PRAPULLA; SUJATHA; PRADEEP, 2007; ROY; BHARDWAJ; YLÄ-HERTTUALA, 2006). Age como regulador da permeabilidade vascular, indutor da proliferação e migração das células endoteliais, bem como promove a expressão de proteínas antiapoptóticas Blc-2 e A1 nessas células (HORTA, 2008; KARAMYSHEVA, 2008; ROY; BHARDWAJ; YLÄ-HERTTUALA, 2006).

As células mesenquimais secretam o VEGF, sendo estimuladas por citocinas, fatores de crescimento, endotoxinas, hormônios e potencializadas em resposta ao óxido nítrico (EL-AZAB; HAZEM; MOUSTAFA, 2012; JOZKOWICZ et al., 2001; VASCONCELOS et al., 2016), EO (CHUA; HAMDY; CHUA, 1998) e hipóxia local (BROGI et al., 1994; VASCONCELOS et al., 2016). Com o restabelecimento do fluxo sanguíneo, os níveis de oxigênio são elevados, diminuindo a expressão do VEGF e inibindo a proliferação de células endoteliais (AIELLO; WONG, 2000; OLSSON et al., 2006). O $VEGF_{165}$ é a isoforma mais frequente e mitogênica (DE LA PAZ et al., 2012; KARAMYSHEVA, 2008), sendo detectável nos tecidos periodontais, nas células endoteliais, em macrófagos, plasmócitos, no epitélio juncional, sulcular e oral (BECIT et al., 2001; PRAPULLA; SUJATHA; PRADEEP, 2007; SANTOS, 2009; VASCONCELOS, 2012).

Autores relatam que o VEGF desempenha um importante papel na progressão da gengivite para a DP por promover a expansão da rede vascular observada na inflamação (BASSIOUNY; ALAYED; ALSALMAN, 2014; BOOTH et al., 1998; OLIVEIRA et al., 2008; SUTHIN et al., 2003). Porém, a literatura não é totalmente unânime quanto a real participação do VEGF no desenvolvimento da DP, podendo ter sua expressão aumentada (BOOTH et al., 1998; JOHNSON; SERIO; DAI, 1999; SUTHIN et al., 2003), diminuída (CHAPPLE; KUMAR; HUNTER, 2000) ou não afetada (ÜNLÜ et al., 2003). Além disso, o VEGF pode estar relacionado tanto com a evolução, quanto com o reparo e regeneração da DP (COOKE et al., 2006; GERBER et al., 1999; R et al., 2014; SUTHIN et al., 2003).

2.2.4 Estresse Oxidativo

Radicais livres (RL) são espécies químicas altamente reativas que contêm um elétron desemparelhado na sua órbita de valência (HALLIWELL; GUTTERIDGE, 1985). Idealmente, durante a cadeia respiratória mitocondrial, todo oxigênio (O_2) deveria sofrer redução tetravalente formando água (H_2O). No entanto, uma pequena porcentagem é reduzida por um, dois ou três elétrons, resultando em EROs, como o ânion superóxido ($O_2^{-\bullet}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($OH^{\bullet}$), respectivamente (ACUÑA-CASTROVIEJO et al., 2001).

Quando há um desequilíbrio entre a produção de EROs e a capacidade do organismo em combatê-las, instala-se um quadro denominado de EO. Tal processo pode causar vários danos celulares e, neste contexto, as macromoléculas como lipídios, proteínas e DNA são altamente suscetíveis (HALLIWELL; GUTTERIDGE, 1985). Estima-se que de 1 a 3 bilhões de EROs são geradas diariamente por célula e, 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 é constituído por antioxidantes enzimáticos e não enzimáticos (BRIGANTI; PICARDO, 2003; LOPEZ-TORRES et al., 1998). Entre os antioxidantes 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 (como a vitamina C), glutationa reduzida (GSH), entre outros (BRIGANTI; PICARDO, 2003; BURTON; INGOLD, 1984; DI MASCIO et al., 1990; MEISTER, 1988; SCHAFER; BUETTNER, 2001; SIES, 1999).

Os danos lipídicos, bem como seus produtos de metabolismo, entre eles o malondialdeído (MDA), podem afetar direta ou indiretamente a homeostase das células e dos tecidos. Como consequência, o aumento da peroxidação da membrana lipídica 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 a peroxidação lipídica é denominado substâncias reativas ao ácido tiobarbitúrico (TBARS), baseado na reação do MDA com o ácido tiobarbitúrico (TBA) (OHKAWA; OHISHI; YAGI, 1979). Estudos relatam e evidenciam que níveis

elevados de MDA na DP crônica, podendo ser considerado um potente marcador do EO induzido pela DP (BALTACIOGLU et al., 2014; DALAI et al., 2013; KHALILI; BILOKLYTSKA, 2008).

Logo, há uma estreita relação entre a produção de EROs e o prejuízo da defesa antioxidante, o dano oxidativo da membrana lipídica celular e os processos inflamatórios ou patológicos degenerativos (BRIGANTI; PICARDO, 2003). Verifica-se que durante o processo inflamatório da DP, as EROs são geradas pela ativação de leucócitos polimorfonucleares (PMN) que, quando presentes em excesso, causam efeitos nocivos ao tecido gengival, ao ligamento periodontal e aos osteoblastos (CHAPPLE; MATTHEWS, 2007; CHAPPLE, 1997; 20 GUSTAFSSON; ASMAN, 1996; SEYMOUR; WHYTE; POWELL, 1986; WADDINGTON; MOSELEY; EMBERY, 2000).

2.3 DOENÇA PERIODONTAL: FATORES EXTRÍNSECOS

2.3.1 Diabetes Mellitus

A DM, uma doença crônica e considerada um grave problema de saúde pública mundial, está sendo designado como a pandemia do século XXI (GUARIGUATA et al., 2014; WILD et al., 2004). A Organização Mundial da Saúde (OMS) estima para 2030, 366 milhões de pessoas acometidas pelo DM, duplicando a prevalência em relação aos 171 milhões de casos identificados no ano de 2000 (WORLD HEALTH ORGANIZATION, 2006). Porém, dados de 2013 já identificaram 382 milhões de pessoas com a doença, elevando as perspectivas de acometidos para 592 milhões nos próximos 25 anos (INTERNACIONAL DIABETES FEDERATION, 2013). O Brasil ocupa o sexto lugar no ranking mundial em relação ao número de portadores da disfunção, com cerca de 11,9 milhões de indivíduos acometidos (INTERNACIONAL DIABETES FEDERATION, 2013). As projeções epidemiológicas brasileiras preveem a elevação desses índices para 19,6 milhões de pessoas em 2030 (WHITING et al., 2011). Tal panorama é de extrema relevância, uma vez que o DM apresenta altos índices de morbidade e mortalidade populacional, representando 9% das causas totais de mortes no mundo (SOUZA; GROSS, 2012).

A DM é uma síndrome de etiologia múltipla, caracterizada por distúrbios metabólicos provenientes da hiperglicemia originada pela disfunção na secreção de insulina ou pela perda progressiva de sua ação, bem como ambos os fatores. A

Associação Americana de Diabetes (ADA) categoriza a doença em quatro classes clínicas: diabetes mellitus tipo 1 (DM1), diabetes mellitus tipo 2 (DM2), outros tipos específicos de diabetes e diabetes gestacional. As mais prevalentes são DM1, que é uma manifestação autoimune agressiva, caracterizada pela destruição das células β pancreáticas, levando à deficiência absoluta de insulina, a qual acomete preferencialmente pacientes jovens e compõe cerca de 15% do total de quadros diabéticos. E a DM2, determinada por variados graus de diminuição da secreção e resistência à insulina, compreende, aproximadamente, 90% dos casos (AMERICAN DIABETES ASSOCIATION, 2013).

Os sintomas clássicos resultantes da hiperglicemia sanguínea são poliúria, polidipsia, polifagia, prurido, fraqueza, fadiga e, em menor escala, náusea, vômito, desidratação, instabilidade cardiovascular, alteração do estado mental, coma e morte (AMERICAN DIABETES ASSOCIATION, 2005). A intensidade dos sintomas é decorrente das complicações causadas pela doença. Estas podem ser de origem metabólica, com intolerância à glicose e metabolismo anormal das proteínas e dos ácidos graxos (KIDAMBI; PATEL, 2008); vasculares, afetando os grandes e pequenos vasos (BROWNLEE, 2001); infecciosa (GROVER; LUTHRA, 2013) e de origem bucal (FISKE, 2004).

Como principais complicações bucais da DM destacam-se a hiposalivação, candidíase, abscessos periapicais, hipocalcificação dentária, distúrbios na cicatrização, atraso na erupção dentária, síndrome da ardência bucal, recessão gengival, aumento na incidência das cáries dentárias e das DP (BASSIM; WARD; DENUCCI, 2007; FISKE, 2004; GALEA; AGANOVIC; AGANOVIC, 1986). Sendo que, a DP acomete cerca de um terço dos indivíduos diabéticos (KIDAMBI; PATEL, 2008) e é referida como a sexta complicação da DM (BASSIM; WARD; DENUCCI, 2007; LÖE, 1993; MARTÍNEZ; FEBLES; ESPORRÍN, 2014).

Estudos evidenciam que a DP pode interferir no controle glicêmico do portador de DM através da liberação de citocinas que atuam nos mecanismos de glicose e lipídios. Ademais, a resistência à insulina pode desenvolver-se em consequência a infecção crônica dos tecidos periodontais (PALMER; SOORY 2005; SCHALLHORN, 2016). Por outro lado, o pobre controle do quadro diabético repercute em maior perda de inserção periodontal e profundidade de bolsa, quando comparado aos quadros de moderado e forte controle da doença (GROVER; LUTHRA, 2013; JAHN, 1994) tanto no DM1 quanto no DM2 (GAROFALO, 2008; MESIA et al., 2016; SCHALLHORN,

2016), levando à progressão e gravidade da DP (ENGEBRETSON, 2014; GROVER; LUTHRA, 2013; KUMAR, 2014; STANKO; IZAKOVICOVA, 2014). No entanto, a patogenia da associação de ambas patologias é ainda indefina (TAYLOR et al., 1998). Algumas alterações biológicas, como microangiopatia, excessiva atividade colagenolítica, modificações na resposta inflamatória do hospedeiro e predisposição genética, tentam explicar esta relação (BOGHOSSIAN et al., 2014; GROVER; LUTHRA, 2013; MADEIRO et al., 2005).

Doenças metabólicas, associadas ao DM, levam a elevados níveis de hiperglicemia e hiperlipidemia, favorecendo a formação de produtos finais de glicosilação avançada (AGEs). Tais compostos podem ser reversíveis, podendo dissociar-se quando os níveis glicêmicos são normalizados, ou irreversíveis se a hiperglicemia permanecer, ficando presentes no plasma e demais tecidos. O acúmulo de AGEs é capaz de provocar alteração no fenótipo dos macrófagos, redução na função dos leucócitos PMN, alteração da produção de citocinas, aumento da destruição tecidual e perda óssea alveolar (BARBOSA, 2013; BROWNLEE, 2001; CLAUDINO et al., 2012; JÚNIOR; MACEDO; ANDRADE, 2007; PALMER; SOORY, 2005; ZIZZI et al., 2013).

A interação entre os AGEs e as células endoteliais resulta no aumento da espessura da parede dos capilares que, associado com diminuição do catabolismo de colágeno, impede a difusão de oxigênio, a eliminação de resíduos metabólicos dos tecidos, a migração de leucócitos e a propagação de fatores imunes. Estas alterações são também encontradas em tecido gengivais e contribuem para evolução da destruição periodontal e prejuízo do processo cicatricial (BROWNLEE, 2001; CLAUDINO et al., 2012; GROVER; LUTHRA, 2013; JÚNIOR; MACEDO; ANDRADE, 2007; ZIZZI et al., 2013). Portanto, a presença dos AGEs provoca destruição tecidual, resultando em DP mais severa e com maior dificuldade de controle glicêmico (BARBOSA, 2013; GROVER; LUTHRA, 2013; MADEIRO et al., 2005).

Estudos mostram que a DM atua como fator modificador para a DP, particularmente em pacientes com pobre controle glicêmico. Vários mecanismos já foram propostos para isso, como: disfunção endotelial, diminuição da quimiotaxia, fagocitose reduzida, aumento da produção de fator-alfa e interleucina-6 e produção de superóxido amplificado por aumento do estresse oxidativo compreendem as alterações vasculares e celulares. Os pacientes diabéticos reduzem a síntese de colágeno, ocorre o desequilíbrio entre a metaloproteinase da matriz tecidual e o

inibidor da metaloproteinase da matriz e retarda a síntese de glicosaminoglicanos. A cicatrização prejudicada verificada nos pacientes diabéticos, tem implicações para os resultados da terapia periodontal (IACOPINO, 2001).

A inter-relação entre DP e DM é um tema amplamente discutido na literatura, todavia, essas alterações histológicas periodontais, na presença do DM, ainda são pouco claras, demostrando a necessidade de estudos nesta esfera (ABREU et al., 2010; BARBOSA, 2013; ENGEBRETSON, 2014; KUMAR, 2014; MARTÍNEZ; FEBLES; ESPORRÍN, 2014; NEGRATO et al., 2013; SANTOS et al., 2012; STANKO; IZACOVIKOVA, 2014; WILLIAMS, 1960).

2.4 DOENÇA PERIODONTAL: ASSOCIANDO FATORES INTRÍNSECOS E EXTRÍNSECOS

2.4.1 DP x Destruição Óssea x VEGF x EO x DM

As EROs e o consequente EO têm sido relacionados com a patogênese de muitas doenças, incluindo o DM (BOLESTA et al., 2013; KOSE et al., 2016; MONEA et al., 2014), e mais recentemente, a DP (BALTACIOĞLU et al., 2014; CHAPPLE; MATTHEWS, 2007; DALAI et al., 2013; ZHANG et al., 2013). Uma grande atenção tem sido dada ao papel das EROs, dos produtos de peroxidação lipídica e do sistema de defesa antioxidante na DP (DALAI et al., 2013), uma vez que, parece haver maior geração de EROs pelos neutrófilos sanguíneos durante a DP em comparação com indivíduos periodontalmente saudáveis (GUSTAFSSON; ASMAN, 1996; MATTHEWS et al., 2007; SEYMOUR; WHYTE; POWELL, 1986). Ohnishi et al. (2009), em um modelo animal em camundongos, demonstraram que as EROs são responsáveis pela perda óssea alveolar na DP. Outra associação revela a redução da capacidade antioxidante frente ao aumento de biomarcadores do EO (AKALIN et al., 2007; BALTACIOĞLU et al., 2006; BARIN et al., 2017; BAUER; BAUER, 1999; CHAPPLE; KANMATTHEWS, 2007; KANZAKI et al. 2017; LIU et al., 2014; MASHAYEKHI et al., 2005; MATTHEWS et al., 2007; THOMAS et al., 2014; TSAI et al., 2005).

Triana, Bernabeu e Febles (2002), em um estudo *in vivo*, relataram que a possível participação do EO na patogenia da DP combinado com enfermidades sistêmicas poderia causar profundo impacto na qualidade da saúde geral e oral dos indivíduos. Assim como, a alta concentração de glicose provenientes da DM pode

elevar o EO e agravar as consequentes complicações, que comumente são demonstradas na cavidade oral como inflamações periodontais (BOLESTA et al., 2013). Existe um aumento do EO na DP crônica com e sem DM, indicando um fator comum no envolvimento do dano tecidual. Assim a destruição periodontal severa está associada com a geração de EROs excessiva, que é positivamente correlacionada com indivíduos diabéticos (PATIL et al., 2016).

Outras evidências sugerem a participação da EROs como potentes indutores pró-angiogênicos, potencializando a ação do VEGF (EL-AZAB; HAZEM; MOUSTAFA, 2012). Autores descrevem a expressão acentuada de VEGF no tecido periodontal de pacientes diabéticos (KELES et al., 2010; KRANTI; MANI; ELIZABETH, 2015; ÜNLÜ et al., 2003).

Sakallıoğlu et al. (2007) utilizaram marcação imuno-histoquímica no tecido gengival de indivíduos com a DP combinada ou não ao DM e observaram que os tecidos gengivais dos pacientes diabéticos apresentavam maior expressão de VEGF, sugerindo uma possível causa para uma maior gravidade da doença nesses pacientes. Pannicker e Mehta (2016) verificaram um aumento do VEGF no fluido crevicular de pacientes com DP crônica com ou sem DM que, ao serem tratados com a RAR, os seus níveis foram reduzidos substancialmente.

O VEGF apresenta grande participação no mecanismo do DM, devido a microangiopatia e a resposta angiogênica aumentada ser característica da doença. A hiperglicemia proveniente do DM provoca anomalias no fluxo sanguíneo, o que reflete na diminuição da atividade de vasodilatadores, como o óxido nítrico; no aumento da atividade de vasoconstritores, como a angiotensina II e no aumento de expressão de fatores de permeabilidade vascular, como o VEGF (BROWNLEE, 2001). Além disso, o VEGF parece afetar os níveis de glicose e a extensão das complicações diabéticas (AIELLO; WONG, 2000; MAHDY et al., 2010; ÜNLÜ et al., 2003). Gyurkovics et al., (2015), em estudo experimental com ratos Wistar, sugerem que a expressão de VEGF é aumentada na gengiva dos animais com DM induzida experimentalmente, podendo contribuir de forma significativa para as mudanças na microcirculação.

Em pacientes diabéticos, a formação de AGEs como resultado de glicosilação de proteínas e lipídios está entre os principais fatores que podem levar ao desenvolvimento da DP e ao aumento do EO gengival (LALLA et al., 2000; SCHMIDT et al., 1996; SOUTHERLAND et al., 2006). O EO exerce efeitos deletérios no DM, pois, a lesão celular oxidativa causada pelos RL contribui para a depleção das defesas

antioxidantes enzimáticas e não enzimáticas, agravando as alterações patológicas da doença (REIS et al., 2008). Como consequência, as células expressam fenótipos inflamatórios e sintetizam maior quantidade de mediadores inflamatórios (interleucinas, prostaglandinas e fator de necrose tumoral α), cujos efeitos podem resultar em ativação de osteoclastos e colagenases, modulando a destruição dos tecidos ósseo e conjuntivo (BEIKLER et al. 2002, BOWERSOX, 1986; CAHILL, 1985). Estes mecanismos podem resultar em diminuição na resistência do hospedeiro, cicatrização prejudicada e resposta inflamatória exagerada, exacerbando a destruição periodontal em pacientes com DM (LALLA et al., 2000). Contudo, tais efeitos variam de acordo com o tipo, duração, controle metabólico da doença, cuidados com os dentes e hábitos (TERVONEN; KARJALAINEN, 1997).

Há limitados estudos na literatura sobre a presença do VEGF nos tecidos periodontais de pacientes diabéticos (ASPRIELLO et al., 2009; GÜNERI et al., 2004; SAKALLIOĞLU et al., 2007; ÜNLÜ et al., 2003). Autores afirmam que a presença de altos níveis de glicose induz a níveis elevados de EO (BROWNLEE, 2001). Este por sua vez, potencializa a ação do VEGF (CHUA; HAMDY; CHUA, 1998), que atua como modulador no desenvolvimento da DP (BASSIOUNY; ALAYED; ALSALMAN, 2014) e é capaz de provocar alterações nos níveis de glicose e expandir as complicações diabéticas (AIELLO; WONG, 2000; MAHDY et al., 2010; ÜNLÜ et al., 2003). O agravo do processo inflamatório presente na DP também contribui para o aumento do EO (CHAPPLE; MATTHEWS, 2007; CHAPPLE, 1997; WADDINGTON; MOSELEY; EMBERY, 2000). Tais estreitas inter-relações tornam sugestivo mais estudos neste sentido (Figura 1).

Figura 1. Desenho esquemático da associação entre DP x VEGF x EO x DM. Fonte: Própria.

2.5 MODELOS EXPERIMENTAIS ANIMAIS

O uso de modelos experimentais tem a finalidade de produzir conhecimento científico aos seres humanos, como o esclarecimento sobre doenças, a elaboração de novas drogas, novas técnicas, novas formulações terapêuticas e vacinas (CHORILLI; MICHELIN; SALGADO, 2007; FAGUNDES; TAHA, 2004; KIRSTEN et al., 2010).

2.5.1 Indução da Doença Periodontal

A DP, diferentemente das demais doenças infecciosas humanas, é comum no reino animal (LISTGARTEN, 1975). Os modelos animais mais utilizados são de cães e ratos (KUHR et al., 2004; TUBB; WILLIAMS; FRAZIER, 1990). Em virtude da maior facilidade de manejo e do custo relativamente baixo, o rato torna-se um instrumento

versátil para o estudo desta doença (BEZERRA et al., 2000; MADDEN; CATON, 1994).

É importante ressaltar que a similaridade anatômica, imunológica e bioquímica dos tecidos periodontais do rato, bem como a semelhança histopatológica e, em parte, microbiológica, no que diz respeito às doenças periodontais, favorecem a indução experimental (KLAUSEN, 1991; SUSIN; RÖSING, 2002). A anatomia dentária do rato é composta por quatro dentes incisivos, que apresentam crescimento contínuo e não têm raízes e, doze molares, em número de três em cada hemi-arcada, com epitélio gengival, sulcular e oral, fibras colágenas, cemento celular e acelular e osso alveolar, semelhantes aos dentes dos humanos, existindo apenas uma diferença, o epitélio sulcular gengival do rato, que é queratinizado (KLAUSEN, 1991; PAGE; SCHROEDER, 1982), tornando possível a reprodução da DP (GALVÃO et al., 2003).

A indução periodontal experimental pode ser feita por colocação de ligadura de seda, elástico e algodão (COX; WILLIAMS-MILLER, 1986; DUARTE et al., 2010; ROVIN; COSTICH; GORDON, 1966), pela introdução de microrganismos patogênicos (FIEHN; KLAUSEN; EVANS, 1992; JORDAN; KEYES; BELLACK, 1972), por injeções de toxinas bacterianas lipopolissacarídeos (DUMITRESCU et al., 2004; LLAVANERAS et al., 1999; WADA et al., 2004) ou pela manipulação dietética (GALVÃO et al., 2003; ROBINSON; HART; PIGOTT, 1991). A técnica preconizada é o uso de uma ligadura, que consiste na inserção de um dispositivo ao redor de um dente, como a finalidade de promover irritação mecânica e aumentar a agregação bactérias através do acúmulo de biofilme supra e subgengival gerando uma resposta inflamatória prejudicial ao periodonto (BENTZEN et al., 2005; DUARTE et al., 2010; GARCIA et al., 2013b; GASPERSIC; STIBLAR-MARTINCIC; SKALERIC, 2002; GYÖRFI et al., 1994). Utilizando este modelo, Bezerra et al. (2000) observaram perda óssea alveolar significante após 4 dias da colocação de ligadura, alcançando um pico máximo aos 7 dias. Histopatologicamente, a presença de infiltrado inflamatório progressivo e aumento do número de osteoclastos comprovaram a existência da DP em apenas uma semana.

Holzhausen et al. (2004) notaram aumento de volume gengival da face vestibular dos primeiros molares inferiores, recobrindo grande parte da ligadura, sendo que esta característica foi facilmente observada a partir do período experimental de 5 dias. Os autores também demonstraram que a colocação da

ligadura favoreceu o acúmulo de grande quantidade de restos alimentares e formação de biofilme bacteriano, confirmando a eficácia deste método na indução periodontal.

2.5.2 Indução da Diabetes Mellitus

Roedores são os animais mais utilizados para a indução da DM experimental, devido ao fácil acesso, manuseio, habitação e menor custo (CHEN; WANG, 2005). A indução da DM experimental em modelos animais pode ser realizada através do uso de substâncias que destroem seletivamente as células β-pancreáticas, produtoras de insulina, como a Aloxana e a Estreptozotocina (STZ) (SZKUDELSKI, 2001). A STZ apresenta maior estabilidade química quando comparada à Aloxana, sendo mais utilizada para este fim (LENZEN, 2008).

A STZ é um agente antimicrobiano, glicosaminanitrosureia, isolado do fungo *Streptomyces achromogenes* utilizado tanto para induzir DM tipo 1 como tipo 2, dependendo da dose, via de administração e concentração (NEGRI, 2005; SZKUDELSKI, 2001). Em ratos, a STZ provoca o bloqueio irreversível, da produção de insulina nas células β do pâncreas, causando grave hiperglicemia, induzindo o DM semelhante ao tipo 1. Uma dose única no rato recém-nascido pode produzir um modelo experimental de DM2, caracterizado por uma deficiência na síntese de insulina no pâncreas, diminuindo sua liberação em resposta à glicose (MARLES; FARNSWORTH, 1995).

Doses elevadas de STZ, consideradas de 160 a 250 mg/kg, apresentam efeito citotóxico direto nas células β de camundongos, causando danos ao ácido desoxirribonucleico (DNA). É importante ressaltar que a toxicidade da STZ, quando administrada em dose única elevada, pode induzir o desenvolvimento de tumores no rim, fígado e pâncreas de ratos (BOLZAN; BIANCHI, 2002; SZKUDELSKI, 2001). Doses baixas de 40mg/kg podem produzir insulinite pancreática, ocasionando inflamação com morte progressiva das células β, levando ao DM (VAREDA, 2013). A diferença entre as dosagens é que, em doses baixas de STZ, o aparecimento da lesão inflamatória nas ilhotas pancreáticas ocorre através da reação imune mediada por células T (LENZEN, 2008), ao invés do dano direto ao DNA devido à ação citotóxica nas células β observado em doses abusivas (RAYAT, 2000; HAGEMAN, BUSCHARD, 1994).

A via de administração de STZ para indução de DM experimental pode ser tanto endovenosa (EV) (DELFINO et al., 2002; KIM et al., 2014) quanto intraperitoneal (IP) (BAHMANZADEH et al., 2016; SILVA et al., 2015; SIMON; WEST, 1992). Por via EV (veia sublingual ou caudal), a dose de 60mg/Kg promove DM severo e estável por 40 semanas (DELFINO et al., 2002). Além disso, Kim et al. (2014) observaram que a dose de 50mg/Kg EV foi suficiente para induzir DM por 20 dias. Já a administração de STZ via IP em ratos Wistar provocou DM leve e instável na dose de 45mg/Kg, porém na dose 60mg/Kg encontrou-se DM duradoura em 100% dos animais (SIMON; WEST, 1992). Bahmanzadeh et al. (2016) observaram que três dias após a administração de 90mg/kg STZ, os animais já apresentavam glicemia superior à 270mg/dL, assim como Silva et al. (2015) que após um único dia de 80mg/kg de STZ, já verificaram um quadro diabético. Os animais que atingem uma glicemia acima de 250 mg/dL são considerados diabéticos (WAYHS et al., 2010).

Existem na literatura, diferentes protocolos em relação ao intervalo de indução do DM e posterior indução da DP, variando entre 7 (ALMEIDA et al., 2008), 14 (NISHIKAWA et al., 2012) e 21 dias (CHANG et al., 2013). Induzir DM e após 14 dias induzir a DP parece ser uma alternativa segura e equilibrada para o estabelecimento e manutenção do modelo experimental de DM em roedores (NISHIKAWA et al., 2012).

2.6 TRATAMENTO PERIODONTAL CONVENCIONAL

O tratamento convencional das DPs caracteriza-se pela remoção mecânica do biofilme dental e de depósitos mineralizados aderido à superfície radicular por meio de procedimentos de RAR. Assim, as superfícies dentárias tornam-se biocompatíveis ocorrendo resolução do processo inflamatório e cicatrização 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).

A efetividade do tratamento periodontal pode ser limitada por características anatômicas que dificultam a adequada descontaminação das superfícies dentárias como fissuras, concavidades e áreas de furca (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). Bem como, a presença de inúmeros periodontopatógenos (FENG; WEINBERG, 2006), com distintos potenciais de patogenicidade variando de sítio para sítio e de indivíduo para indivíduo, tornam a DP uma infecção mista de difícil controle (DZINK et al., 1985).

A presença de doenças sistêmicas também pode prejudicar a resolução do quadro clínico, devido à fragilidade das respostas imuno-inflamatórias (BOGHOSSIAN et al., 2014; KULKARNI; KINANE, 2014). Evidências apontam que pacientes com DM apresentam DP mais severa e não respondem ao tratamento periodontal da mesma forma que pacientes não diabéticos (ALTAMASH; KLINGE; ENGSTRÖM, 2016; TERVONEN; KARJALAINEN, 1997). O prejuízo da resposta leucocitária dos indivíduos com DM leva à uma diminuição da habilidade microbicida de leucócitos PMN e falência na entrega de componentes do sistema imune, humoral e celular. Então, ocorre uma depressão da quimiotaxia de leucócitos, assim como defeitos na fagocitose e morte bacteriana, aumentando a suscetibilidade à infecção dos pacientes diabéticos (BROWNLEE, 2001; CUTLER et al., 1991; OLIVER; TERVONEN, 1994).

Frente a tais complicações, o uso de antibióticos (SLOTS, 2004) e da TFDa vem sendo propostos como estratégias coadjuvantes ao tratamento periodontal convencional (BERAKDAR et al., 2012; DAI; HUANG; HAMBLIN, 2009; GURSOY et al., 2013; NOVAES et al., 2012; PRATES et al., 2011). Deve-se considerar que antibióticos administrados sistemicamente podem apresentar repercussões também sistêmicas, reações de hipersensibilidade e infecções oportunistas (CASSELL; MEKALANOS, 2001). A rápida emergência da resistência bacteriana frente ao uso indiscriminado e prolongado de antimicrobianos tem se tornado um problema atual (CASSELL; MEKALANOS, 2001). As bactérias que sofrem mutação na presença de antibióticos para favorecer sua sobrevivência podem tornar-se predominantes na população microbiana (ARDILA; GRANADA; GUZMÁN, 2010; GENCO, 1981; HEITZ-MAYFIELD, 2009). O desenvolvimento de patógenos multirresistentes, a heterogeneidade e a disposição dos microrganismos em camadas no biofilme possibilita a proteção dos mesmos frente aos agentes antibacterianos, podendo levar esta terapia ao fracasso (PRESHAW, 2004a; PRESHAW, 2004b). Recentes revisões sistemáticas têm apresentado benefícios clínicos limitados em diabéticos submetidos à RAR associada a antibióticos sistêmicos (GRELLMANN et al., 2016; SANTOS et al., 2015).

Neste contexto, a TFDa é apontada como uma alternativa terapêutica coadjuvante promissora, apresentando como vantagem sobre as demais a baixa probabilidade de desenvolver resistência bacteriana, possuir ação local, não ser invasiva, ter amplo espectro de atuação e não possuir dose-limite (ALVARENGA et al., 2015; BERAKDAR et al., 2012; DAI; HUANG; HAMBLIN, 2009; GURSOY et al., 2013; NOVAES et al., 2012; PRATES et al., 2011), ideal para infecções localizadas e associadas a complicações sistêmicas (GOULART et al., 2010; HAMBLIN; HASAN, 2004; MAISCH, 2007; WAINWRIGHT et al., 1997). Estudos experimentais animais (BARIN et al., 2017; PILLUSKY et al., 2017) e ensaios clínicos randomizados têm demonstrado efeitos adicionais da TFDa associada à RAR em comparação com apenas RAR (ALWAELI; AL-KHATEEB; AL-SADI, 2013; BETSY, et al., 2014; BRAUN, et al., 2008; GIANNELLI, et al., 2012).

2.7 TRATAMENTO PERIODONTAL COADJUVANTE

2.7.1 Terapia Fotodinâmica antimicrobiana

A TFDa é definida como uma reação fotoquímica, oxigênio-dependente na qual a ativação de um corante, conhecido como Fs, por uma luz visível e de comprimento de onda apropriado, leva a geração de EROs, principalmente oxigênio singleto ou 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 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 Fs no estado tripleto pode sofrer dois tipos de reações (Figura 2). Na reação tipo I, o Fs reage diretamente com um substrato, como as bactérias ou moléculas do meio, produzindo RL e superóxido. 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, mas 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 da concentração de substrato e de oxigênio, e do tipo de Fs utilizado (CASTANO; DEMIDOVA; HAMBLIN, 2004).

A TFDa, associada ao tratamento periodontal convencional, apresenta como vantagens ser uma terapia específica à célula alvo, não apresentar efeito colateral, iniciar sua atividade somente quando o Fs é exposto à luz e não favorecer a seleção de cepas resistentes, muito comum com o uso indiscriminado de antibióticos (GURSOY et al., 2013), teoricamente muito benéfica em casos de comprometimento sistêmico associado. O uso da TFDa em pacientes diabéticos promove a angiogênese e aumenta o aporte de oxigênio no local, contribuindo para a cicatrização tecidual (MEALEY; OCAMPO, 2007). Bem como, reduz a inflamação gengival em pacientes com DM e DP (OBRADOVIC et al., 2012).

Recente revisão sistemática avaliou a eficácia do uso adjuvante do laser e da TFDa na melhoria dos resultados clínicos periodontais e dos níveis de HbA1c de pacientes com DP crônica e DM2. Foram selecionados seis estudos dos quais dois mostraram resultados significativamente melhores com laser adjuvante à RAR, em comparação com apenas RAR, enquanto que quatro estudos demonstraram resultados comparáveis entre laser, TFDa e RAR. Dois estudos mostraram redução significativa dos níveis de HbA1c com laser e TFDa, em comparação com RAR, enquanto três estudos mostraram níveis percentuais similares. Ainda clinicamente é discutível se terapias adjuvantes à RAR são mais eficazes em comparação com apenas RAR na melhoria da condição clínica e do controle glicêmico em pacientes com DP e DM (ABDULJABBAR et al., 2016).

Em modelos de DP experimental em animais, o uso coadjuvante da TFDa à RAR tem demonstrado benefícios adicionais em comparação ao tratamento mecânico somente (BARIN et al., 2017; DE OLIVEIRA et al., 2016; PILLUSKY et al., 2017). O desenho experimental de indução da DP por colocação de ligadura no primeiro molar inferior de ratos vem sendo utilizado para estudos de TFDa em ratos sistemicamente normais (CARVALHO et al., 2011; DE ALMEIDA et al., 2008; GARCIA et al., 2013b), com DM (DE 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., 2013b). Inúmeras variáveis foram avaliadas nestes estudos e, dentre os métodos utilizados para mensurá-las, destacam-se a análise histológica (CARVALHO et al., 2011), histométrica (ALMEIDA et al., 2008; BOTTURA et al., 2011; GARCIA et al., 2011), bioquímica (BARIN et al., 2017; PILLUSKY et al.,

2017), imuno-histoquímica (GARCIA et al., 2013) e radiográfica (FERNANDES et al., 2010).

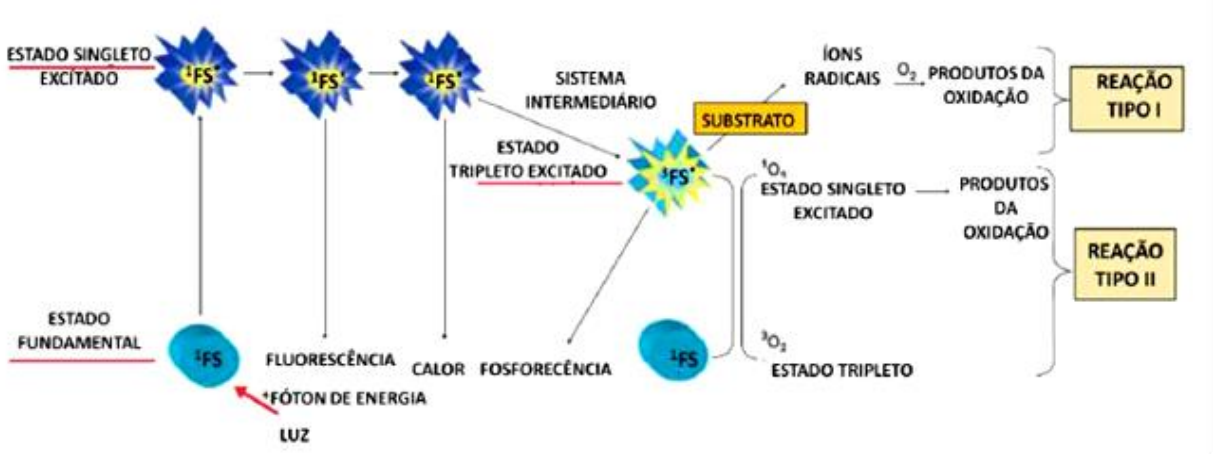

Figura 2 - Mecanismo de ação da TFDa; reações Tipo I e Tipo II. Fonte: (Soukos; Goodson, 2011).

2.7.2 Azul de Metileno

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 é utilizada, porém, o 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). Muitos ensaios clínicos randomizados, comparando a RAR com a terapia combinada (RAR+TFDa) utilizando como Fs o AM, foram realizados (BALATA et al., 2013; BETSY et al., 2014; GE et al., 2011; GIANELLI et al. 2012). As revisões sistemáticas apontam resultados estatisticamente significantes a favor da terapia combinada, mas ainda com benefício clínico questionável (ABDULJABBAR et al., 2016; SGOLASTRA et al., 2014; SGOLASTRA et al., 2013a; b; c).

Possibilidades para melhorar o efeito da TFDa estão sendo investigadas. A alteração do solvente no qual o Fs é dissolvido torna-se uma questão oportuna, pois até o presente momento os demais pesquisadores utilizam como Fs o AM dissolvido em água, porém há evidências de que esta solubilização resulta em limitada produção

de oxigênio singleto de meia-vida curta (4 µs) (MEISEL; KOCHER, 2005) e baixo potencial de difusão (OCHSNER, 1997). A produção e meia-vida do oxigênio singleto podem ser influenciadas pelo solvente no qual o Fs é dissolvido (GEORGE; KISHEN, 2007). Estudos recentes demostraram melhores propriedades fotofísicas e fotoquímicas em Fs dissolvidos em solvente menos polares que a água, com maior penetrabilidade tecidual e aumento da meia-vida do oxigênio singleto (MEISEL; KOCHER, 2005).

George e Kishen (2007) demonstraram *in vitro* que a inclusão do etanol aumentou o efeito antimicrobiano da TFDa frente a biofilmes de *Enterococcus faecalis* e *Aggregatibacter actinomycetemcomitans*. Prochnow et al. (2016) verificaram que a solubilização do AM em um solvente contento 20% de etanol aumentou o efeito antimicrobiano da TFDa contra biofilme de *P. aeruginosa in vitro*. Dutra et al. (2013) demonstraram *in vitro* que a produção de EROs oriunda da reação tipo I e tipo II foi maior na solubilização 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 na solução. Quando há presença de dímeros do Fs, que correspondem a formação de uma molécula maior, ocorre maior atividade para transferências eletrônicas com o substrato (mecanismo da reação do tipo I) (PATIL; PAWAR; TALAP, 2000) devido a menor capacidade de capturar energia quando o Fs está agregado. 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).

Contudo, existem apenas os nossos estudos *in vivo* considerando a potencialidade do Fs modificado por um solvente menos polar que a água, o etanol, demostrando êxito na tentativa de melhorar o desempenho desta terapia no tratamento periodontal (BARIN et al., 2017; PILLUSKY et al., 2017).

3 ARTIGO 1 - BIOCHEMICAL, HISTOLOGICAL AND IMMUNOHISTOCHEMICAL RESPONSES OF ANTIMICROBIAL PHOTODYNAMIC THERAPY IN TREATMENT OF PERIODONTITIS IN DIABETIC RATS

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Biochemical, histological and immunohistochemical responses of antimicrobial photodynamic therapy in treatment of periodontitis in diabetic rats

Responses of photodynamic therapy in diabetic rats with periodontitis

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Biochemical, histological and immunohistochemical responses of antimicrobial photodynamic therapy in treatment of periodontitis in diabetic rats

ABSTRACT

Objectives: To evaluate the effect of antimicrobial photodynamic therapy (aPDT) responses using methylene blue (MB) photosensitizers solubilized in ethanol as adjuvant in periodontal treatment associated with a systemic disorder, as diabetic mellitus (DM). In addition, to verify the relationship of vascular endothelial growth factor (VEGF) in the disease and the repair process of periodontitis associated with DM.

Methods: Eighty Wistar rats received streptozotocin intraperitonial injections to induce DM and after 14 days some of the rats received ligatures. Animals were divided in: NC (negative control; no periodontitis); PC (positive control; periodontitis without any treatment); SRP (periodontitis and scaling and root planing); aPDTI (periodontitis and SRP+aPDT+MB solubilized in water) and aPDTII (periodontitis and SRP+aPDT+MB solubilized in ethanol). After 7 days, the ligature was removed and animals received treatment, then rats were euthanized at 7 and 15 days and biochemical, histological and immunohistochemical responses were evaluated.

Results: At 7 and 15 days, aPDTII showed lower inflammatory and VEGF intensities; at 7 days, lower amount of blood vessels; and at 15 days, lower oxidative damage (as well as the NC group), when compared to SRP alone. The aPDTII did not show differences when compared to the aPDTI.

Conclusion: The aPDT used as adjuvant treatment was able to facilitate and accelerate the periodontal healing. VEGF intensity could have greater influence on during disease process of periodontitis.

Clinical relevance: DM is a public health problem worldwide. Thus is of profound interest to study alternatives to improve prognoses of diabetics who may be affected by periodontitis. The aPDT presents a biostimulating effect, which leads to a cascade of photobiological events that could be benefic on periodontal healing.

1. Introduction

Periodontitis is an infectious-inflammatory disease that affects the tissues of protection and support of the dental element. The destruction of periodontal tissues is caused by host response to microorganisms presents and their products [1]. The destructive process have been associated with (1) loss of homeostatic balance between reactive oxygen species (ROS) and antioxidant defense systems [2]; (2) expression of inflammatory mediators as growth factors [3]; (3) interaction of systemic diseases [4]; or with the combination of these factors [5,6].

During the pathogenesis of periodontitis, polymorphonuclear leukocytes (PMNs) act as primary mediators of the host response. Activated PMNs produce a large amount of ROS that combined with inability to defend antioxidant systems, generating oxidative stress (OS), resulting in destruction of periodontal tissue [7,8]. This also evidences that periodontal inflammation might be associated with systemic oxidative damage [9,10] Recent studies reveal that OS plays an important role in the pathogenesis of oral and systemic diseases, being able to be connected simultaneously to both [11,12].

During the periodontitis progression, the periodontal vasculature is affected [13]. Studies show that inflamed tissues decrease oxygen levels and increase the expression of mediators, such as growth factors, which can regulate angiogenesis. Angiogenesis is defined as the process by which new blood vessels are produced by sprouting of established vessels [14,15]. The vascular endothelial growth factor (VEGF) is the main angiogenic protein, that potentially enhances microvascular permeability, stimulates endothelial cell proliferation, induces the expression of proteolytic enzymes and the migration of endothelial cells [16] and regulator of osseous homeostasis [17]. VEGF contributes to extension of inflammation [18] and during the periodontal maintenance and repair has been associated to physiological angiogenesis. The searches remain divergent on regarding the expression of VEGF in the etiopathogenesis of periodontitis, reported as increased [19,20] decreased [21] or unaffected form [3] .

Diabetes mellitus (DM), which is being mentioned as a major systemic risk factor for periodontitis [22]. Many biological mechanisms have been proposed to explain the role of DM in periodontitis, such as endothelial dysfunction, decreased chemotaxis, reduced phagocytosis, increased alpha-factor and interleukin-6 and production of amplified superoxide by increased OS and vascular and cellular alterations [23]. The deleterious effects of DM act on systemic (as OS), inflammatory-immune and

vasculature responses (as VEGF), corroborating to deteriorated healing [24]. Thus, it is a great challenge to apply an effective treatment for the recovery of periodontal tissues in diabetic patients [4].

Scaling and root planing (SRP) is the gold standard treatment for periodontitis [25], however, in the presence of DM, it may not have the expected result, particularly in patients with poor glycemic control [26]. As a way to improve the prognosis of these patients, through a more effective tissue recovery, adjuvant therapies to SRP are an interresting strategy. Antimicrobial photodynamic therapy (aPDT) emerges promisingly. aPDT is an oxygen-dependent photochemical reaction in which the activation of a dye, known as photosensitizer (Ps), by a visible light of the appropriate wavelength excites a molecule of Ps that changes from its current state to excited, leading to the generation of ROS extremely toxic and lethal for microbial cells [27]. Clinicals studies [28,29] and sytematic reviews [30,31] shown statistically significant results in favor of combination therapy, nevertheless in diabetic patients, studies indicated that aPDT did not provide additional benefits in the clinical parameters [32,33].

Possibilities to improve the effect of aPDT are being investigated and the solvent in which the Ps is diluted becomes a timely issue. Research on aPDT carried out to date uses as Ps methylene blue (MB) diluted in water [34]. Recent *in vitro* studies show better photophysical and photochemical properties in solubilization of Ps in ethanol, culminating in a major antimicrobial effect [35,36]. To date, only our *in vivo* studies have considered the potentiality of ethanol-modified MB Ps and applied in aPDT adjuvant to SRP in periodontitis, which demonstrated systemic and local reduction of OS, better tissue responses facilitating and accelerating the repair of periodontal tissue [37,38].

Currently, DM is a major public health problem worldwide, referred to as the 21st century pandemic [39]. Periodontitis affects about one-third of these individuals, and their manifestation is more severe [24]. It is of great interest to study alternatives to improve the performance of peridodontal treatment for this growing number of diabetic individuals. In this sense, this study aims to evaluate for the first time the aPDT using MB Ps solubilized in ethanol as adjuvant periodontal treatment associated with systemic disorder, as DM, evaluating the biochemical, histological and immunohistochemical responses. In addition, the following objective is to verify the relationship of VEGF in the disease and repair process of periodontitis associated with DM.

2. Materials and Methods

2.1. Ethical Considerations

The experimental protocol (Fig. 1) followed ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines and were approved by Committee on Animal Research of the Federal University of Santa Maria, Brazil (027/2013).

2.2. Animals

Eigthy rats (male, *Rattus norvegicus albinus*, Wistar) which were 90 days old (250-300 g) were housed (5 animals per cage) with food and water *ad libitum*. The room presented controlled temperature (23 ± 1ºC), relative air humidity close to 60% and exhaust air, noise control (maximum 85 dB), and standard light-dark cycle (12:12 h). Before experimentals procedures, the animals were allowed to acclimatize to the laboratory environment for a 2-week-period.

2.3. Diabetic Induction

Diabetes was induced in all the experimental groups after six hours of fasting by intraperitoneal injection of streptozotocin (STZ, Sigma Chemical Company, St. Louis, MO, USA) 50 mg / kg diluted in Na citrate (0.1 M at pH 4,5) according to the body weight of each animal [40]. Fasting blood was extracted from the tail veins of all animals on the same day prior to STZ injection, after 7 days to confirm the highest glucose level and on days of induction of periodontitis, periodontal treatment and euthanasia. Blood glucose levels were measured using a glucose monitor (Accu-check Active, Roche Diagnosis, Sandhofer, Germany). Animals with glucose levels above 250 mg / dL were considered diabetic [41].

2.4. Periodontitis Induction

After 14 days of STZ injection [42], diabetic rats were randomly assigned into the five experimental groups: NC (negative control; no periodontitis; n = 10); PC (positive control; periodontitis without any treatment; n = 10); SRP (periodontitis and

scaling and root planing; n = 20); aPDT I (periodontitis and SRP + aPDT + MB solubilized in water; n = 20) and aPDT II (periodontitis and SRP + aPDT + MB solubilized in ethanol; n = 20). PC, SRP, aPDT I and aPDT II groups received a cotton ligature (Ethicon 4-0, Johnson & Johnson, São Paulo, SP, Brasil), under anesthesia (ketamine/xylazine, 70 and 6 mg/ kg, intramuscular injection, respectively), in the mandibular right first molar in the submarginal position. After 7 days, all ligatures were removed and in the baseline the animals received the treatment for periodontitis according to their group [43].

2.5. Randomization and Blinding Operator

A computer program (Random Allocation Software, version 1.0, May 2004) was used to randomly allocate animals in experimental groups. A single operator performed all SRP procedures prior to knowing whether it would apply to aPDT. The formulations used for aPDT had the same color and the same syringes. The animals were treated in the order of the randomization sequence generated. Thus, the operator was blinded to treatment groups.

2.6. Scaling and Root Planing Protocol

The mandibular right first molar was subjected to SRP with manual 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 scaled with the same curettes through cervico-occlusal traction movements [43].

2.7. Antimicrobial Photodynamic Therapy Protocol

The aPDT employed two different solvents to dilute 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 with 20% ethanol. The MB formulation was gently and slowly dropped into the periodontal pocket around the mandibular right first molar using a syringe (1 mL) and a needle (BD® UltrafineTM, U-100, 0.5 mL, 8 mm x 0.3 mm) without a bevel. In this study, the pre-

irradiation time used was 1 minute and corresponds to the time elapsed between the MB application and its activation by light. After 1 minute of MB application, low-level laser therapy was applied to three points at each buccal and lingual sides of the tooth. The laser employed was an indium-gallium-aluminum-phosphorous (TheraLase®, DMC Equipments, São Carlos, SP, Brazil), wavelength 660 nm, continuous emission mode, power output of 35 mW transmitter, with spot size of 0.0283 cm^2. The laser was activated for 4 s in each point (4.94 J / cm^2) and 49.400 W / m^2 of the irradiance. The tooth received a total energy density of 29.64 J / cm^2 [37,38].

2.8. Blood Collection

At 7 and 14 days after the treatment, the animals were anesthetized (isoflurano 2-3%, inhaled) and blood collection was performed by cardiac puncture under. The heparinized syringe was held with the right hand and punctured on the third intercostal space (left) of the animal. The needle was inserted in order to perceive the perforation of the pericardium. The blood was aspirated slowly with the plunger until 5 mL was reached [38]. The blood was placed in falcon tube for centrifugation (1300 g for 15 min) in order to obtain plasma for the lipid peroxidation and antioxidant defenses levels through glutathione reduced (GSH) and vitamin C (VIT C).

2.9. Gingival Connective Tissue Collection

Buccal gingival connective and epithelial tissues from periodontitis induction region was removed and soaked in 10% neutral buffered formalin (pH 7.2) for 24 hours. The biopsies embedded in paraffin was serial sections cutted with a thickness of 6 µm in a mesial-distal direction and staning with hematoxylin and eosin (H&E). The blocks containing the subsequent sections were used for immunohistochemical.

2.10. Bone Alveolar Collection

Then the mandibular specimens were fixed soaked in 10% neutral buffered formalin (pH 7.2) for 48 hours [44] and then decalcified with ethylenediamine tetraacetic acid 10% (EDTA) buffered with sodium hydroxide (pH 7.4) for 13 weeks. The decalcified mandibule tissues were neutralized, then dehydrated, embedded in

paraffin, and serially sectioned using a microtome. Serial paraffin sections (4µm) were obtained in the mesial-distal direction. After excluded the first section that the furcation region was evident, serial sections were obtained and staning with H&E.

2.11. Biochemical Analysis

2.11.1. Evaluation of Lipid Peroxidation Plasma Levels

Lipid peroxidation was evaluated by quantifying TBARS through the pink chromogen produced by the reaction of TBA to MDA at 100°C, measured spectrophotometrically at 532 nm [45].

2.11.2. Evaluation of GSH Plasma Levels

GSH were determined after plasma reaction with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, Proquímios®, Rio de Janeiro, RJ, Brazil), according to Ellman (1959) [46] with modifications [46,47]. The protein fraction plasma was precipitated with 20% trichloroacetic acid, followed by centrifugation. A standard curve was constructed to estimate the GSH content.

2.11.3. Evaluation of Vit C Plasma Levels

VIT C was assessed as described by Galley et al. (1996) [48], with modifications [47,48]. 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 3 h at 37°C. A sulfuric acid solution (65%) was added and the samples measured spectrophotometrically at 520 nm. A standard curve was used to calculate the VIT C content.

2.12. Histological Analysis

2.12.1. Gingival Connective Tissue

The presence and degree of inflammatory cell response was evaluated from biopsia of the buccal gingiva using 100x magnification, and then graded by a 4-point scoring system, such as absent (0) when characterized by absence of inflammatory cells in the connective tissue; mild (1) when presenting sparse inflammatory cells distribution in connective tissue; moderate (2) when found dense accumulation of

inflammatory cells in isolated areas, sparse distribution in other areas of connective tissue and severe (3), when dense aggregation of inflammatory cells throughout the connective tissue was observed [49].

The number of sectioned blood vessel profiles was counted in three individual fields of 60 x 60 μm^2 area per section at an objective magnification of 400x [21]. The diameter of two randomly selected sectioned blood vessel profiles per section was measured at an objective magnification of 100x [21]. The mean of measurements was used for data analysis.

2.12.2. Measurements of Linear Bone Loss

The vertical linear distance between the cementum and the apex of the alveolar bone crest of the furcation region in the mandibular right first molar was measured to determine bone loss (μm) by a 100x magnification [50], with modifications.

2.13. Immunohistochemical Analysis

VEGF immunoreactivity was evaluated employing the VG1 monoclonal mouse anti-human (Dako, Glostrup, Denmark), which recognizes the 121, 165 and 189 isoforms of VEGF. After cooling, the gingival connective tissues embedded in paraffin were sectioned in thickness of 2 μm. The process of dewaxing and hydration of the slides occurred in PT Link machines (Dako, Glostrup, Denmark) with deionized water and ready solutions of low pH and high pH. For the immunohistochemical reaction, the entire EnVision Flex™+/ HRP kits (Dako, Glostrup, Denmark) protocol was followed, together with the use of concentrated (to be diluted) and flex (already diluted) antibodies according to their respective pH. The primary VEGF antibody was diluted in 1:100, according to standardization.

The evaluation was based according to the brown staining within the cell cytoplasm, which was accepted as positive staining, under magnification of 400x. Immunoreactivity was graded in 3 groups, by using a modification of the scale previously described: grade (0): absence of staining; grade (1): light to moderate amounts of staining, not completely filling the cytoplasm, light brown in color; or grade (2): intense staining of the cytoplasm, completely filling the cell, very dark brown [51,52]. Additionally, the staining of PMNs neutrophils was used as an internal positive control, since these cells also express VEGF [53].

All the histological and immunohistochemical analysis were performed through a light microscope (Binocular Optical Microscope ZEISS, Axio Lab.A1, Germany), the images were captured by a camera (AxioCam, ERc 5S, Germany) with monitor coupled, for the measurements were used the software ZEN 2011 (blue edition) 1.0, Carl Zeiss MicroImaging GmbH by a blind operator.

2.14. Intra-examiner Reproducibility

The examiner was calibrated through two measurements of ten specimens with an interval of one week. The intra-class correlation coefficient values were 0.85 and 0.95 for inflammatory infiltrate and immunohistochemical analysis, respectively.

2.15. Statistical Analysis

In order to verify the homogeneity of the data was used Levene's test. Data from biochemical analysis, vessel number and diameter of blood vessels were analyzed by two-way ANOVA, followed by Duncan's post-hoc, where appropriate (Statistica 8.0 software for Windows). The bone loss was analyzed by one-way ANOVA followed by Bonferroni's post-hoc. All data were expressed as means ± SEM and *P* value less than 0.05 was considered as statistically significant.

Biochemical and bone loss data were expressed quantitatively, histological and immunohistochemical data such as inflammatory and VEGF intensity were shown descriptively, number and diameter of blood vessel are shown in both forms.

3. Results

The experimental model of diabetes was successfully induced, all animals had glucose levels greater than 250 mg / dL during all periods of the experiment. Periodontitis was successfully indecued (Table 1). Bone loss was significantly higher in PC when compared to NC group.

3.1. Biochemical Analysis

Date of biomarkers of OS showed in Fig. 2. PC group showed the higher plasmatic lipid peroxidation levels at 7 and 15 days, compared to all other experimental groups ($P < 0.05$). Between 7 and 15 days, there was a reduction of plasmatic lipid peroxidation of PC group ($P < 0.05$). At 7 days, there was no statistical significant difference between the groups of treatment, which showed lipid peroxidation levels higher than NC group ($P < 0.05$). Interestingly, in the 15 days, only aPDT groups showed lipid peroxidation levels statistically similar to the NC groups ($P < 0.05$).

In relation to the antioxidant defense status (Fig. 2), at 7 and 15 days the groups that received the ligature presented high levels of GSH compared to the NC group ($P < 0.05$). At 7 and 15 days the treated groups presented higher levels of VIT C compared to the PC group and lower levels than the NC grop ($P < 0.05$).

3.2. Histological and Immunohistochemical Analysis

7 days

NC group. Discrete bone loss was observed in the furcation region (Table 1). In the gingival connective tissue there was absence of inflammatory infiltrate (80% of cases) (Fig. 3A) and rare cases of mild intensity (20%). In the Table 2, the number of blood vessels was not expressive (Fig. 4A) and the diameters found were large (Fig. 4B). The absence of VEGF staining was more observed (80%) (Fig. 5A).

PC group. Alveolar bone loss was evidenced at furcation region (Table 1), according to gingival connective tissue that showed mild (40%) and moderate (40%) mononuclear inflammatory cell infiltration and some severe cases (20%) (Fig. 3B). In relation to number (Fig. 4C) and diameter of blood vessels (Fig. 4D), the behavior was similar to the NC group (Table 2). This group showed the highest VEGF intensity of this study, presenting most of the cases with intense staining of very dark brown (60%) (Fig. 5C).

SRP group. Moderate inflammatory infiltration (40%) (Fig. 3C) and numerous congested blood vessels were also noted (Fig. 4E). The diameters of blood vessels (Fig. 4F) were smaller in comparison to the control groups (Table 2). The intensity of the VEGF was mild (50%) (Fig. 5E) and absence (30%), presenting some cases of intense staining (20%).

aPDT I group. The inflammatory infiltrate was in most cases mild (70%) (Fig. 3D) and absence (30%), the number (Fig. 4G) and diameter (Fig. 4H) of the vessels was also similar at SRP group (Table 2). The VEGF was less expressive than SRP, cases comprised absence (30%) or light staining (70%) (Fig. 5G).

aPDT II group. The histological findings was similar in aPDT I group. The inflammation infiltrate was absence (40%) (Fig. 3E) and in some cases mild (60%). Smaller number (Fig. 4I) of blood vessels was observed when compared to the SRP group (Table 2), the diameters were equals (Fig. 4J). VEGF intensity was mostly absence staining (30%) and light staining (70%) (Fig. 5I), equal to aPDT I group.

15 days

NC group. There was no change in inflammatory infiltrate and the number and diameter of the blood vessels (Table 2), as well as the immunoreactivity of VEGF (Fig. 5B).

PC group. The inflammatory intensity remained with moderate (40%), however part of the group presented mild (40%) and absence (20%) mononuclear inflammatory cell infiltration and no severe cases were recorded. The intensity of VEGF remained well marked at light (40%) and intense (40%) staining (Fig. 5D), with few cases of absence of staining (20%). No increase were observed in blood vessel numbers or change in diameters (Table 2).

SRP group. Major changes in inflammatory and VEGF intensity (Fig. 5F) were not observed, although more cases infiltrate (30%) and staining (40%) absence, were presented, respectively. On the other hand, a difference in the number of vessels was

observed by the decrease of the amount (Table 2). The unchanged diameters and remain smaller than the control groups.

aPDT I group. In relation to the vascular behavior (Table 2), gingival connective tissue was similar to the histological findings of the SRP group. Nevertheless, the infiltrate remains light and more cases with absence (50%) of infiltration of mononuclear inflammatory cells were found. Most of the VEGF intensity reached absence (80%) of staining (Fig. 5H).

aPDT II group. Inflammatory, vascular (Table 2) and immunohistochemical (Fig. 5J) findings were equal to the aPDT I group.

4. Discussion

In this study, the model of periodontitis induction was successfully performed in Wistar rats by placing a submarginal ligature in the mandibular right first molar. Our findings demostrated statistically significant differences in alveolar bone loss in the furcation region between the PC group and the NC group at 7 days. It was also complementing by the presence of moderate and severe inflammatory infiltrate in PC group, while in NC group showed absence inflammatory. According, studies showed alveolar bone loss in the region of furcation and severe inflammatory infiltrate in ligature-induced periodontitis in rats, while healthy groups showed a periodontal tissue with few inflammatory cells [50,54]. The biological mechanisms involved in bone resorption respond to signs of inflammatory cells and initiate bone degradation in order to maintain a peripheral distance from the inflammatory infiltrate [55], so it is clear that the greater the inflammatory grade, the greater the bone loss response.

Experimental periodontitis also was able to increase the plasmatic lipid peroxidation in the PC group, compared to the NC group [12]. Diabetic subjects could have higher MDA levels in their periodontal tissues, suggesting an increased lipid peroxidation in case of DM [6]. In agreement with our findings, meta-analysis [56], clinical [57] and experimental [58,59] studies also demonstrated an increase in lipid peroxidation levels in periodontitis. Considering antioxidants system, periodontitis-induced higher GSH levels in the PC group in all evaluated time-points compared to NC groups. This may be related to the attempt of this antioxidant defense in

neutralizing high levels of MDA observed in the PC group. The increase of GSH levels against lipidi peroxidation caused by periodontitis has been previously reported in erythrocytes from rats with periodontitis [38], confirming our results. On the other hand, in the PC group, VIT C plasma levels decreased in the presence of periodontitis comparing with NC group. VIT C is the main hydrophilic antioxidant present in blood, which acts primarily by scavenging ROS, and can completely protect against peroxidative damage and OS [60]. For being the front line, was more consumed [37], our study confirms these findings.

The association of periodontitis and DM caused a dark-brow immunostaining of VEGF in PC group. Studies have shown that this association can increase VEGF levels, which contribute to the pathogenesis and progression of gingivitis to periodontitis [51,56], confirmed in our study and supported by data of inflammatory intensity and bone loss. On the other hand, the NC group did not present immunostaining. Sakallıoğlu et al. [62] described that the pronounced expression of VEGF in the periodontal tissue of diabetic patients may be a possible cause for greater severity of the periodontal disease in these patients. This may occur because VEGF appears to affect glucose levels and the extent of diabetic complications that may contribute significantly to changes in microcirculation [63], as well as constant hyperglycemia may stimulate further release of VEGF [64] and ROS [12]. In parallel, the hypoxia of periodontal tissue caused by inflammation also increases the intensity of VEGF [19,20] and its maintenance may expand active inflammatory process, causing greater release of ROS and periodontal tissue damage [65]. VEGF is the main angiogenic inducer [15], but in PC group we have not observed the increase in the number of vessles, which at first sight seems controversial. This can be explained by the fact that VEGF is also a potent inducer of vascular permeability which is associated to the greater inflammatory intensity, in according to other authors [66,67], showing increased expression, confirming our results. VEGF is able to modify the contractile response of the pericytes in inflammatory state, therefore it allows a greater passage between them, resulting in a large flux of fluid and macromolecules from the vasculature to the interstitium [67,68], the permanence of this effect exacerbates the inflammatory response and causes tissues insults. This fact allied to deleterious effects coming from DM on vasculature, inflammatory and immune response improve periodontitis severity becoming difficult to repair [24]. Due to this fact we believe, there has been no expansion of the network of blood vessels, because all the attention and

energy are being consumed for the minimal maintenance of the body and in its defense against the harmful agents. The vessels diameter of the PC group were equal to the NC group, however for different reasons, the remodeling in the NC group occurs physiologically, although in the PC, confirmed by other authors, this increase can be given by thickening of the basement membrane and loss of pericytes [68].

The SRP group showed lower bone loss and was able to significantly decrease lipid peroxidation in diabetic rats at 7 days when compared at PC group. Mlachkova and Popova [69] also demonstrated the effectiveness of SRP as periodontal treatment in moderate and chronic periodontitis. GSH levels were more consumed in 7 days compared to the aPDT I group, as a way to control circulating ROS and to prevent oxidative damage. GSH is considered the most important nonenzymatic antioxidant agent, having an important role in regulation pathways that insure whole body homeostasis [6]. However, SRP group showed higher intensity of inflammatory infiltration and VEGF at 7 and 15 days, compared to the aPDT groups. Prates et al. [54] verified that the number of inflammatory cells in the SRP group was significantly higher than in the SRP+aPDT group in rats with ligature, confirming our findings. There was more neoformation of blood vessels, justified by the increase of VEGF, and the observation of small diameters characteristic of neovascularization [37]. In view of the local and systemic damages observed, we believe that an environment of permanent inflammation was installed, where there was oxygen restriction and greater release of VEGF, so there was a “greater effort” of the tissues to reach the repair. This may have occurred due to the presence of remaining mononuclear bacteria, not removed manually. Thus, the SRP procedure has presumably limitations due to the persistence of periodontal pathogens and subsequent recolonization [70].

In this context, the search for new approaches to improve the effectiveness of periodontal therapy is needed. The aPDT emerges as an alternative for reduction of microbial remaining, with minimal side effects [71]. Therefore, the advantages of this therapy make it a promising adjuvant method to conventional periodontal treatments, particularly diabetic one. The aPDT I and aPDT II groups, obtained very similar biochemical, histological and immunohistochemical responses. When comparing aPDTs groups to SRP group, we observed that the laser facilitated and accelerated local and systemic repair, due to the lower inflammatory intensity, VEGF expression, vascular formation (aPDT II) and oxidative damage, equal NC group at 15 days. Randomized controlled clinical trials [28,29] and systematic reviews [30,31] have

shown statistically significant results in favor of combination therapy; however, in diabetic patients, studies have indicated that aPDT with clinical questionable benefits [32,33]. Interestingly, we note that there are important differences in the tissues responses with the adjuvant use of aPDT.

The proposed modification of the Fs in this study was based on previous research that demonstrated better photophysical and photochemical properties of Fs dissolution in less polar solvents than water, such as ethanol, providing greater tissue penetrability and increased half-life of singlet oxygen, thus increasing the antimicrobial power [34]. Prochnow et. al. [36] evidenced that MB containing 20% ethanol increased the antimicrobial effect of aPDT against *P. aeruginosa* biofilm *in vitro*. To date, only our *in vivo* studies have considered the potentiality of ethanol-modified MB Ps applied adjuvant to SRP in periodontitis [37,38], which demonstrated systemic and local reduction of OS, better tissue responses facilitating and accelerating the repair of periodontal tissue. However, with the involvement of DM, the responses to MB containing ethanol were not as expressive. It may have been subtly perceived due to the fact that this group has expressed fewer number vessels in short-term, we believe this represents a "lesser need for repair" to recover from damages in the periodontal tissues. In general, the benefic adjuvant action of aPDT is evident, especially in the presence of systemic impairment, corroborating with other authors, which evidenced acceleration in tissue repair [72], inhibition of the production of inflammatory mediators and angiogenesis [73], favoring the healing process. Recently, Fahimipour et al. [74] found that the oral mucosa wound of diabetic rats treated with low laser therapy also had a lower number of PMNs cells and vascular neoformation, reinforcing our data.

Whereas, the involvement of VEGF in disease and repair process of periodontal tissues in diabetic individuals still scarce [5,21,51]. Based on our study, there is strong evidence of greater participation of VEGF in progression of periodontitis associated with DM, although VEGF also appeared in the treated groups in a lesser extent, because angiogenesis occurs also in physiological phases, as repair [15]. The intensity of the VEGF, degree of bone loss and inflammatory intensity show a possible relationship. Further investigation regarding VEGF and periodontitis in diabetic patients is necessary and perhaps in the future may be based on therapies that involve its control or otherwise instigate its VEGF application, for the benefit of the patient.

5. Conclusion

Our results demostrated that aPDTs, independently of the formulations, were able to facilitate and accelerate the periodontal healing, could have better effects on periodontal healing than SRP alone.

The higher expression of VEGF was observed in diabetic rats with periodontitis showing that VEGF could have a greater influence on during disease process of periodontitis.

Compliance with Ethical Standards

This research was approved by Committee on Animal Research of the Federal University of Santa Maria, Brazil (027/2013). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Declaration of interests

The authors declare that they have no conflict of interest.

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Table 1 - Analysis of bone loss. The linear distance between the cementum and the apex of the alveolar bone crest of the furcation region.

	Bone loss (µm)				
	NC	**PC**	**SRP**	**aPDT I**	**aPDT II**
7th day	156.17±21.34^{c}	399.44±29.58^{a}	257.15±31.92^{b}	284.90±23.64^{b}	285.60±33.04^{b}

Data are mean ± SEM. Different lower case letters (a–c) indicate significant difference among periodontal treatment in the same evaluation time ($P < 0.05$). NC: negative control; PC: positive control; SRP: scaling and root planing; aPDT: antimicrobial photodynamic therapy.

Table 2 - Effect of aPDT with Ps diluted in differents solvents, used as an adjuvant to SRP, in the periodontal treatment on the number and diameter of blood vessels of the gingiva connective tissue.

Blood vessels				
Number (unit)			**Diameter (µm)**	
	7th day	**15th day**	**7th day**	**15th day**
NC	1.60±0.24^{c}	1.33±0.23^{b}	15.48±0.70^{a}	14.56±1.16^{a}
PC	1.86±0.16^{c}	1.73±0.19^{b}	14.94±1.63^{a}	15.12±0.50^{a}
SRP	4.25±0.48^{a}	2.80±0.30^{a*}	12.37±0.78^{b}	12.17±0.85^{b}
aPDT I	3.62±0.34^{ab}	2.83±0.27^{a}	11.56±0.31^{b}	10.66±0.57^{b}
aPDT II	2.81±0.28^{b}	2.92±0.29^{a}	10.73±0.59^{b}	11.26±0.39^{b}

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 ($P < 0.05$). NC: negative control; PC: positive control; SRP: scaling and root planing; aPDT: antimicrobial photodynamic therapy.

Legends of Figures

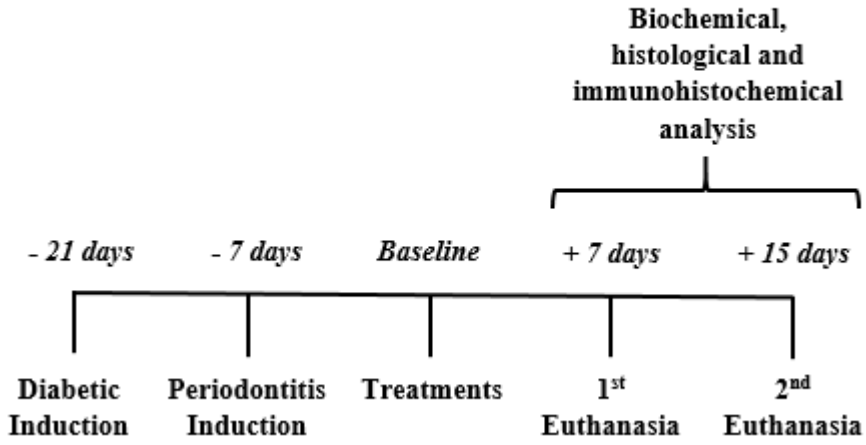
Fig. 1 – Experimental desing.

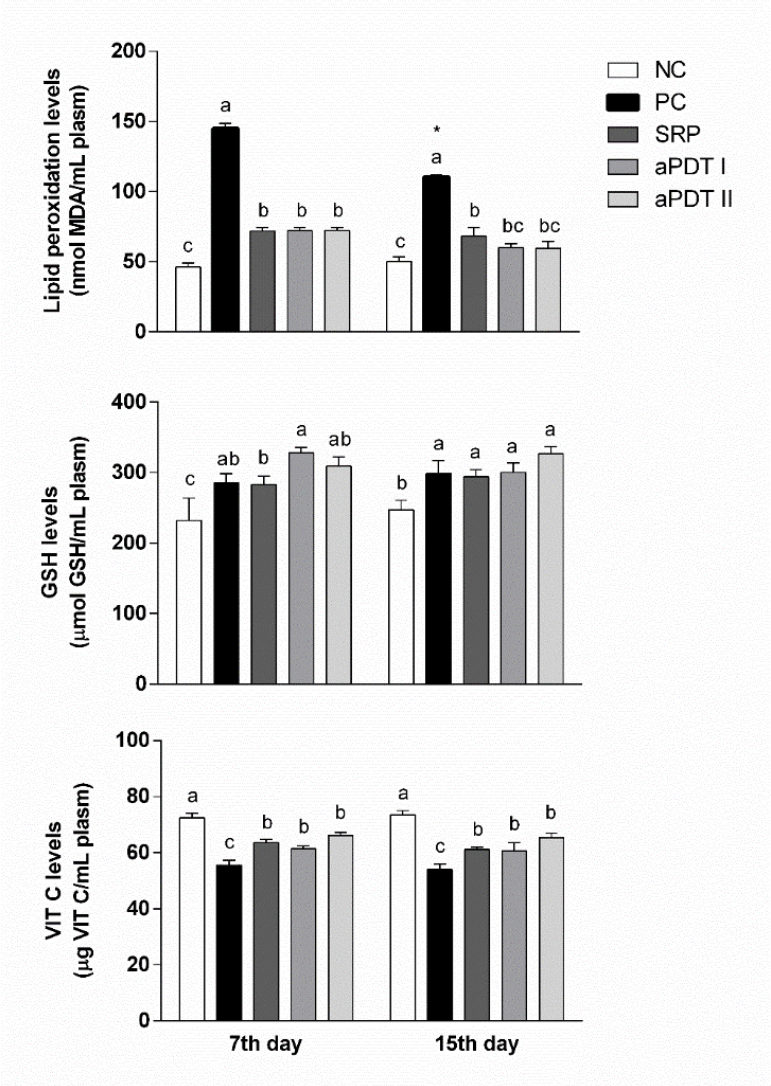
Fig. 2 - Analysis of oxidative stress biomarkers in diabetic rats, by lipid peroxidation (A), GSH (B) and VIT C levels (C). Different lower case letters (a-c) indicate significant difference among periodontal treatment in the same evaluation time. *Indicates significant difference of 7 day evaluation in the same periodontal treatment ($P<0.05$). NC: negative control; PC: positive control; SRP: scaling and root planing; aPDT: antimicrobial photodynamic therapy.

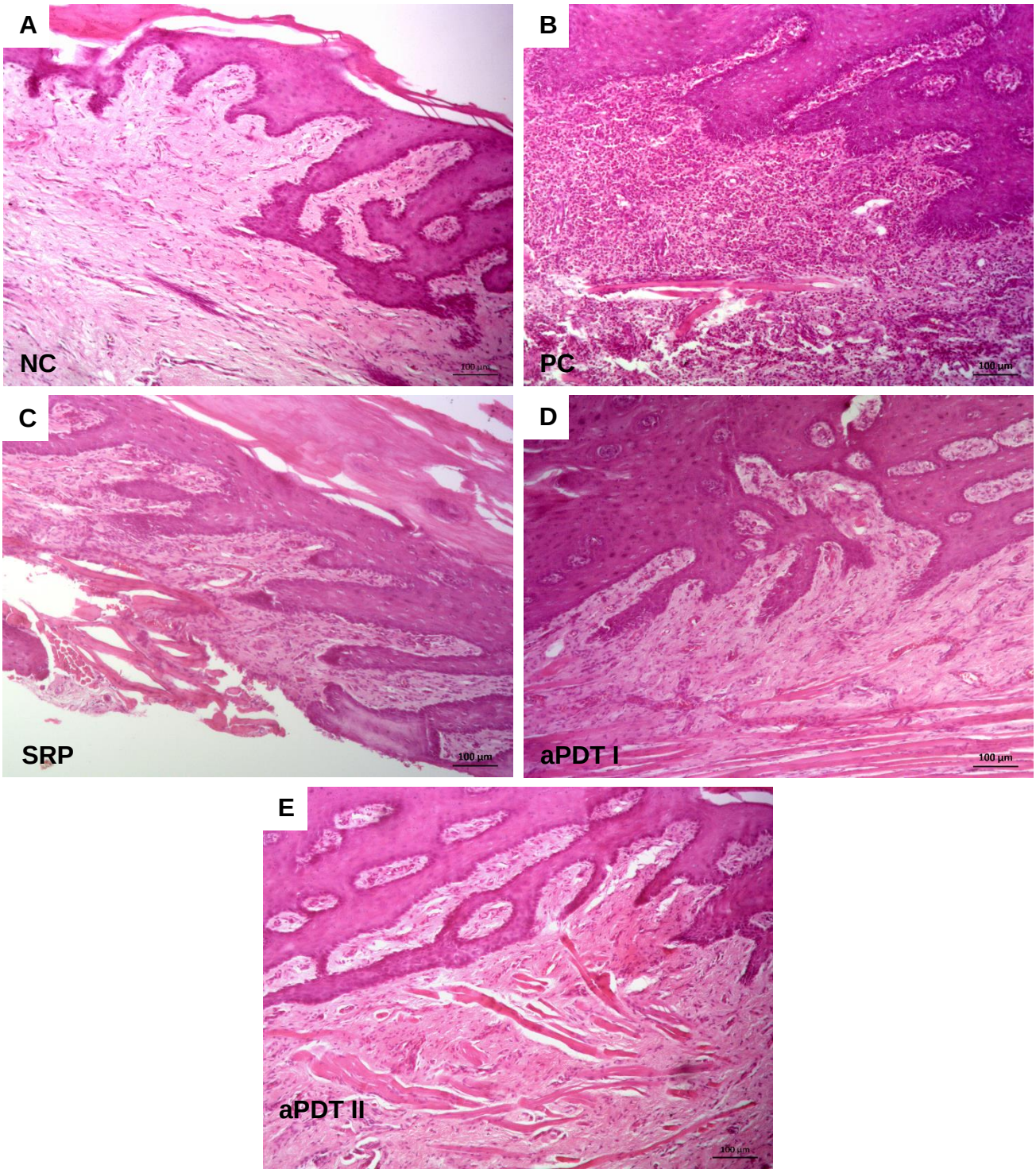
Fig. 3 - In the gingival connective tissue the diabetic rats, at 7 days, the intensity of inflammatory infiltrate was graded at an objective magnification of 100x. A (score 0); B (score 3); C (score 2); D (score 1); E (score 0). H&E- staining.

Fig. 4 - At 7 days, in the gingival connective tissue of diabetic rats, the numbers *(arrows)* and diameters of blood vessels were evidenced at an objective magnification, 400x and 100x, respectively. H&E-staining.

Fig. 5 - At 7 and 15 days, the immunohistochemical reactivity for VEGF in the gingival connective tissue of diabetic rats was evidenced at an objective magnification of 400x. A (score 0), B (score 0); C (score 2), D (score 2); E (score 1), F (score 1); G (score 1), H (score 0) and I (score 1), J (score 0), respectively. Immunohistochemical staining.


Biochemical, histological and immunohistochemical analysis
- 21 days
- 7 days
Baseline
+ 7 days
+ 15 days
Diabetic Induction
Periodontitis Induction
Treatments
1st Euthanasia
2nd Euthanasia


Lipid peroxidation levels (nmol MDA/mL plasm)
GSH levels (µmol GSH/mL plasm)
VIT C levels (µg VIT C/mL plasm)
NC
PC
SRP
aPDT I
aPDT II
7th day
15th day


A
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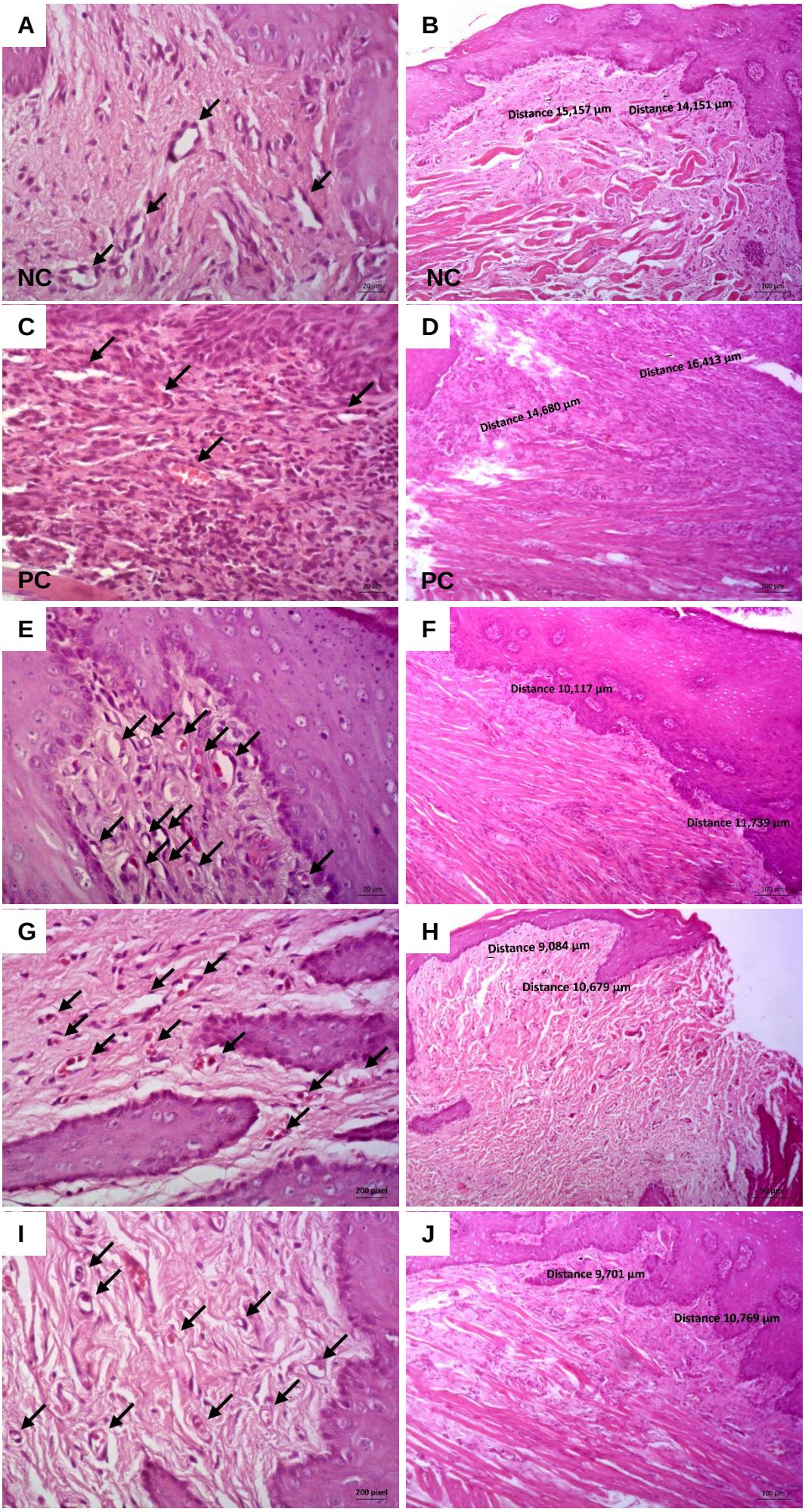
100 µm
B
PC
100 µm
C
SRP
100 µm
D
aPDT I
100 µm
E
aPDT II
100 µm


A
NC
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Distance 15,157 µm
Distance 14,151 µm
NC
C
PC
D
Distance 16,413 µm
Distance 14,680 µm
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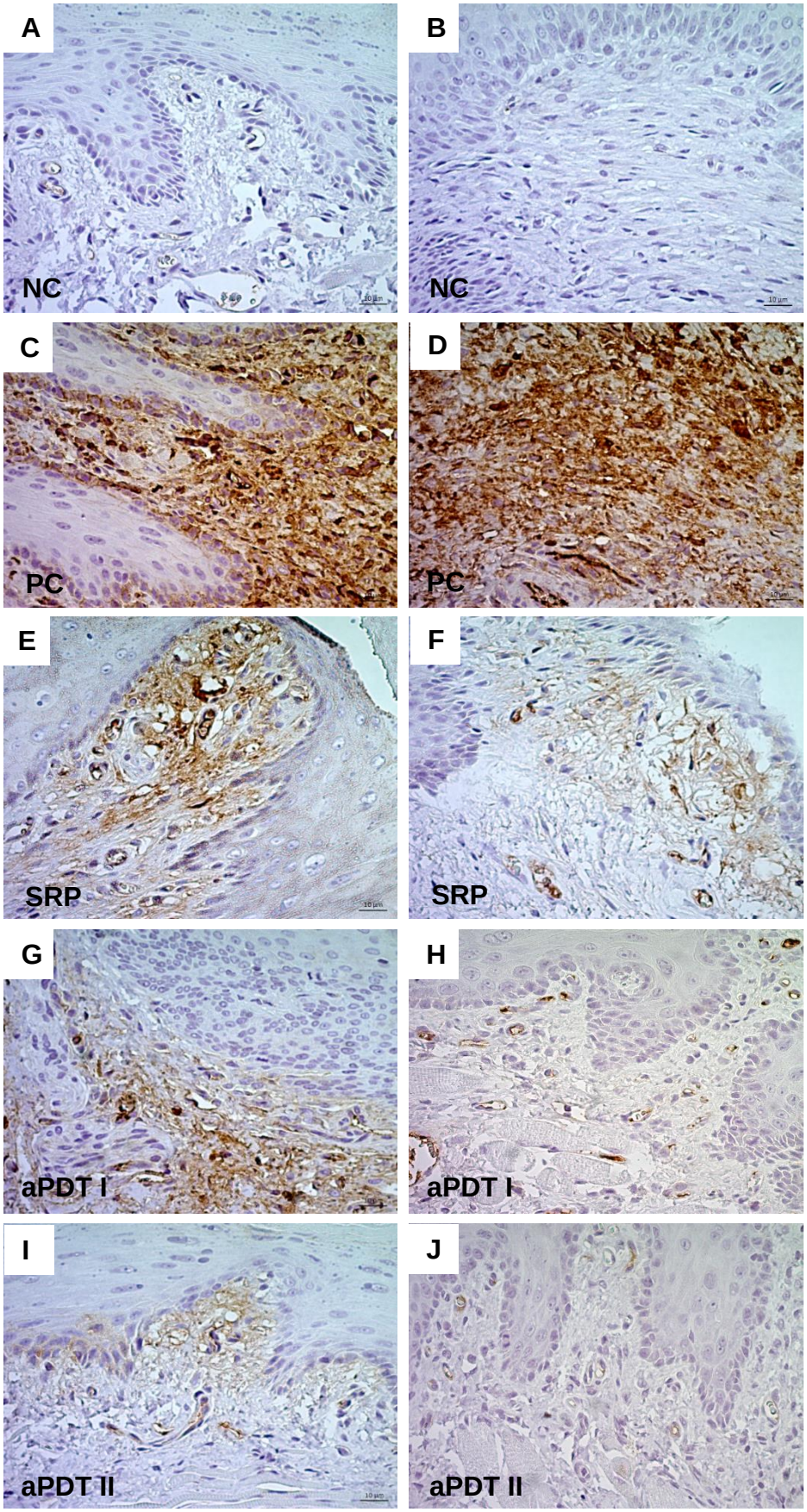
A
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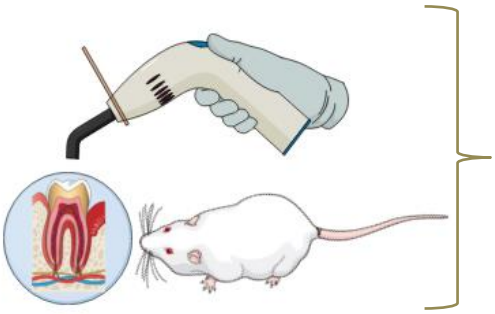
Abstract Graphic



aPDT I and II provided better tissue responses. **VEGF** occurs in advanced of periodontitis.

4 ARTIGO 2 - EFFECTS OF ANTIMICROBIAL PHOTODYNAMIC THERAPY ON THE PERIODONTAL TISSUES: HISTOMORPHOMETRIC AND IMMUNOHISTOCHEMICAL ANALYSIS

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Effects of antimicrobial photodynamic therapy on the periodontal tissues: histomorphometric and immunohistochemical analysis

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Abstract The purpose of the present study is to investigate effects of antimicrobial photodynamic therapy (aPDT) in periodontal tissue when it is used as an adjuvant treatment for periodontitis with photosensitizer (Ps) methylene blue (MB) solubilized at ethanol 20%. One hundred and twenty Wistar rats were randomly divided into five experimental groups: negative control (NC, no periodontitis, n=15) and positive control (PC, with periodontitis, without any treatment, n=15). The other three groups had periodontitis and were treated with scaling and root planing (SRP, n=30); SRP + aPDT + MB solubilized in water (aPDT I, n=30); SRP + aPDT + MB solubilized in ethanol (aPDT II, n=30). Periodontitis was induced by placing a cotton ligature around one mandibular right first molar of each animal. The ligature was removed after 7 days and the treatments were applied. At 7, 15 and 30 days rats were euthanized and bone and gingival tissues were immediately collected for histomorphometric and immunohistochemical analysis. The SRP group showed a delay in relation to elimination of the inflammatory infiltrate; aPDTs groups showed absence of inflammatory infiltrate in 7 days; and aPDT II showed almost total absence of leukocyte infiltration at 30 days, surpassing NC group. Treated groups showed no difference and low staining in relation to VEGF, while PC group showed intense immunopositivity. The aPDT provided better responses to the periodontal tissue compared to SRP alone in short-term. aPDT II demonstrated greater repair capacity in short-term and especially in long-term. VEGF was very intense in periodontitis in PC group, showing a relation at the inflammatory degree and disease progress.

Keywords Ethanol. Inflammation. Periodontal diseases. Photosensitizing agents. Therapeutics. Vascular endothelial growth factor.

Introduction

Periodontal disease is an inflammatory disorder of the gingival tissue induced by bacteria residing in the plaque biofilm on the subgingival tooth surface. The host's immune response to these bacteria can lead to disease progression and consequent pocket formation, loss of insertion, bone destruction and eventually possible tooth loss [1]. In periodontitis, specially, the infiltration of inflammatory cells causes endothelial damage and microcirculatory failure, resulting local hypoxia. This condition and the activity of cytokines, growth factors and endotoxins, stimulates the production of vascular endothelial growth factor (VEGF) [2]. It is one of the most powerful substances known to induce endothelial cell growth and increase vascular permeability, playing a central role in the regulation of angiogenesis. VEGF is involved in physiological and pathological biologic processes [3]. Current data suggest that this growth factor is also essential in the regulation of inflammatory periodontal disease [4, 5].

All inflammatory cascade that causes damage to the periodontal tissues has as trigger point the permanent and residual bacteria. The reduction or elimination of these etiological factors is the main purpose of periodontal treatment. Scaling and root planing (SRP) is the *golden standard* treatment for periodontitis [6]; however, this conventional mechanical treatment can fail to eliminate periodontopathogenic bacteria in hard-to-reach areas, such as furcation regions, bone fissures and concavities [7]. In this sense, the antimicrobial photodynamic therapy (aPDT) is a promising adjuvant modality that can suppress periodontal pathogens and increase the effectiveness of manual mechanical treatment [8, 9]. The advantages of this therapy is low probability of developing bacterial resistance, local action, non-invasive behavior, large spectrum of action and no limit dose. aPDT consists of combining three elements: a photosensitizer (Ps), light and oxygen. Thus, the photochemical reaction generated releases cytotoxic products to microorganisms causing cell death [10].

Periodontitis induced in rats demonstrated a good response to aPDT, there was a reduced expression of inflammatory cytokines, growth factors in the gingival tissues and decreased neutrophil migration. All these factors manifested in a significant reduction of bone resorption. Inhibition of bone loss and reduction of inflammatory infiltrate is a main indicator of successful periodontal therapy [11–13]. However, until this date, systematic reviews still suggest that the use adjunctive of aPDT provides modest benefits and is not stable over the time when comparing to SRP [14, 15].

Therefore, strategies to optimize the aPDT effect on periodontal outcomes are necessary. Because PS is one of the main components of aPDT and there is a possibility of changing its formulation, this can be a key point to increase the effectiveness for therapy [16, 17]. The research on aPDT carried out so far uses as methylene blue (MB) diluted in water [18]. Recent *in vitro* studies showed better photophysical and photochemical properties in MB PS solubilized in ethanol, culminating in a better antimicrobial effect [19].

Several inflammatory mediators, cytokines, hormones and growth factors may act directly or indirectly during the inflammatory process, stimulating host cells and also leading to damage to the periodontal tissue [20]. Thus, it is of interest to verify the histological and immunohistochemical effects on periodontal tissues treated with aPDT using MB Ps solubilized in ethanol. Our hypothesis consists in the adjuvant use of aPDT containing the reformulated Ps to stimulate periodontal tissue repair comparing the effects to the use of only SRP and aPDT with MB Ps solubilized in water.

Material and Methods

Animals

One hundred and twenty male Wistar rats (275 ± 25g and 3 months of age) were used. They were housed at five 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), a 12:12 light:dark cycle and had free access to water. Before starting the experimental protocols, animals underwent an acclimatization period of 15 days. The experimental protocols followed ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines and were approved by Committee on Animal Research of the Federal University of Santa Maria, Brazil (027/2013).

Experimental Period

The ligature remained for a period of 7 days, then it was removed and treatments were applied. At 7, 15 and 30 days after baseline [21], 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).

Randomization and Blinding Operator

A computer program (Random Allocation Software, version 1.0, May 2004) was used to randomly allocate animals. One study team member performed the randomization process. Rats were randomly divided into five experimental groups presented in Table 1. In order to induce experimental periodontitis the animals were over again randomized into the 4 groups (PC, SRP, aPDT I and aPDT II). An operator performed the SRP and aPDT procedures. After the SRP procedures, the operator was informed if the animals received the additional irrigation with saline solution or aPDT. The formulations used for aPDT presented the same coloration and equal syringes. The animals were treated in the order of the randomization sequence generated. Thus, the operator was masked to the treatments groups during the SRP procedures.

Table 1 Description of experimental groups

Groups	Description of experimental groups	Total animals (n)
NC	No periodontitis; without ligatures.	15
PC	Periodontitis; with ligatures; without any treatments.	15
SRP	Periodontitis; with ligatures; treated with SRP and irrigation with 1 mL of saline solution.	30
aPDT I	Periodontitis; with ligatures; treated with SRP and aPDT with MB (0.01%) solubilized in ultra-pure water.	30
aPDT II	Periodontitis; with ligatures, treated with SRP and aPDT with MB (0.01%) solubilized in ultra-pure water, ethanol and carboxymethylcellulose (77:20:3).	30

NC: negative control; PC: positive control; SRP: scaling and root planing; aPDT**:** antimicrobial photodynamic therapy; MB: methylene blue

Periodontitis Induction Protocol

One mandibular right first molar of each animal received the 4-0 cotton ligature (Ethicon 4-0, Johnson & Johnson, São Paulo, SP, Brasil) was inserted into the gingival sulcus, under general anesthesia (ketamine/xylazine, 70 and 6 mg/ kg, intramuscular injection, respectively). The ligature was removed from rats after 7 days [21], and the animals of the SRP, aPDT I and aPDT II groups received the periodontal treatments. The animals of NC group were manipulated likewise all other groups.

Scaling and Root Planing Protocol

The SRP protocol was manual 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 [21].

Antimicrobial Photodynamic Therapy Protocol

The aPDT employed the MB Ps (Sigma Aldrich®, St. Louis, MO, USA). The MB (0.01%) was solubilized in ultra-pure water (Milli-Q) for aPDT I; and in ultra-pure water (Milli-Q), ethanol (Cromoline®, Diadema, SP, Brazil), and carboxymethylcellulose (Sigma Aldrich) in the proportion of 77:20:3, respectively, for the aPDT II. The laser used was an Indio-Gallium-Aluminum-Phosphorous (InGaAlP, Thera Lase - DMC, São Carlos, SP, Brazil) with a wavelength of 660 nm, a fiber spot size of 0.02827 cm^2, and continuous emission mode.

MB formulations were slowly applied into the periodontal pocket around the mandibular right first molar using a syringe (1 mL) and insulin needle (BD® UltrafineTM, U-100, 0.5 mL, 8 mm X 0.3 mm). After 1 minute, the laser was applied to three equidistant points in the tooth on buccal and lingual sides. In each spot the laser was applied by 4 s (0.14 J / point), the tissues received 0.84 J of energy and a total energy density of 29.64 J / cm^2 [16, 17].

Histomorphometric Analysis

Bone Loss

From each experimental group: NC (n = 5); PC (n = 5); SRP (n = 10); aPDT I (n = 10) and aPDT II (n = 10) specimens were obtained in each time interval (7, 15 and 30 days). The samples of the mandibles collected were immediately soaked in 10% neutral buffered formalin (pH 7.2) for 48 hours [22] and then decalcified with ethylenediamine tetraacetic acid (EDTA) 10% buffered with sodium hydroxide (pH 7.4) for 13 weeks. The decalcified mandibular tissues was neutralized, after were dehydrated, embedded in paraffin, and serially sectioned (4 μm) in the mesial-distal direction. After excluded the first section that the furcation region was evident, serial sections were obtained and staining with hematoxylin and eosin (H&E).

The linear distance between the cementum and the apex of the alveolar bone crest of the furcation region was measured to determine a histometric bone loss (μm) by means of a linear measurement in the mandibular right first molar at an objective magnification of 100x [23], with modifications. The linear bone loss represents the average value obtained from two measurements for each rat of each experimental group after 7 days of ligature removal. In order to prove the induction of periodontitis, only the animals of the NC and PC groups were evaluated for linear bone loss [17]. A blind examiner evaluated the experimental groups with an interval of one week and mean values were calculated.

Inflammatory Infiltrate

The gingival connective tissue from buccal region of periodontitis induction was removed and soaked in 10% neutral buffered formalin (pH 7.2) for 24 hours and then embedded in paraffin. Serial sections were cut with a thickness of 6 μm in a mesial-distal direction and staining with H&E. The intensity of inflammatory infiltrate was graded as absent, mild, moderate and severe at an objective magnification of 100x [24]. The specimens were evaluated by a single blinded examiner and characterized as absent (0) when it is characterized by absence of inflammatory cells in the connective tissue; mild (1) when presenting distribution sparse inflammatory cells in connective tissue; moderate (2) when found dense accumulation of inflammatory cells

in isolated areas, sparse distribution in other areas of connective tissue and severe (3), when it presents dense aggregation of inflammatory cells throughout the connective tissue.

Immunohistochemical Analysis

The blocks containing the subsequent sections of gingival connective tissue were used for immunohistochemical. VEGF immunoreactivity was evaluated employing the VG1 monoclonal mouse anti-human (Dako, Glostrup, Denmark), which recognizes the 121, 165 and 189 isoforms of VEGF. After cooling, the tissues embedded in paraffin were sectioned in thickness of 2 μm. The process of dewaxing and hydration of the slides occurred in PT Link machines (Dako, Glostrup, Denmark) with deionized water and ready solutions of low pH and high pH. For the immunohistochemical reaction, the entire EnVision FlexTM+/ HRP kits (Dako, Glostrup, Denmark) protocol was followed, together with the use of concentrated (to be diluted) and flex (already diluted) antibodies according to their respective pH. The primary VEGF antibody was diluted in 1:100, according to standardization.

VEGF staining was evaluated by a single blinded examiner. The evaluation was based according to the brown staining within the cell cytoplasm, which was accepted as positive staining, under magnification of 400x. Immunoreactivity was graded in 3 groups, by using a modification of the scale previously described, grade (0): absence of staining; grade (1): light to moderate amounts of staining, not completely filling the cytoplasm, light brown in color; or grade (2): intense staining of the cytoplasm, completely filling the cell, very dark brown [25]. Additionally, the staining of polymorphonuclear neutrophils was used as an internal positive control, since these cells also express VEGF [26].

All the histomorphometric and immunohistochemical analysis were performed through a light microscope (Binocular Optical Microscope ZEISS, Axio Lab.A1, Germany), the images were captured by a camera (AxioCam, ERc 5S, Germany) with monitor coupled, for the measurements were used the software ZEN 2011 (blue edition) 1.0, Carl Zeiss MicroImaging GmbH.

Intra-examiner Reproducibility

The examiner was calibrated through two measurements of ten specimens with an interval of one week. The intra-class correlation coefficient values were 0.85 and 0.95 for histomorphometric and immunohistochemical analysis, respectively.

Results

Histomorphometric and Immunohistochemical Analysis

The analyzed areas for histomorphometric measures were gingival connective tissue and furcation region. The immunopositivity for VEGF was observed in gingivals epithelial and connective tissues, in chronic inflammatory cells as well as in endothelial cells. The protein also showed nuclear and cytoplasmic immunoreactivity and a diffuse pattern of expression. The percentage of tissues responses analyzed follows in the Table 2.

Table 2 Analysis of gingival connective tissue, considering inflammatory and VEGF intensity

	Inflammatory infiltrate (grade (%))			VEGF (grade (%))		
	7th day	**15th day**	**30th day**	**7th day**	**15th day**	**30th day**
NC	0 (20)	0 (40)	0 (60)	0 (90)	0 (100)	0 (90)
	1 (80)	1 (60)	1 (40)	1 (10)	1 (00)	1 (10)
	2 (00)	2 (00)	2 (00)	2 (00)	2 (00)	2 (00)
	3 (00)	3 (00)	3 (00)			
PC	0 (00)	0 (20)	0 (40)	0 (20)	0 (40)	0 (80)
	1 (10)	1 (20)	1 (60)	1 (60)	1 (60)	1 (20)
	2 (70)	2 (40)	2 (00)	2 (20)	2 (00)	2 (00)
	3 (20)	3 (00)	3 (00)			
SRP	0 (00)	0 (40)	0 (60)	0 (80)	0 (90)	0 (90)
	1 (100)	1 (60)	1 (40)	1 (20)	1 (10)	1 (10)
	2 (00)	2 (00)	2 (00)	2 (00)	2 (00)	2 (00)
	3 (00)	3 (00)	3 (00)			
aPDT I	0 (30)	0 (60)	0 (60)	0 (80)	0 (90)	0 (90)
	1 (70)	1 (40)	1 (40)	1 (20)	1 (10)	1 (10)
	2 (00)	2 (00)	2 (00)	2 (00)	2 (00)	2 (00)
	3 (00)	3 (00)	3 (00)			
aPDT II	0 (20)	0 (40)	0 (90)	0 (70)	0 (90)	0 (90)
	1 (80)	1 (60)	1 (10)	1 (30)	1 (10)	1 (10)
	2 (00)	2 (00)	2 (00)	2 (00)	2 (00)	2 (00)
	3 (00)	3 (00)	3 (00)			

The intensity of inflammatory infiltrate was graded as absent (0); mild (1); moderate (2) and severe (3). The immunoreactivity was graded in absence of staining (0); light to moderate staining (1) and intense staining (2). NC: negative control; PC: positive control; SRP: scaling and root planing; aPDT**:** antimicrobial photodynamic therapy

Histomorphometric Analysis

NC group. At 7 days a very discrete bone loss was observed in the furcation region [17] (Fig. 2A). In gingival connective tissue most of the inflammatory intensity was mild (Fig. 3A). At 15 days and 30 days, it was observed gradual increase of absence cases of inflammatory infiltrate (Table 2).

PC group. At 7 days, periodontal breakdown, characterized by alveolar bone loss was evidenced at furcation region [17] (Fig. 2B). The gingival connective tissue showed inflammatory infiltrate of moderate characteristics (Fig. 3B) and also some severe cases. At 15 days, it was not observed severe cases, most of cases still remained in moderate grade and there was inflammation absence. At 30 days, the number of mild grade and absence increased. No moderate cases was found (Table 2).

SRP group. At 7 days, in all cases, mild inflammatory intensity (Fig. 3C) was observed. At 15 and 30 days, there was a gradual increase of cases with absence of inflammatory infiltrate (Table 2).

aPDT I group. At 7 days, the inflammatory infiltrate was mild in most cases (Fig. 3D) and cases with absence inflammatory infiltrate were observed. At 15 and 30 days, we observed the similar results for SRP group (Table 2).

aPDT II group. At 7 days, very similar responses were observed to aPDT I (Fig. 3E), as well as at 15 days. At 30 days, aPDT II group presented almost all cases with total absence of inflammatory infiltrate (Fig. 3F), surpassing even the NC group (Table 2).

Immunohistochemical analysis

NC group. At 7 (Fig. 4A), 15 and 30 days was observed the absence of immunostaining by VEGF, with very few exceptions (Table 2).

PC group. At 7 days, the highest VEGF intensity of this study was observed, most cases presented light to moderate amounts of staining and some cases with severe staining (Fig. 4B). At 15 days, the behavior of this factor was similar to 7 days. However, at 30 days, was observed a decreased of this immunostaining (Table 2). Interestingly, only in this group and scattered by the experimental time there was in isolated cases the coloration of gingival epithelial tissue (Fig. 4C).

SRP group. At 7 days, most cases showed absence of immunostaining (Fig. 4D). At 15 and 30 days, almost all the cases reached total absence staining (Table 2).

aPDT I group. At 7 days, similar to SRP group and some light staining cases (Fig. 4E), 15 and 30 days this growth factor behavior was equal to SRP group (Table 2).

aPDT II group. At 7 days, more light staining cases (Fig. 4F), 15 and 30 days, the immunostaining was equal to SRP and aPDT I group (Table 2).

Discussion

In the present study, we reproduced a previously reported model of experimental periodontitis in rats caused by the ligature presence in subgingival position in mandibular right first molar [16, 17]. This induction model is characterized by accumulation of plaque, flattening and displacement of the gingival crest, increased proliferation of epithelium into underlying connective tissue and infiltration of mononuclear inflammatory cells [27]. In our study, PC group, showed moderate inflammatory infiltration and significant alveolar bone loss in furcation region. On the other hand, NC presented mild inflammation and discrete bone alveolar loss. This statement is in agreement with previous histopathological reports that showing progressive bone alveolar loss and presence of mononuclear inflammatory intense in ligature-induced periodontitis in rats [21, 28, 29].

Gingival tissues are often in a state of injury and repair that involve repetitive cycles of production of chemotactic and inflammatory reactions [30]. The continued presence of bacteria on the surface of the subgingival tooth leads to bone loss, i.e. periodontitis [1]. Especially at this stage, infiltration of inflammatory cells can cause endothelial damage and suppress oxygen levels. Local hypoxia stimulates the release of VEGF, which is indirectly related to the inflammatory degree [2]. In our study, in gingival tissue, at 7 days after removal of the ligature, the PC group presented the higher immunostaining by VEGF, in parallel, in the same period it presented the highest inflammatory density and important bone loss. The investigators speculated that VEGF might be an important factor in the progression of gingivitis to periodontitis through its role in promoting the expansion of the vascular network observed in inflammation [31–33], confirming our findings at 7 days. However, within 30 days after removal of the ligature, there was a mitigation of this parameters, which seems to have been a "spontaneous recovery" of the periodontal tissues, reducing the intensity of inflammation and VEGF. Chapple et al. (2000) [34] suggested that chronic untreated periodontal disease is characterized by extensive vasculature remodeling in a selective increase of vessels with larger diameters and reduced expression of VEGF, situation confirmed by our study and complemented by Barin et al. [17], in which it was observed an increase of the number and diameter of the blood vessels in the PC group in the same experimental period, justifying the findings.

The damage to the periodontal tissues has as trigger point the permanent and residual bacteria and the main objective of periodontal therapy is significantly reduce these periodontopathogens for establishing the disease as well as the reduction of its inflammatory signs [1]. The conventional therapy for treating periodontitis is SRP. In most cases, this treatment successfully reduces the bacterial load with an excellent therapeutic effect [35]. The SRP group presented mild inflammatory infiltrate in 7 days, over time the number of absence inflammatory infiltrate appeared, ratifying this information. In this study, we believe that SRP proves to be effective in treatment of periodontal disease, nevertheless, this conventional mechanical treatment can fail in hard-to-reach areas, such as furcation regions, bone fissures and concavities [7], requiring complementary tools.

In this sense, the aPDT emerges promisingly as adjuvant therapeutically modality [10]. In the present work, *at 7 days the aPDT I and aPDT II presented very similar histological and immunohistochemical responses*, as for the inflammatory infiltrate, both presented the most cases of mild grade and *some cases with absence of the mononuclear cells*, situation not observed in the SRP group. Cases of leukocyte absences in SRP group was only found at 15 days, this are reinforced by Barin et al. [17] that showed to decrease the systemic oxidative damage released by the presence of periodontitis in only also 15 days, in the SRP group, being able to have a response effect even though a late one. On the other hand, the laser could be able to remove remaining biofilm from areas not accessed by manual techniques and accelerate events involved in tissue repair [36]. Pillusky et al. [16] also contributed to our results, demonstrating the capacity of aPDT II to induce the maturation from type III to type I collagen earlier than other treatments.

The Ps is a pivotal component of aPDT, which act at the surface of several types of bacterial to increase their permeability and allow a significant amount of Ps to accumulate at the level of the cytoplasmic membrane [37]. The changing possibility in Ps formulations can be a key point to increase the effectiveness for therapy. In short-time, the parameters evaluated in this study were very similar to the solutions of MB in water or in ethanol. Although, *at 30 days we observed that, interestingly, in the aPDT II almost all the cases presented abstention of leukocyte infiltrate, surpassing the NC group*. These findings, reinforced by Barin et al. [17], showed that this same group at 30 days presented total vitamin C recovery, an important antioxidant in organism defense, reaching the same levels of the NC group, as also recovered the GSH,

confirming that in the long-term there was a better local and systemic response of this therapy. The set of these outcomes confirm our initial hypothesis. On regarding VEGF, there was no immunostaining at all periods, with very few exceptions. A very similar behavior to other treated groups and NC group was observed.

Studies have not been able to present a clear trend for understanding the major role of VEGF in promoting the progression or the healing of periodontal disease [32, 38]. In this work, a *high VEGF immunostaining was observed in periodontitis without treatmen, tha PC groupt*. In contrast, the expression of this protein reached *lower levels in the healthy gingival tissue and in the treated groups in the repair phase*. These outcomes suggest that VEGF is associated with the severity and progress of inflammation with consequently bone loss [32]. Additionally, previous evidences have reported the role of VEGF also on osteoblasts and osteoclasts recruitment which can act on regulation of osseous homeostasis [39]. Contrary, Cetinkaya et al. [38] found a higher expression of VEGF during the healing stage of periodontal disease, when compared to the destruction stage. Other studies [34, 40] reported higher concentrations of VEGF in gingival fluid collected from healthy sites. These results still indicate conflicting roles of VEGF in the pathogenesis of periodontal diseases, and this factor has not yet been well elucidated.

The VEGF was detectable in periodontal tissues within vascular endothelial cells, plasma cells, and macrophages and in junctional, sulcular, and gingival epithelium [40]. In this study, the staining of VEGF was more frequent in inflammatory infiltrate cells present in the lamina propria and diffuse by cytoplasmic of the gingival connective tissue. This fact corroborates to the findings of other authors who demonstrated VEGF in monocytes and macrophages [4, 40]. Furthermore, macrophages may be the determinants if tissues enter in the state of destruction or inflammatory proliferation, minimizing local oxygen levels and release of growth factors such as VEGF may occur [41, 42], *demonstrating the interaction inflammation-VEGF*, which was observed by us.

However, there was a notable regional variation in the intensity of immunostaining for VEGF only in the PC group, with staining in gingival epithelial tissue, like some studies also point out [30, 39], reinforcing the staining in non-vascular sources [43]. The changes in the vascularity of the periodontal connective tissues in untreated advanced periodontitis may be, in part, a consequence of altered expression of angiogenic activity by the epithelium. In turn, this may reflect to the epithelial

response to microbial flora [34], which may prove our observations because the PC group is the most affected and represents the mechanism for biological defense epithelium. The discrepancy in the location of VEGF may also be justified by the difference in antibody protocols. In our study, we applied anti-human monoclonal antibodies for the parallel detection of three VEGF isoforms: VEGF-A (VEGF-121), VEGF-B (VEGF-165) and VEGF (clone VG1) diluted 1:100 and we obtained epithelial staining. Artese et al. [30], provided no source of the antibodies applied to localize VEGF and even used different dilution than 1:50, also finding staining in epithelial tissue. In divergence [44, 45], studies with the same dilution and VEGF, failed to detect this immunostaining in epithelial tissue.

Conclusions

The present study suggests that the aPDT provided better responses to the periodontal tissues, verified by the greater number of cases with absence of inflammatory infiltrate, compared to SRP alone in short-term. aPDT II, containing MB Ps solubilized in ethanol, showed almost total absence of leukocyte infiltration at 30 days, demonstrating its greater repair capacity also in long-term. Additional studies should be performed to evaluate laser actions and the different Ps, especially their responses on the periodontal tissues.

On regarding VEGF, between the treated groups and NC group, there was no difference and few staining, but in PC group, there was increase of this protein, showing a possible relation with the inflammatory degree and the bone loss, that is, with the disease advance. Still, one of the research that deserves to be explored is the real role of VEGF in gingival disease. Further research on the importance of VEGFs for diseases will continue to be performed and, consequently, the scope for the use of therapeutic anti-VEGF approaches will grow. Therefore, the challenge will be to develop more effective ways to prevent pathophysiological angiogenesis during advance of periodontitis.

Compliance with Ethical Standards

Conflicts of Interest

The authors declare that they have no conflict of interest.

Statement of Human Rights

This article does not contain any studies with human participants performed by any of the authors.

Statement on the Welfare of Animals

This research was approved by Committee on Animal Research of the Federal University of Santa Maria, Brazil (027/2013). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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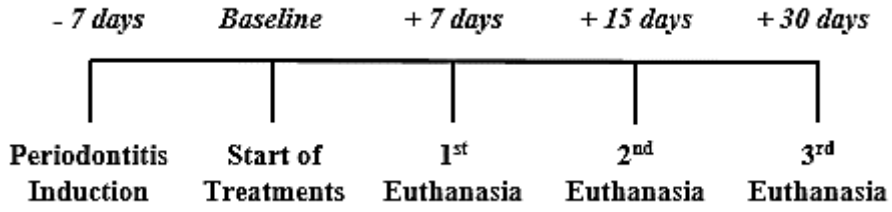

Fig. 1 Experimental procedures

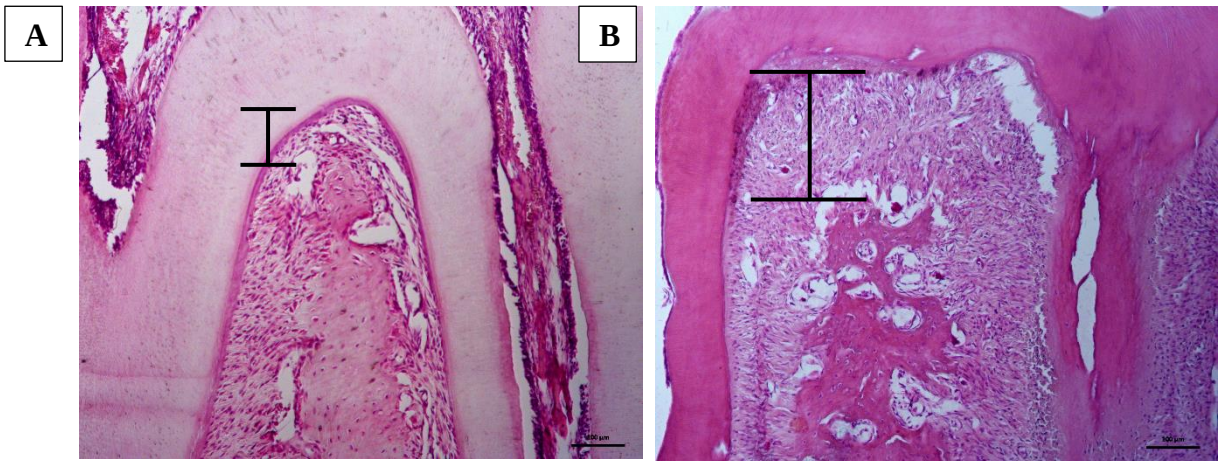

Fig. 2 Linear measure of bone loss in the furcation region of the mandibular right first molar in 7 days, at an objective magnification of 100x. A) Negative Control; B) Positive Control. H&E-staining

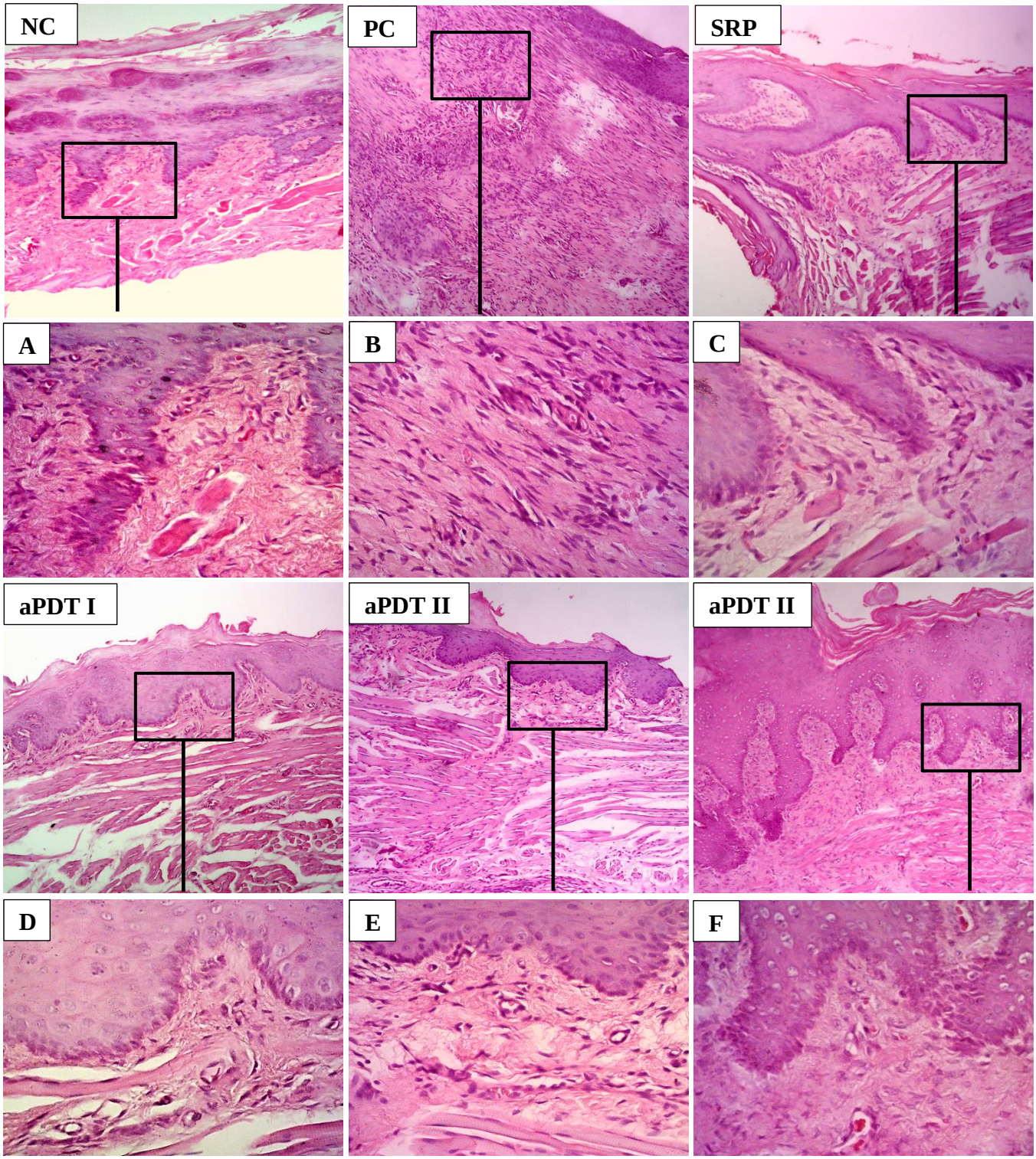

Fig. 3 In the gingival connective tissue at 7 days, the intensity of inflammatory infiltrate was observed under an objective magnification of 100x, followed by 400x magnification of the highlighted area, A (1); B (2); C (1); D (1); E (1). At 30 days, F (0). H&E staining

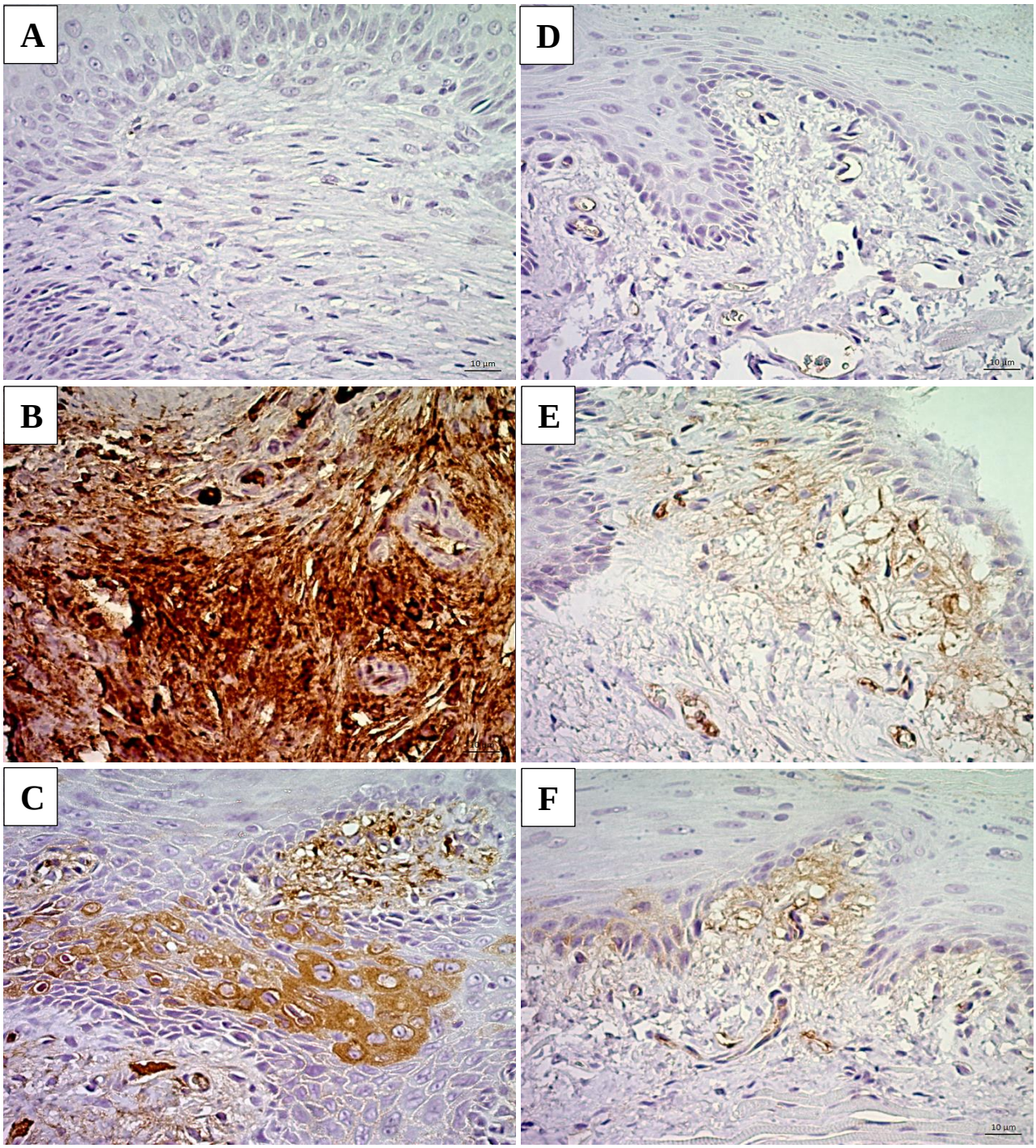

Fig. 4 Immunoreactivity for VEGF in the gingival connective tissue at 7 days under magnification of 400x. A (0); B (2); D (0); E (1); F (1). Staining of epithelial gingival tissue in the PC group (C). Immunohistochemical staining

5 DISCUSSÃO

Os modelos experimentais animais têm a finalidade de produzir conhecimento científico, através do esclarecimento sobre doenças, elaboração de novas drogas, novas técnicas e novas formulações terapêuticas (CHORILLI; MICHELIN; SALGADO, 2007; FAGUNDES; TAHA, 2004; KIRSTEN et al., 2010), como no caso do presente estudo. A similaridade anatômica, imunológica e bioquímica dos tecidos periodontais dos ratos, bem como a semelhança histopatológica no que diz respeito à DP, favorecem a indução experimental desta (KLAUSEN, 1991; SUSIN; RÖSING, 2002). Assim, torna-se uma ferramenta viável para reprodução dos resultados, podendo serem estendidos aos seres humanos. Evidentemente, todos os achados devem ser analisados com cautela antes de serem extrapolados.

A indução experimental da DP pode ser realizada por colocação de ligadura de seda, elástico e algodão (COX; WILLIAMS-MILLER, 1986; DUARTE et al., 2010; ROVIN; COSTICH; GORDON, 1966), pela introdução de microrganismos patogênicos (FIEHN; KLAUSEN; EVANS, 1992; JORDAN; KEYES; BELLACK, 1972), por injeções de toxinas bacterianas lipopolissacarídeos (DUMITRESCU et al., 2004; LLAVANERAS et al., 1999; WADA et al., 2004) ou pela manipulação dietética (GALVÃO et al., 2003; ROBINSON; HART; PIGOTT, 1991). A técnica preconizada aqui foi a colocação de uma ligadura de algodão, que consiste na inserção de um dispositivo ao redor de um dente, com a finalidade de promover irritação mecânica e aumentar a agregação bacteriana gerando uma resposta inflamatória prejudicial ao periodonto (BENTZEN et al., 2005; DUARTE et al., 2010; GARCIA et al., 2013b; GASPERSIC; STIBLAR-MARTINCIC; SKALERIC, 2002; GYÖRFI et al., 1994). Este método, bem como os períodos de indução das doenças e das eutanásias foram baseados em estudos prévios (BARIN et al., 2017; GARCIA et al., 2014; PILLUSKY et al., 2017), que demostraram a possibilidade de investigação dos desfechos escolhidos. Realmente, em ambos estudos, conseguimos a reprodução da DP, confirmada sempre pela comparação entre os grupos controle positivo e negativo, sob parâmetros de perda óssea (com diferença estatística significativa) e infiltrado inflamatório, fato que fornece consistência aos nossos achados.

Também foi possível a reprodução da DM experimental em ratos, com STZ, pois esta droga provoca o bloqueio irreversível da produção de insulina nas células β do pâncreas, causando grave hiperglicemia, induzindo a DM semelhante ao tipo

1(MARLES; FARNSWORTH, 1995). Assim, conseguimos atingir um quadro de doença sistêmica, em que os animais além de apresentarem permanente hiperglicemia (a glicemia foi aferida semanalmente e previamente aos procedimentos), também demonstraram características clínicas como perda de peso, aspecto menos vistoso, poliúria, debilidades e até mesmo óbito. Embora minucioso cuidado tenha sido adotado durante o experimento, com constante aferição da temperatura, aquecimento, proteção ocular e objetos de enriquecimento ambiental, houve a perda de dois animais, que não resistiram aos procedimentos devido suas fragilidades. Podemos considerar que a DM induzida foi de característica não controlada.

A presente Tese apresentou os resultados de dois experimentos, frente a alteração do Fs AM solubilizado em etanol, considerado a novidade do estudo, na TFDa adjuvante ao tratamento padrão ouro (RAR) no controle da DP, com ou sem o envolvimento da DM. O tema foi escolhido no intuito de melhorar o mecanismo de ação desta terapia e permitir melhores respostas periodontais, culminando para um reparo mais rápido e efetivo, tanto em situações de apenas DP quanto associada à doença sistêmica considerada. A literatura vigente já demostra alguns benefícios da terapia combinada (BETSY et al., 2014; GIANNELLI et al., 2012). Ge et al. (2011) demonstraram que TFDa associada à RAR resultou em redução significativa de sangramento à sondagem em bolsas inicialmente de 5 mm ou mais quando comparada à RAR somente. Theodoro et al. (2012) verificaram que TFDa associada à RAR reduziu significativamente alguns patógenos periodontais, embora nenhum benefício clínico tenha sido verificado. Esses dados ainda são inconsistentes e parecem não permitir grandes mudanças clinicamente, o que é nosso objetivo final. Com isso, aliado aos estudos *in vitro*, que demostram melhores propriedades alterando o Fs (PROCHNOW et al., 2016), acreditamos na possibilidade de melhorar as repostas teciduais frente a aplicação da TFDa com este novo protocolo. Felizmente, foi o que se observou, ainda que por vezes de forma discreta, em ambos os estudos.

No primeiro estudo, que continha o fator agravante da DM, foi verificada as respostas sistêmicas e locais geradas pela indução e tratamento da DP. Como principais resultados, foi verificado que o grupo que continha etanol no Fs apresentou, em 7 e 15 dias, menor intensidade inflamatória local, menor intensidade de VEGF, menor quantidade de vasos sanguíneos (em 7 dias) e menor dano oxidativo (igual ao grupo CN ao atingir os 15 dias), quando comparado ao grupo que recebeu apenas a

RAR. Em relação aos dois grupos que receberam a terapia adjuvante, não houve diferença expressivas em todos os desfechos considerados. A menor neoformação de vasos percebida, induz a pensar, que houve uma maior recuperação e estabilização dos tecidos à curto prazo no grupo do etanol, não necessitando de tanto aporte sanguíneo para eliminação do processo inflamatório e grandes reparações. Os vasos identificados nesse grupo foram de pequeno calibre e isso pode ser explicado por serem vasos recém formados (CETINKAYA et al., 2007), os quais ainda não sofreram o processo de remodelamento, em que células endoteliais se unem as já existente e consequentemente aumentam a sua luz. Aqui, aponta-se uma das possíveis limitações do estudo, a análise do diâmetro dos vasos sanguíneos, pois embora, em cada lâmina considerou-se o que era o predominante, a seleção, conforme metodologia seguida é dada de forma arbitrária. Então, este achado é um tanto quanto impreciso. Como forma de padronizar e minimizar o viés, a área selecionada foi definida sempre como a margem subepitelial.

De forma geral, fica evidente que o uso de TFDa, independentemente da solubilização do Fs, em comparação apenas com a RAR, foi capaz de facilitar e acelerar a cura periodontal, apresentando benefícios adicionais da terapia combinada em pacientes sistemicamente comprometidos, suportado por outros autores (ALMEIDA et al., 2008). Assim, as ações fotobiológicas do laser evidenciam aceleração no reparo tecidual (WOODRUFF et al., 2004), inibição da produção de mediadores inflamatórios e promoção da vasodilatação local e angiogênese (HOURELD; ABRAHAMSE, 2007), favorecendo o processo de cicatrização. Recentemente, Fahimipour et al. (2016) descobriram que a ferida da mucosa oral de ratos diabéticos tratados com baixa terapia a laser também apresentava um menor número de células polimorfonucleares e neoformação vascular, reforçando nossos dados, principalmente em relação ao grupo do Fs com etanol. Em contrapartida, autores apontam ainda como discutível o uso das terapias adjuvantes à RAR em comparação com apenas RAR na melhoria da condição clínica e do controle glicêmico em pacientes com DP e DM (ABDULJABBAR et al., 2016; AL-ZAHRANI et al., 2009). Acrescentados por revisões sistemáticas que mostram resultados estatisticamente significantes a favor da terapia combinada, mas ainda com benefício clínico questionável (SGOLASTRA et al., 2013a). Neste panorama formado, o nosso estudo se encaixa pela investigação da possibilidade de melhorar o efeito da TFDa, através da alteração do solvente no qual o Fs é dissolvido. Até o presente, só há estudos do

nosso grupo de pesquisa, considerando a potencialidade do Fs modificado por etanol *in vivo*, porém sem o envolvimento da DM (BARIN et al., 2017; PILLUSKY et al., 2017). De acordo com os achados aqui descritos e os embasamentos prévios, a solubilização do Fs em etanol pode ser considerado como possível potencial para o tratamento periodontal em pacientes com DM. É evidente que mais estudos neste sentido devem ser realizados para fortalecer esta nova linha de pesquisa, com a intenção de testes em humanos, pois sabe-se que há segurança para execução deste procedimento. Além disso, novas peculiaridades podem ser percebidas e ajustadas, a fim de sempre melhorar o prognóstico desses pacientes, visto que, a DM é um importante problema de saúde pública e possui previsões alarmante quanto ao número de indivíduos a serem acometidos, por isso merece especial atenção.

Em relação à técnica imuno-histoquímica do VEGF (Apêndice A), os resultados analisados mostraram maior intensidade em ratos diabéticos com DP, já os com DM e sem DP, não evidenciaram imunomarcação. Acredita-se que a DP possa ser responsável pela maior presença do VEGF durante o curso da doença. Pois, poderia estar interagindo com a DM e corroborando para maior liberação de EO e todo aquela cadeia entre DP x VEGF x DM x EO, culminando com a grande perda periodontal observada, infiltrado inflamatório moderado e altos níveis de peroxidação lipídica no grupo controle positivo (com ambas as doenças), evidenciando o agravo do quadro clínico. Há limitados estudos na literatura sobre a presença do VEGF nos tecidos periodontais de pacientes diabéticos (ASPRIELLO et al., 2009; GÜNERI et al., 2004; SAKALLIOĞLU et al., 2007; ÜNLÜ et al., 2003). Autores afirmam que a presença de altos níveis de glicose, induz a níveis elevados de EO (BROWNLEE, 2001), que potencializa a ação do VEGF (CHUA; HAMDY; CHUA, 1998), já a DP (BASSIOUNY; ALAYED; ALSALMAN, 2014). também contribui para o aumento do EO e agravo da doença (CHAPPLE; MATTHEWS, 2007; CHAPPLE, 1997; WADDINGTON; MOSELEY; EMBERY, 2000). Tais estreitas inter-relações tornam sugestivo mais estudos neste sentido.

Já o segundo estudo, que possuía o mesmo desenho e grupos experimentais do estudo acima, exceto pela ausência da DM e mais um período de análise em 30 dia, foi observado as respostas locais do tecido periodontal geradas pelos tratamentos. Como principais resultados, os grupos da TFDa, independente do Fs utilizado, apresentaram casos de ausência de infiltrado leucocitário já a partir dos 7 dias, enquanto o grupo que foi tratado apenas com a RAR, conseguiu atingir alguma

ausência do infiltrado inflamatório apenas ao alcançar os 15 dias. Evidenciando mais uma vez que, a TFDa forneceu melhores respostas teciduais em curto prazo de tempo, resultado também observado no primeiro estudo. Pesquisas recentes reforçam tais achados, demonstrado o efeito benéfico da TFDa nos tecidos periodontais, tanto na redução de espécies bacterianas quanto a favor da resolução do processo inflamatório (FERNANDES et al, 2010; CARVALHO et al, 2011). Estudos experimentais animais (BARIN et al., 2017; DE OLIVEIRA et al., 2016; PILLUSKY et al., 2017) e ensaios clínicos randomizados também têm demonstrado efeitos adicionais da TFDa associada à RAR em comparação com apenas RAR (ALWAELI; AL-KHATEEB; AL-SADI, 2013; BETSY, et al., 2014; BRAUN, et al., 2008; GIANNELLI, et al., 2012). Entretanto, não significa que o tratamento padrão ouro não é efetivo, muito pelo contrário, ele funciona sim, porém revela um “atraso” em relação à terapia combinada.

Interessantemente, o grupo com solubilização do Fs em etanol aos 30 mostrou ausência quase total de infiltração mononuclear, superando os dados do grupo controle negativo, que possuía alguma presença leucocitária. Fato, que denota uma maior capacidade de reparo também a longo prazo, experimentada somente por este grupo. Ademais, aliado com os dados sugestivos de benefícios que esta solubilização poderia proporcionar, pelo estudo supracitado, fica claro que há bem mais que uma intenção de melhores respostas, há a detecção destas nos tecidos periodontais e sistêmico, apresentado também por estudos prévios (BARIN et al., 2017; PILLUSKY et al., 2017), o que se torna um incentivo para prosseguir as investigações nesta esfera.

Em relação ao VEGF, entre os grupos tratados e o controle saudável, não houve diferenças e não houve coloração, porém, novamente, no grupo controle positivo, foi evidenciado imunopositividade intensa. Aqui não havia a DM, o que nos leva a crer que o elo principal envolvido com o VEGF é a presença da DP, e a partir desta as inter-relações podem ser ativadas (representados pela Fig. 2). Revelando assim o principal papel do VEGF envolvido com a DP, mostrando relação positiva com o alto grau inflamatório e de destruição óssea, sendo considerado como participante da progressão da doença. Corroborando com demais estudos em que o VEGF desempenha um importante papel na progressão da gengivite para a periodontite (BASSIOUNY; ALAYED; ALSALMAN, 2014; BOOTH et al., 1998; OLIVEIRA et al., 2008; SUTHIN et al., 2003).

6 CONCLUSÃO

O presente estudo propôs investigar um possível aprimoramento do uso da TFDa, adjuvante à RAR, na tentativa de melhorar o seu efeito no tratamento periodontal, detectado sistêmica e localmente nos tecidos periodontais, com ou sem o envolvimento da DM, através da inovadora solubilização do Fs AM em etanol. A reprodução experimental animal das enfermidades crônicas DP e DM possibilitaram tais condições de avaliação.

Dentre os limites do estudo, podemos concluir que a TFDa com Fs AM adicionado ao etanol permitiu melhores respostas a curto e longo prazo, em relação aos desfechos avaliados. Novas pesquisas a cerca disso devem ser executadas para fortalecer e proporcionar melhorias nesta linha, sugere-se a avaliação sobre novos desfechos e o desenvolvimento de um estudo clínico.

O uso combinado da TFDa com RAR fornece benefícios adicionais quando comparadas apenas à RAR, representado no estudo por menores infiltrações leucocitárias (em ambos os estudos), menor grau de VEGF, menor estresse oxidativo, dentre outros. A TFDa possibilitou respostas teciduais sistêmica e locais de reparo e homeostasia tecidual de forma mais acelerada. Assim, fica claro que a biostimulação da luz com o Fs levou à uma série de eventos fotobiológicos que repercutiram em efeitos vantajosos sobre a reparação periodontal.

Na mesma perspectiva, ainda identificamos que o VEGF está mais relacionado com o processo de avanço da DP, confirmado pela associação com os dados de perda óssea e maior grau de infiltração leucocitária. Assim, considera-se a DP como o ponto chave para maior expressão do VEGF, independetemente da presença ou não da DM, o que pode levar à um maior dano tecidual. Este recente campo de pesquisa está merecendo ser explorado para consolidar o real papel do VEGF na DP. Estudos sobre a importância deste fator de crescimento devem continuar a serem gerados no intuito de, talvez, propor o uso de abordagens terapêuticas anti-VEGF. Este poderá ser um desafio futuro e uma forma mais efetiva de prevenir a angiogênese fisiopatológica durante o avanço da DP, com ou sem envolvimento sistêmico.

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APÊNDICE A – TÉCNICA DE IMUNO-HISTOQUÍMICA DO ANTICORPO VEGF (APC/SP)

APC APOIO EM PATOLOGIA CIRÚRGICA	**Título** IMUNO-HISTOQUÍMICA	**Código** POP – APC -005	**Página** 1 de 5

IMUNOHISTOQUÍMICA

REAGENTES

Os reagentes são armazenados em local apropriado e conforme as especificações técnicas e compatibilidade química.

Água deionizada

Álcool 100%

Anticorpos primários monoclonais ou policlonais (conforme bulas)

Hematoxilina de Harris – Queel

Kit ENVISION FLEX da DAKO

Xilol

Meio de Montagem - Coversleeper

EQUIPAMENTOS

PT LINK – marca: DAKO

COVERSLEEPER – marca: DAKO

REAÇÃO IMUNO- HISTOQUÍMICA – PROCEDIMENTO PASSO A PASSO

Cortes

- Os blocos foram entregues à área de histotécnica após a triagem (identificação), e colocados no freezer para gelar;
- Após resfriamento do bloco, foram realizados cortes de 2 micras em micrótomo manual;
- Os cortes foram colocados em banho maria à +/-65°C e “pescados” nas lâminas já identificadas com o número do caso;
- Estas lâminas foram colocadas em estufa à 60°C por 40 minutos para o início da desparafinização.

Preparação

Período noturno:

- As lâminas foram colocadas em berços de coloração devidamente numeradas para serem distribuídas nos tanques dos ptLink (equipamento em comodato DAKO) e de acordo com o PH determinado.
- Fecharam-se os ptLink, fez-se a programação na tela do Menu de cada equipamento, verificou-se horário, dia da semana, temperatura e programação de início do processo.
- Finalmente verificamos se o NO-BREAK estava ativo corretamente, a fim de garantirmos **o início do processo no período da manhã**.

Processo da imunohistoquímica - Período da manhã

- O processo de desparafinização e hidratação das lâminas ocorrem nas máquinas ptLink com água deionizada e soluções prontas de pH baixo e pH alto que atingirão uma temperatura de aprox. 180ºC num período de 72 minutos a partir das 4:00AM em soluções de pHs ALTO e BAIXO.

- A continuidade do processo se dá com uso de todo o kit Envision Flex da Dako para a realização da reação imunohistoquímica, juntamente com uso de anticorpos concentrados (a serem diluídos) e *flex* (já diluídos) de acordo com seus respectivos ph's.

Vide protocolo resumido do uso do Kit Envision Flex, conforme figura abaixo:

Abaixo

Dako

Protocolo EnVision™ *FLEX*

Etapa	Tempo
1. EnVision™ *FLEX* Peroxidase Block	5 minutos
Solução de Lavagem	*5 minutos*
2. Anticorpo primário FLEX RTU	20 minutos
Solução de Lavagem	*5 minutos*
3. EnVision™ *FLEX* /HRP	20 minutos
Solução de Lavagem	*2 x 5 minutos*
4. EnVision™ *FLEX* Substrate Working Solution	2 x 5 minutos
Solução de Lavagem	
5. EnVision™ *FLEX* Hematoxilyn	5 minutos
Água destilada ou deionizada	
Solução de Lavagem	5 minutos
Água destilada ou deionizada	

Obs.: Quando realizados os dois protocolos simultaneamente, após a etapa 2 deixar as lâminas em solução de lavagem enquanto se incuba o "**Linker**" do protocolo **EnVision™** ***FLEX*** **+**. Após isto, todas as etapas serão semelhantes para ambos os protocolos.

Protocolo EnVision™ *FLEX* +

Etapa	Tempo
1. EnVision™ *FLEX* Peroxidase Block	5 minutos
Solução de Lavagem	*5 minutos*
2. Anticorpo primário FLEX RTU	20 minutos
Solução de Lavagem	*5 minutos*
3. EnVision™ *FLEX*+ (Linker)	15 minutos
Solução de Lavagem	*5 minutos*
4. EnVision™ *FLEX* /HRP	20 minutos
Solução de Lavagem	*2 x 5 minutos*
5. EnVision™ *FLEX* Substrate Working Solution	2 x 5 minutos
Solução de Lavagem	
6. EnVision™ *FLEX* Hematoxilyn	5 minutos
Água destilada ou deionizada	
Solução de Lavagem	5 minutos
Água destilada ou deionizada	

Abaixo segue também a descrição detalhada do processo resumido na figura 1:

- Abrir as tampas do PT LINK e deixar esfriar por 30 minutos para que não haja choque térmico nas lâminas;
- Retirar os carrinhos do PTLINK;
- Mergulhar 10 vezes em solução tampão e deixar descansar por 5 minutos;
- Trocar o tampão, repetir o procedimento e deixar mais 5 minutos em descanso;
- Retirar os carrinhos com as lâminas da solução, tirar dos carrinhos uma a uma, secar nos arredores do fragmento com campo cirúrgico, fazer um círculo com a caneta hidrofóbica DAKO PEN ao redor do fragmento delimitando-o, e distribuir as mesmas nas caixas acrílicas lado a lado. Em seguida pipeta-se 100ul de peroxidase block e deixar por 5 minutos em descanso;
- Em seguida lavar com solução tampão (WASH) as lâminas e deixar também por 5 minutos em descanso;
- Retirar o excesso de solução tampão, levantando uma lâmina por vez, devolver na caixa novamente para pipetar o anticorpo primário já diluído, conforme

- designado nas lâminas previamente etiquetadas. O processo de incubação os anticorpos devem seguir as instruções abaixo:

INCUBAÇÃO COM ANTICORPO PRIMÁRIO

✓ Diluiu-se o anticorpo primário VEGF com diluente DAKO, de acordo com o título já padronizado de 1:100.

✓ Enxugou-se as lâminas que estavam na água deionizada com campo cirúrgico ou papel toalha, tomando o cuidado de não estragar fragmento ou esfregaço citológico.

✓ Delimitou-se a área do corte com caneta Dako Pen.

✓ Pingou-se 100µl do anticorpo primário em cima do corte.

✓ Colocaram as lâminas dentro de uma caixa de acrílico ou recipiente plástico, e incubamos por 30 minutos em temperatura ambiente.

✓ Lavamos com tampão Dako na própria cuba, utilizando pissete.

- Após a reação com os anticorpos primários, lavar novamente as lâminas nas caixas acrílicas com solução tampão (WASH) e deixar novamente em solução tampão por 5 minutos;
- Retirar o tampão levantando as lâminas, levantando uma lâmina por vez, devolver na caixa novamente e pipetar o polímero HRP e deixar incubar por 20 minutos;
- Após, entrar novamente em processo de lavagem com a solução tampão por 2 vezes e descansar de 5 minutos em cada etapa;
- No caso de anticorpos (anti mouse ou anti rabbit) incubar o linker por 15 minutos e depois lavar com tampão;
- Na próxima etapa entrar em processo de revelação com o cromógeno Substrate Working Solution (equivalente ao DAB), onde pipeta-se 2 vezes e fica em reação por 7 minutos em cada uma das vezes;

- Depois, lavar as lâminas com solução de lavagem (tampão wash) e colocá-las em carrinhos que serão levados às cubas de hematoxilina por 30 segundos em descanso, para depois serem lavadas em água corrente;
- Em seguida, cada carrinho é mergulhado na bateria de 4 cubas de álcool absoluto e 8 cubas de xilol, até serem trocadas de carrinhos e levadas à máquina de montagem de Lâminas (COVERSLEEP – DAKO).

ANTICORPO PRIMÁRIO:

Dako

Monoclonal Mouse
Anti-Human
Vascular Endothelial Growth Factor
Clone VG1
Code No. M7273

For research use only. Not for use in diagnostic procedures.

Recommended use

Monoclonal Mouse Anti-Human Vascular Endothelial Growth Factor, Clone VG1, is recommended for use in immunocytochemistry. The antibody labels the VEGF-121, VEGF-165, and VEGF-189 isoforms of vascular endothelial growth factor (VEGF) (1).

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

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

CARTA DE APROVAÇÃO

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

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

Número do Parecer: 027/2013

Pesquisador Responsável: Prof. Dra. Cristiane Cadermatori Danesi

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

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

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

DATA DA REUNIÃO DE APROVAÇÃO: 05/09/2013

Santa Maria, 05 de setembro de 2013.

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

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 NO PERIÓDICO JOURNAL OF DENTISTRY.

JOURNAL OF DENTISTRY

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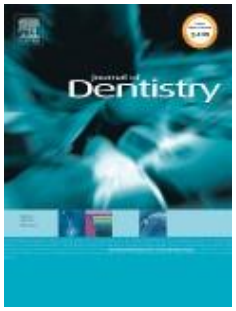

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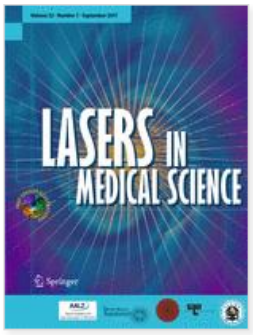

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3. This effect has been widely studied [1-3, 7].

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Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

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Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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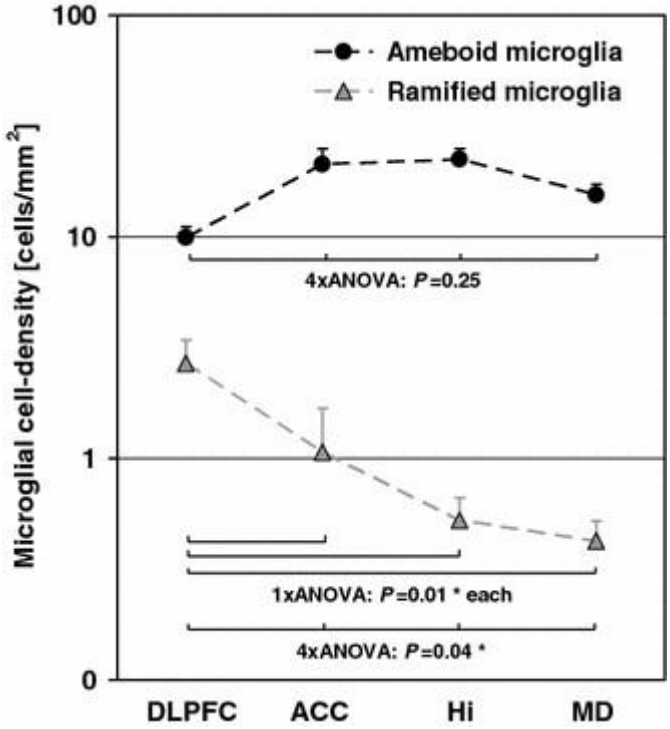

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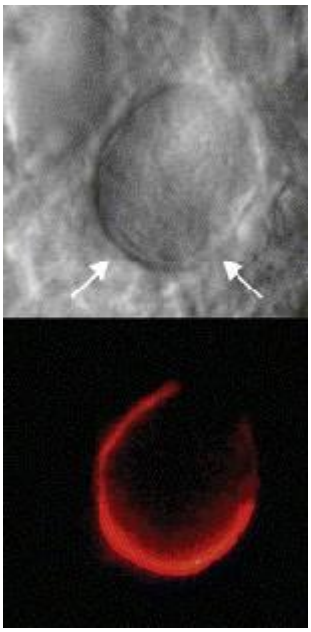

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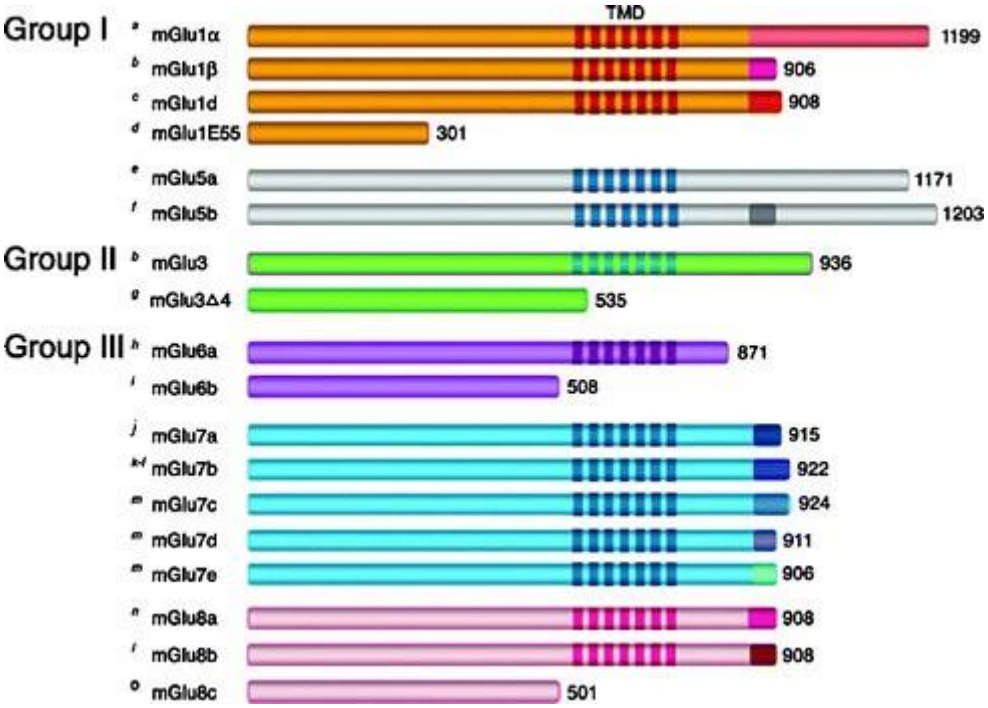

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