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EFEITO DOSE-DEPENDENTE DA TERAPIA FOTODINÂMICA ANTIMICROBIANA SOBRE PARÂMETROS OXIDATIVOS E HISTOLÓGICOS NA LÍNGUA DE RATOS

Santa Maria, RS, Brasil
2016

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Trabalho de conclusão de curso apresentado ao Curso de Graduação em Odontologia, área de concentração em Patologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do **Grau de Cirurgiã-dentista.**

Orientadora: Profª. Drª. Cristiane Cademartori Danesi

Co-orientadora: Drª. Raquel Cristine Silva Barcelos

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Aprovado em 01 de julho de 2016:

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Santa Maria,
Julho/2016.

DEDICATÓRIA

Dedico este trabalho à minha mãe, Sandra Torbes, por toda dedicação e amor desde o meu nascimento, por ser meu maior exemplo de amor a profissão.

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“Há uma força mais poderosa que o vapor, a eletricidade
e a energia atômica: a VONTADE.”
(Albert Einstein)

RESUMO

EFEITO DOSE-DEPENDENTE DA TERAPIA FOTODINÂMICA ANTIMICROBIANA SOBRE PARÂMETROS OXIDATIVOS E HISTOLÓGICOS NA LÍNGUA DE RATOS

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A Terapia Fotodinâmica antimicrobiana (TFDa) é definida como uma reação fotoquímica utilizada com o intuito de causar destruição de micro-organismos. Esta terapia tem sido amplamente empregada na Odontologia, porém há duas importantes limitações do seu uso: (I) ainda não existem protocolos de segurança estabelecidos, (II) poucos estudos de toxicidade em relação as doses fornecidas para as variadas terapêuticas. Nesse sentido, diferentes doses de TFDa foram administradas na língua de ratos saudáveis e as alterações teciduais oxidativas e histológicas foram investigadas, a fim de estimar o dano tecidual. Quarenta Wistar machos (±250g) foram distribuídos aleatoriamente em oito grupos experimentais (n=5): grupo controle (grupo CT), que não foi submetido à TFDa e os demais grupos testados com as seguintes doses de energia luminosa de 18J/cm^2 (grupo L18), 24J/cm^2 (grupo L24), 30J/cm^2 (grupo L30), 70J/cm^2 (grupo L70), 122J/cm^2 (grupo L122), 275J/cm^2 (grupo L275) e 400J/cm^2 (grupo L400). Seis horas após a exposição à TFDa, a língua dos animais foi removida para as análises bioquímicas de parâmetros de estresse oxidativo através da quantificação dos níveis de espécies reativas de oxigênio, dos níveis de peroxidação lipídica e carbonilação proteica, além da atividade da catalase e dos níveis de glutationa reduzida da língua e para análise histológica do infiltrado inflamatório. Os resultados sugerem que existe uma janela de segurança entre as diferentes doses da TFDa, a qual pode ser empregada com segurança, sem danificar o tecido lingual saudável.

Palavras-chave: Terapia Fotodinâmica Antimicrobiana. Infiltrado Inflamatório. Estresse Oxidativo.

ABSTRACT

DOSE-DEPENDENT EFFECTS OF ANTIMICROBIAL PHOTODYNAMIC THERAPY ON THE HISTOLOGICAL AND OXIDATIVE PARAMETERS IN THE TONGUE IN RATS

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Antimicrobial Photodynamic Therapy (aPDT) is defined as a photochemical reaction used in order to cause destruction of microorganisms. This therapy has been widely used in dentistry, but there are two important limitations of its use: (i) there are no established security protocols, (ii) little quantity of toxicity studies regarding the doses for therapeutic varied. Accordingly, different aPDT doses were administered in the tongue of healthy rats and oxidative and histological tissue changes were investigated in order to estimate the tissue damage. Forty male Wistar rats (± 250g) were randomly divided into eight experimental groups (n = 5): control group (CG group), which was not submitted to aPDT and the other groups tested with the following light energy doses of doses 18J / cm^2 (L18 group), 24J / cm^2 (L24 group), 30J / cm^2 (L30 group), 70J / cm^2 (L70 group), 122J / cm^2 (L122 group), 275J / cm^2 (L275 group) and 400J / cm^2 (L400) group. Six hours after exposure to aPDT, the tongues of the animals were removed for biochemical analysis of oxidative stress parameters by measuring the levels of reactive oxygen species, lipid peroxidation levels and carbonylation protein also the activities of catalase (CAT), reduced glutathione (GSH) levels tongue and histological analysis of inflammatory infiltrate. The results suggest that there is a security window between different doses of aPDT, and this may be employed safely without damaging healthy tissue lingual.

Keywords: Antimicrobial Photodynamic Therapy. Inflammatory Infiltrate. Oxidative Stress.

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LISTA DE ABREVIATURAS E SIGLAS

TFD	Terapia Fotodinâmica
TFDa	Terapia Fotodinâmica antimicrobiana
EROs	Espécies Reativas de Oxigênio
GPx	Glutationa Peroxidase
CAT	Catalase
SOD	Superóxido Dismutase
GSH	Glutationa Reduzida

SUMÁRIO

1. INTRODUÇÃO

1.1. TERAPIA FOTODINÂMICA ANTIMICROBIANA: HISTÓRICO

A luz, como agente terapêutico, tem sido empregada há muito tempo e é datada desde a Antiguidade, quando era utilizada para o tratamento de doenças da pele como psoríase, vitiligo e câncer. Nesse contexto, o médico grego Heródoto enfatizava a importância da exposição à luz solar para restauração da saúde (ACKROYD et al., 2001).

O estudo sobre fototoxicidade começou em 1897 com o início dos estudos de Oscar Rabb sobre os efeitos letais do cloridrato de acridina associado à luz visível sobre o protozoário *Paramecium caudatum*. Em 1900, o cientista alemão demonstrou que a incidência de luz sobre corantes pode induzir a morte de células (RABB, 1900). Raab notou que a exposição do *Paramecium caudatum* à acridina e posterior exposição à luz culminava na morte deste organismo. Na verdade, a Terapia Fotodinâmica (TFD) é considerada como tendo a sua origem em 1900 com esse experimento clássico. Em 1904, a TFD foi introduzida como terapia médica como a luz induzindo a inativação de células, micro-organismos ou moléculas (TAPPEINER; JOLDLBAUER, 1904). Mais tarde, Raab e seu supervisor Hermann Von Tappeiner definiram o termo "terapia fotodinâmica" e a aplicaram com sucesso no tratamento de tumores cutâneos usando eosina. A partir desse conceito, (MACHADO, 2000; SIMPLICIO; MAIONCHI; HIOKA, 2002; ALMEIDA et al., 2004; AGOSTINIS et al., 2004), como nós conhecemos hoje, foi fundada a TFD. Desde então, o desenvolvimento de outros estudos, como o realizado por Dougherty e colaboradores resultou em uma técnica não-invasiva para o tratamento do câncer e outras doenças (MITTON; ACKROYD, 2001; DOUGHERTY et al., 1998).

1.2. PRINCIPAIS COMPONENTES DA TERAPIA FOTODINÂMICA ANTIMICROBIANA: O FOTOSSENSIBILIZADOR E A FONTE DE LUZ

Atualmente, vários fotossensibilizadores estão sendo empregados nos estudos da Terapia Fotodinâmica Antimicrobiana (TFDa). Já são mais de 400 compostos conhecidos com propriedades fotossensibilizantes, incluindo corantes, fármacos, cosméticos, produtos químicos e muitas substâncias naturais. Dentre esses, destacam-se os fotossensibilizadores da

classe das fenotiazinas, como azul de metileno, azul de toluidina, clorinas, e forfirina, que absorvem luz de comprimentos de onda entre 550 e 700nm (SHACKLEY, 1999). Utiliza-se um tempo de pré-irradiação, que é aquele em que o fotossensibilizador entra em contato com os micro-organismos para que possa penetrar ou, ao menos, se ligar à membrana plasmática e, consequentemente, aumentar os efeito deletérios ao alvo proposto (GUTKNECHT; NORBERT et al., 2000).

O fotossensibilizador deve ser biologicamente estável, fotoquimicamente eficaz, possuir toxicidade mínima às células normais, seletividade pelas células-alvo, ser administrado de forma local, tópica ou no interior da cavidade e, após um tempo ideal, ser irradiado com luz em dose e comprimento de onda adequado. Os principais alvos desses fotosensibilizadores parecem ser componentes do DNA e da membrana celular, causando aumento de sua permeabilidade (WAINWRIGHT et al., 1997).

Quanto à fonte de luz, a TFDa envolve vários parâmetros de dosimetria de luz, tais como: comprimento de onda, potência, tempo de exposição, taxa de fluência, fluência (dose), número de tratamentos e intervalos; por isso, não é fácil definir um protocolo para essa técnica (MAROTTI et al., 2009). Nesse sentido, há vários esforços para que um protocolo clínico seja estabelecido pelas vantagens que esta terapia apresenta devido seu mecanismo de ação. Desta forma, é improvável que os micro-organismos desenvolvam resistência à TFD (SOUKOS; GOODSON, 2011). Além do não desenvolvimento de resistência bacteriana, tem sido demonstrado que micro-organismos resistentes à fármacos são suscetíveis a TFDa (MAISCH, 2009). Por esses motivos, essa terapia tem ressurgido como uma alternativa aos regimes antimicrobianos, os quais permitem o estabelecimento de patógenos oportunistas por meio do desenvolvimento da resistência dos micro-organismos (WILSON, 1994). Nesse contexto, o uso da TFDa vem sendo difundido na Odontologia, na expectativa de auxiliar na resolução dos efeitos adversos das terapias antimicrobianas tradicionais, podendo figurar como adjuvante nos tratamentos odontológicos.

As primeiras fontes de luz utilizadas na TFDa foram lâmpadas convencionais, que emitiam luz não coerente e policromática, com um forte componente térmico associado (ACKROYD et al., 2001). O desenvolvimento dos lasers de diodo de baixa intensidade com luz monocromática e coerente facilitou a associação com fotossensibilizadores com banda de absorção ressonante com o comprimento de onda emitido pelo laser. A dose de radiação é facilmente calculada e a área de irradiação é controlada focalizando o tratamento. A luz pode

ser transmitida por meio de fibra óptica que podem receber adaptações para melhor acessar a lesão alvo com microlentes e difusores (GUTKNECHT; DE PAULA EDUARDO, 2004).

1.3. MECANISMO DE AÇÃO DA TERAPIA FOTODINÂMICA ANTIMICROBIANA

A TFDa foi inicialmente aprovada, em 1999, para o tratamento de lesões cutâneas pré-cancerosas no rosto e couro cabeludo (BABILAS et al., 2005). Tal terapia baseia-se na concepção de que um agente que absorve a luz conhecido como fotossensibilizador, é ativado pela luz de comprimento de onda apropriado, na presença de oxigênio e gera espécies reativas de oxigênio (EROs) que são citotóxicos para os micro-organismos (DE MELO et al., 2013). A TFDa é baseada na tríade: fonte de luz, fotossensibilizador e oxigênio, uma vez que a energia absorvida pelo corante é transferida à molécula de oxigênio, dando origem à reação oxidativa (SHACKLEY, 1999).

A TFDa baseia-se no princípio de que uma substância fotoativável (fotossensibilizador) liga-se à célula-alvo e pode ser ativado por uma luz de comprimento de onda adequado. Durante este processo, EROs são formadas (entre elas, o oxigênio singleto) que, em seguida, irão produzir um efeito tóxico para as células (MARTINETTO et al., 1985). Em outras palavras, a molécula do fotossensibilizador absorve a luz irradiada com comprimento de onda específico e passa de um estado de baixa energia para um estado mais excitado (singleto), que irá sofrer transição para o estado tripleto (maior energia). O estado tripleto pode reagir com o oxigênio e produzir endogenamente o oxigênio singleto e outros radicais livres, causando uma rápida destruição seletiva do tecido alvo (KONOPKA; GOSLINSKI 2007).

Há dois mecanismos pelos quais o fotossensibilizador no estado tripleto pode reagir com as biomoléculas: reações do tipo I e do tipo II. As reações do tipo I envolvem a formação de EROs através da transferência de elétron entre o fotossensibilizador no estado tripleto excitado, gerando ânion superóxido. Na reação do tipo II, o fotossensibilizador no estado tripleto reage com o oxigênio e produz oxigênio singleto que pode interagir com uma variedade de substratos biológicos como resultado da sua reatividade química elevada, induzindo danos oxidativos e danificando a parede e a membrana celular (PERUSSI, 2007).

Os micro-organismos para os quais o oxigênio singleto é letal incluem vírus, bactérias, protozoários e fungos. Os locais de lesão celular inicial da TFDa está intimamente relacionado com a localização do fotossensibilizador. Assim, a reação realiza-se em espaço limitado, levando à uma resposta localizada e tornando-a ideal para aplicação em sítios localizados sem afetar órgãos (MOAN; BERG 1991). O efeito antimicrobiano da TFDa não ocorre na ausência da luz, do oxigênio e do fotossensibilizador (MAROTTI et al., 2009).

1.4. A TERAPIA FOTODINÂMICA ANTIMICROBIANA E O ESTRESSE OXIDATIVO

O desequilíbrio entre a produção de EROs e sua desintoxicação pelo sistema de defesa antioxidante pode conduzir ao estresse oxidativo (EBADI; SRINIVASAN; BAXI, 1996). Além disso, sabe-se que o estado oxidativo do tecido é controlado por uma interacção complexa entre a pró- oxidante e mediadores antioxidantes (GONÇALVES et al., 2013). Quando o equilíbrio oxidante-antioxidante é perturbado em favor de EROs , o estresse oxidativo ocorre, o que culmina em dano tecidual (CHAPPLE, 1997; PISOSCHI; POP, 2015).

Para combater os efeitos do estresse oxidativo, as células possuem o sistema de defesa antioxidante, que compreende antioxidantes enzimáticos e não enzimáticos (BRIGANTI; PICARDO, 2003). Entre os antioxidantes enzimáticos,a glutationa peroxidase (GPx),a catalase (CAT) e a superóxido dismutase (SOD) desempenham um papel central. Os anti-oxidantes não enzimáticos presentes nas células são o α-tocoferol,a ubiquinona,o β-caroteno, o ácido ascórbico e a glutationa reduzida (GSH) (BRIGANTI; PICARDO, 2003).

1.5. A TOXICIDADE DA TERAPIA FOTODINÂMICA ANTIMICROBIANA

Pelo fato da TFDa estar baseada na produção de EROs, necessita cautela em seu uso, pois apesar de inúmeros estudos comprovarem sua efetividade na eliminação de patógenos, ainda são escassos os estudos a respeito da segurança desta terapia. A TFD pode provocar uma série de reações adversas intrínsecas, como causada pela toxicidade de algum fotossensibilizador ou apresentar toxicidade causada pelo protoloco de iluminação ou ainda pela combinação de droga e luz (CHEVALIER et al., 2013).

Alguns estudos documentaram danos causados pela TFDa como fotosensibilidade (JOSEFSEN; BOYLE, 2012) gerando desconforto, eritema, bolhas, despigmentação e descamação à nível clínico (ALEXIADES-ARMENAKAS, 2006). Outros que demonstram o efeito tóxico da TFDa através da peroxidação lipídica (DAICOVICIU et al., 2008; FILIP et al., 2008) que leva à perda da integridade da membrana celular e, consequente aumento do fluxo iônico (GIROTTI, 2001), podendo ocasionar necrose ou apoptose nos tecidos circundantes e lesões macroscópicas (CASTANO; DEMIDOVA; HAMBLIN, 2004; SHARMAN; ALLEN; VAN LIER, 1999).

Existe um estudo em que foi testada a segurança da TFD em ratos, 24 horas após a iluminação os animais apresentaram resposta inflamatória significativa na perna irradiada, com sinais clínicos de edema e necrose podendo ser vistos mesmo após 72h da utilização da terapia (ROCHA et al., 2015). Portanto a segurança no uso da TFDa ainda não pode ser confirmada devido à falta de evidências adequadas, bem como em função da falta de uma padronização de dosimetria, pois os estudos existentes utilizaram diferentes condições de tratamento (FONTANA et al., 2013), o que dificulta as comparações e tornando os resultados existentes conflitantes (TAO et al., 2014).

1.6. A TERAPIA FOTODINÂMICA ANTIMICROBIANA NA ODONTOLOGIA

A TFDa tem mostrado resultados promissores em diversos estudos nas variadas áreas da Odontologia, como na Periodontia, Endodontia e Estomatologia. Na Endodontia os micro-organismos desempenham um importante papel nas infecções endodônticas, nas quais os metabólitos tóxicos são responsáveis pelo desenvolvimento e pela persistência de lesões apicais. Um dos objetivos do tratamento endodôntico é a máxima desinfecção do sistema de canais radiculares, bem como a prevenção da sua reinfecção. Comumente, para cumprir esse objetivo, utiliza-se a terapia convencional, que consiste na limpeza e modelagem do sistema de canais radiculares por meio de limas manuais ou rotatórias, concomitantemente com a irrigação de substâncias químicas auxiliares e, em alguns casos, complementa-se com uma medicação intracanal. Porém, a eliminação dos micro-organismos patogênicos nem sempre é atingida na prática clínica, gerando o insucesso do tratamento endodôntico. Diante disso, a TFDa surge como um novo método de desinfecção com significativa redução da carga microbiana (SOUKOS, 2006; STUART et al., 2006; FOSCHI et al., 2007).

O efeito antimicrobiano da TFDa foi testado em associação com o tratamento endodôntico convencional. As amostras microbiológicas foram realizadas após acessar o canal radicular, a realização da endodontia e a TFDa. Ao final da primeira sessão, o canal radicular foi preenchido com hidróxido de cálcio e, depois de uma semana, a TFDa foi realizada. Os resultados sugeriram que a TFDa associada ao tratamento endodôntico reforça a diminuição da carga bacteriana (GARCEZ et al., 2008). O mesmo resultado foi encontrado ao utilizar essa associação depois de desenvolvida a resistência antimicrobiana por uso de antibióticos. Nesse estudo, todos os pacientes tiveram pelo menos um micro-organismo resistente ao antibiótico. A terapia endodôntica isolada produziu uma redução significativa no número de espécies microbianas, enquanto a combinação do tratamento endodôntico com a TFDa eliminou todas as espécies, podendo ser uma abordagem adequada para o tratamento de infecções orais (GARCEZ et al., 2010).

A Periodontia utiliza a raspagem e o alisamento radiculas como padrão ouro no tratamento das doenças periodontais mais severas, porém há inúmeros estudos que buscam novas abordagens para o tratamento periodontal. O objetivo do tratamento é restaurar a compatibilidade biológica de superfícies periodontais (SAXENA; PANT; GOVILA, 2015). Nesse contexto a TFDa surge como uma abordagem não-invasiva para o controle da infecção e tem sido usada como coadjuvante à terapia mecânica. Esta nova abordagem terapêutica parece promissora principalmente no tratamento não cirúrgico da periodontite agressiva (SOUKOS, 2003; DE OLIVEIRA, 2007).

Na área da estomatologia, a candidíase é considerada a doença universal dentre as infecções oportunistas e seu tratamento convencional consiste na utilização de antifúngicos. A TFDa, neste contexto, emerge como uma proposta de tratamento substitutivo ao convencional e alguns estudos mostraram a efetividade desta terapia na inativação de *C. albicans* (MARTINS et al., 2011).

Com base na revisão de literatura exposta, fica evidente que a TFDa é uma terapia promissora na área da Odontologia e, assim, é de suma importância que mais estudos sejam realizados avaliando seus riscos, benefícios e segurança de uso, estabelecendo técnicas e protocolos de uso para as mais variadas aplicações. Neste sentido, o presente estudo objetivou investigar os efeitos da TFDa aplicada em diferentes doses na língua saudável de ratos sobre parâmetros de estresse oxidativo e inflamatório.

2. MANUSCRITO CIENTÍFICO

Os resultados obtidos para este trabalho de conclusão de curso apresentam-se sob a forma de um manuscrito científico, o qual se encontra aqui estruturado, a fim de facilitar o entendimento do assunto. Os itens Materiais e Métodos, Resultados, Discussão e Referências, encontram-se inclusos no manuscrito, o qual se encontra em fase final de redação. O manuscrito será submetido para publicação no periódico Laser in Medical Science.

Antimicrobial photodynamic therapy dose-dependent effects on morphology and oxidative response in the tongue of rats

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Abstract

Antimicrobial photodynamic therapy (aPDT) is a local treatment for oral infection, simple and inexpensive with little risk of microbial resistance, which can be employed alone or in association with traditional methods of oral care. However, literature lacks studies about the safety assessment of oral aPDT. In this sense, this study aimed to investigate the aPDT dose-dependent response on healthy tongue of rats. Forty rats were randomly distributed into eight experimental groups according to the aPDT dose and control group (no aPDT exposed). Six hours after the aPDT, the tongue of animals was removed for biochemical and histological analysis. Reactive oxygen species and lipid peroxidation levels showed no differences between groups. Besides that, aPDT was able to increase catalase (CAT) activity in all groups and L400 group showed the highest CAT activity compared to other groups. The L18 and L24 groups showed the lowest reduced glutathione levels compared to other groups, whose levels were similar. After aPDT, L30, L70, L122, L275 and L400 groups showed an increase in the inflammatory infiltrate in a dose-dependent manner compared to other groups, whose amount were similar. These findings suggest that there is a window of aPDT doses with methylene blue as a photosensitizer that can be employed safely without cause damages to the healthy tongue.

Keywords: Inflammatory infiltrate; Oxidative stress; Tissue toxicity; Laser

Introduction

Reactive oxygen species (ROS), especially the singlet oxygen, have been a focus of intense investigation due to their important role in biological processes [1]. ROS can effectively oxidize lipids and proteins of cells leading to cell damage, gene regulation and even cell death [2]. The imbalance between the ROS production and their detoxification by the antioxidant defense system can lead to oxidative stress [3]. To counteract ROS harmful effects, cells have the antioxidant defense system, which comprises enzymatic and non-enzymatic antioxidants [4]. Among the enzymatic antioxidants, glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) plays a central role. The non-enzymatic antioxidants present in cells comprises α-tocopherol, ubiquinone, β-carotene, ascorbic acid and reduced glutathione (GSH) [4]. However, ROS plays a dual role, its release is the photodynamic therapy mechanism of action, which associates the visible light (commonly a diode laser) and a photosensitizer [5]. Due the selective destruction of the bacteria and their by-product by this therapy [6], it is called antimicrobial photodynamic therapy (aPDT) [7]. Recently, aPDT has received increased attention in dental clinic and in research field, since it is a form of non-invasive treatment and shows inability to develop bacterial resistance. Besides that, the aPDT is specific to the target cells and its activity is initiated only when exposed to the light [8].

In aPDT, a photosensitive agent is activated by a light source at an appropriate wavelength in the presence of oxygen, undergoing transition from low-energy-level ground state to a higher-energy triple state. In the tripled state, the photosensitizer molecules can pass their electron or energy to surrounding biomolecules via two mechanisms. The type I mechanism operates with a local substrate producing cytotoxic radicals and the type II mechanism works with molecular oxygen [9-10]. The ROS released by aPDT can induce cellular components oxidation, such as macromolecules, lipids, proteins and DNA. This process can result in membrane transport system inactivation, inhibition of plasma membrane enzyme activities, lipid peroxidation, and cell death [11].

The aPDT can be employed as a local treatment for oral infection (periodontal diseases, endodontic therapy, dental caries, peri-implantitis), which can be employed by itself or as an adjuvant tool to traditional methods. Moreover, aPDT protocol can be related to advantages, such as, low-cost, simple and nontoxic procedure, besides being promptly

reproducible [12]. Nevertheless, the assessment of oral aPDT safety is still scarce in the literature. Unfortunately, there are numerous well-documented problems about ROS generation that may lead to the loss of cell membrane potential and integrity, leading to an increased ionic influx that destroys the cellular organelles initiating apoptosis [13-14]. In this context, the purpose of this study was to investigate the dose-dependent response of aPDT employing methylene blue as photosensitizer on the healthy tongue of rats.

Materials and methods

Animals

Forty Male Wistar rats (3 months of age) were used. Groups of four animals, from the breeding facility of Universidade Federal de Santa Maria (UFSM, RS, Brazil) were kept in Plexiglas cages with free access to food (Supralab®, Alisul Alimentos LTDA, São Leopoldo, RS, Brazil) and water in a room with controlled temperature (23±1°C) and 12h light/dark cycle. This study was approved by the Animal Ethical Committee of Universidade Federal de Santa Maria (027/2013-UFSM), affiliated to the Council for the Control of Animal Experiments (CONCEA), following international norms of animal care and maintenance.

Experimental Procedure

After one week of acclimatization, the rats were randomly allocated into eight experimental groups (n=5) according to the oral treatment, designated as control (C; saline solution), L18 group, L24 group, L30 group, L70 group, L122 group, L275 group, and L400 group, according to the Figure 1. The aPDT employed methylene blue (MB) (Vetec Química Final®, Rio de Janeiro, Brazil) 0.01% dissolved in bidistilled water, it has been widely used as a photosensitizer [15-16], which produce a limited singlet oxygen amount associated with a short half-life (4μs) [16] , low potential of diffusion [17], and thus it was chosen as photosensitizer in this study.

Under anesthesia (ketamine/xylazine, 60 and 15 mg/kg, intramuscular, respectively), MB solution was gently poured on the tongue using a syringe (1mL) and a needle (BD® UltrafineTM, U-100, 0.5mL, 8mm x 0.3mm). After 1 minute, low-level laser therapy was applied once on the tongue. The laser employed was an indium-gallium-aluminum-phosphorous (TheraLase®, DMC Equipment, São Carlos, SP, Brazil), wavelength 660nm, continuous emission mode, power output of 30mW transmitter, with spot size of 0.0283cm^2. One operator blinded to the experimental groups performed the aPDT procedures. The laser

light was applied during 16s in the L18 group (energy density of 18J/cm^2), 22s in the L24 group (energy density of 24J/cm^2), 28s in the L30 group (energy density of 30J/cm^2), 65s in the L70 group (energy density of 70J/cm^2), 114s in the L122 group (energy density of 122J/cm^2), 259s in the L275 group (energy density of 275J/cm^2), and 376s in the L400 group (energy density of 400J/cm^2). The aPDT doses used in this study were based on previous studies from other research group with energy fluence ranging from 18J/cm^2 to 400J/cm^2 [18]. The device probe was positioned about 3mm from the tongue surface.

Preparation of tongue samples

After six hours aPDT protocols, animals were euthanized under anesthesia (ketamine/xylazine, 60 and 15 mg/kg, intramuscular, respectively) and the total tongue was removed from each rat for biochemical and histological analysis (Fig.1). For biochemical assays, half of each tongue was homogenized with Tris-HCl buffer (10mM; pH 7.4) followed by centrifugation at 3640 g for 15 min. and the supernatants were used.

Biochemical analysis

ROS levels were quantified using the oxidant sensing fluorescent probe, 2,7-dichlorofluorescein diacetate (DCHF-DA, Sigma Aldrich®, São Paulo (SP), Brazil) [19]. The oxidation of the DCHF-DA to dichlorofluorescein (DCF) was determined at 488nm for excitation and 525nm for emission. Tongue samples were incubated for 1h until fluorescence measurement. DCF-ROS levels were corrected by protein content [20] and expressed as percentage of control group.

Lipid peroxidation was estimated through the pink chromogen produced by the reaction of thiobarbituric acid with malondialdehyde (MDA) formed during the lipid oxidation from tongue homogenates, and measured spectrophotometrically at 535nm [21]. Results were expressed as nmol MDA/g tissue.

Protein carbonyl (PC) levels were measured according to Yan, Traber and Packer [22]. Tongue samples were mixed with 2,4-dinitrophenylhydrazine (10mM DNPH, Sigma Aldrich®, São Paulo-Brazil) for 1h. Denaturing buffer (150mM sodium phosphate buffer, pH6.8, 3% SDS), heptane (99.5%) and ethanol (99.8%) (all reagents from Vetec Química Final®, Rio de Janeiro-Brazil) were added sequentially. Isolated protein was washed twice with ethyl acetate/ethanol (Vetec Química Final®, Rio de Janeiro-Brazil) 1:1 (v/v) and

suspended in buffer. Each sample was measured at 370nm against the corresponding HCl sample (blank). Total carbonylation of proteins was calculated according to Levine et al. [23].

Estimation of tongue antioxidant defenses

Catalase (CAT) activity was spectrophotometrically quantified according to Aebi, 1984 [24], monitoring the disappearance of hydrogen peroxide (H_2O_2) (Proquímios®, Rio de Janeiro, Brazil) in the homogenates at 240nm. The enzymatic activity was expressed in K/mg protein/min. Protein content was determined by the method of Lowry, Rosebrough,and Randall (1951) [20].

Reduced glutathione (GSH) levels were determined after reaction of the homogenates with 5,50-dithiobis-(2-nitrobenzoic acid) (DTNB, Proquímios®, Rio de Janeiro (RJ), Brazil). The yellow chromogen formed was read at 412nm, according to Boyne and Ellman [25]. A standard curve using GSH was plotted in order to calculate the GSH content in each sample, expressed as μmol GSH/g tissue.

Histological analysis

For histological analysis, tongue samples were fixed in 10% neutral buffered formalin for 24 hours and them embedded into paraffin. The specimens were sectioned (5μm), deparaffinized and stained with hematoxylin and eosin (H&E) for light microscopic evaluation (Binocular Optical Microscope ZEISS, Axio Lab.A1, Germany) five images (100x) were taken per section and the images were transferred to a monitor with a camera apparatus (AxioCam, ERc 5S, Germany). The interexaminer and intraexaminer reproducibility revealed a high correlation (Kappa>0.85).

Analysis of tongue inflammatory infiltrate

Tongue sections were stained with H&E to measure the inflammatory infiltrate through scores defined according to the density and arrangement of inflammatory cells. This event was assessed by a scoring system (from 0 to 3) according to Oliver, Holm-Pedersen and Löe (1969) [26] In tongue, the inflammatory infiltrate was graded as absent (0), mild (1), moderate (2) and/or severe (3) in three fields of view (100x). The scores will be considered absent (0) when it is characterized by absence of inflammatory cells in the tissue; mild (1) when inflammatory cells are present and sparse distributed in the tissue; moderate (2) when is found dense accumulation of inflammatory cells in isolated areas and sparse distributed in

other areas of connective tissue; and severe (3) when it is present a dense aggregation of inflammatory cells throughout the tissue.

Statistical analysis

Levene's test was applied in order to verify the homogeneity of the data. Biochemical and histological assessments were analyzed by one-way ANOVA followed by Duncan's multiple range test when appropriate (Software package Statistica 8.0 for Windows was used). All data are expressed as mean±standart error media (SEM). A value of $P<0.05$ was considered statistically significant for all comparisons made.

Results

Oxidative damage measured by ROS generation, lipid peroxidation and protein carbonyl levels in rat tongue as shown in Fig. 2:

One-way ANOVA showed no effect of aPDT in different doses on ROS generation (Fig 2a).

Duncan's test showed that L400 group increased lipid peroxidation and PC levels in relation to control group and to the other aPDT doses, whose levels were similar (Fig. 2b and 2c).

Antioxidant defenses determined by CAT activity and GSH levels in rat tongue as shown in Fig 3:

One-way ANOVA of CAT activity revealed a significant influence of aPDT [$F(7,40)=20.47$, $P<0.0000$]. Post-hoc test showed higher CAT activity in all groups after aPDT when compared to control group (Fig 3a). The L400 group showed a greater increase in CAT activity compared to the other experimental groups.

One-way ANOVA of GSH levels revealed a significant influence of aPDT [$F(7,40)=7.26$, $P<0.0000$]. Post-hoc test showed lowest GSH levels in L18 and L24 groups when compared to the other groups, whose values were similar (Fig 3b).

Effects of aPDT on histopathological evaluation in rat tongue mucosa as shown in Figure 4 and Table 1:

One-way ANOVA showed an effect of aPDT in different doses on inflammation degree [$F(7,40)=6.19$, $P<0.0000$]. Post-hoc test showed an increase in inflammation degree

from L30 to L400 groups, whose amounts were similar to each other and greater than control group (Table 1).

Discussion

Several studies have been shown the therapeutic action of aPDT in the field of dentistry, for malignancies [27], non-oncological diseases as halitosis [28] and candidiasis [29] in the tongue. The aPDT is based on the selective killing of target cells by light, which is activated by a photosensitizer initiating the photochemical generation of singlet oxygen and other cytotoxic ROS. These species induce irreversible bimolecular oxidative alterations damaging cellular sites and leading to the direct destruction of target cells [30]. The increased production of ROS can culminate in oxidative stress, which is frequently defined as an increased production and/or a decreased scavenging of them [31]. High ROS levels are also related to the loss of integrity and potential of cell membranes, causing an ionic imbalance, which initiates cellular apoptosis or necrosis [13-14]. The responses of aPDT applied to the healthy tongue of rats are still scarce in the literature.

In this sense, the aim of this study was to investigate the aPDT dose-dependent response employing MB as photosensitizer on healthy tongue of rats. To the best of our knowledge, these findings are the first report about the aPDT dose-dependent toxicity potential on the tongue. Here, the toxicity was estimated by oxidative stress parameters and histological analysis in the tongue of rats.

The aPDT mechanism of action is ROS release [5]. Interestingly, we did not observe an increase in ROS generation in the tongue 6h after aPDT in all studied doses compared to control group. According to the outcomes of the present investigation, we can affirm that aPDT induced an increase in tongue lipid peroxidation levels only at the highest dose tested, i.e. L400 group, observed by MDA levels, as well as induced an increase in protein carbonyls levels. The two main constituents of cell membranes are lipids and proteins, thus they may be damaged by ROS [32], although this was not observed in this study. Most of the ROS have a very short half-life [33], and thus we can infer that 6h after aPDT may have been sufficient time for endogenous antioxidant scavengers neutralized them. In this way, the photosensitizers selectivity to the tissue and the short half-life of ROS aPDT-induced ensure that the phototoxic damage can be restricted to target cells (lesion), thereby saving the healthy tissue [34], which is in accordance with our results.

Normally, cellular oxidative injuries are prevented by endogenous antioxidant defense system [35]. In the present study, the aPDT was related to alterations in CAT activity and GSH levels in the tongue of rats. Thereby, the aPDT until the dose of 300J/cm^2 showed similar amount of ROS, lipid peroxidation and protein carbonyls when compared to control group, reflecting no induction of oxidative stress in the rat tongue. In addition, we found an increased CAT activity in all groups after aPDT procedure, which demonstrates the responsiveness of the antioxidant defense system forward this therapy in the tongue of rats. Moreover, the consumption of non-enzymatic antioxidant observed in L18 and L24 groups, as represent by GSH levels, targeted the homeostasis of the tongue. This outcome is consistent with the results about ROS, lipid peroxidation and protein carbonyls levels observed in these groups. In other words, these findings may have occurred at the expense of tongue GSH consumption, which can be avoiding oxidative damage on cellular lipids and proteins, since GSH is one of the main endogenous antioxidants [36].

Our findings demonstrated that from the dose of the 30J/cm^2, aPDT induced an inflammatory infiltrate in the tongue of rats as compared to the control, L18 and L24 groups. Inflammation is an essential immune response characterized by swelling, redness, pain, heat, and impaired function. An acute inflammatory response controlled is necessary to fight off stimuli and insults noxious and overcome injury [37], which persists for only a short period of time and it is usually beneficial to the host [38]. Across studies, the adverse events from aPDT appear to be transient and mild, which include local inflammatory reactions, hyperemia, stinging sensations or mild burning, pruritus, sloughing, scarring, local necrosis, erythema and hyperpigmentation, and are tolerated by the most of patients [39]. Since we did not observe oxidative damage to lipids and proteins, we can hypothesize that the acute inflammation was follow by the end of inflammatory response. Thus, the process resulted in a transition to the homeostatic state, i.e., inflammation resolution [40].

According to our findings, the antioxidant enzymes can attenuate ROS, leading to inflammation resolution. Several factors can influence the photodamage aPDT-induced such as the type, dose, application site and incubation time of the photosensitizer. In addition, the wavelength of light, the light power density, the light energy fluence and the availability of oxygen can influence as well [12]. We tested the aPDT dose-dependent response *in vivo* for the first time, in order to evaluate its effects on oxidative stress parameters, as well as on histological analysis in the healthy tongue of rats. In general, no cytotoxic effects were observed on the tongue, suggesting that aPDT may be employed since it displays a safe range of doses, which is in accordance with Fontana, Lerman, Grecco, de Souza Costa, Amiji,

Bagnato, and Soukos [12]. Thus, the findings demonstrated that aPDT appears to be a safe and well-tolerated treatment.

This hypothesis is supported by our results, which were obtained after 6h from the different doses of aPDT exposure on the healthy tongue: (i) ROS levels were not increased; (ii) overall, no damages to lipids and proteins were observed; and (iii) the responsiveness of antioxidant defense system was increased, i.e., CAT activity. Taken together these outcomes, we can infer that aPDT is safe as treatment to several diseases, demonstrated by the absence of oxidative damage and recovery of antioxidant system on the healthy tongue of rats.

In summary, no oxidative insult to lipids and proteins were observed in the healthy tongue of rats submitted to multiple doses of aPDT, indicating that this treatment when used acutely can be exciting antioxidant defense mechanisms, thus avoiding the oxidative damage induced by ROS from aPDT. Based on preliminary findings of this study, additional research are necessary to confirm the relationships described here, in order to improve knowledge about the aPDT safety, especially after its use after repeated doses.

Compliance with Ethical Standards

Funding

The work was not supported by any funding.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

Animal care and study protocols were approved by the Animal Ethical Committee (027/2013), affiliated to the Council for the Control of Animal Experiments, following international norms of animal care and maintenance. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

The manuscript does not contain clinical studies or patient data.

Informed Consent

For this type of study, formal consent is not required.

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Legends

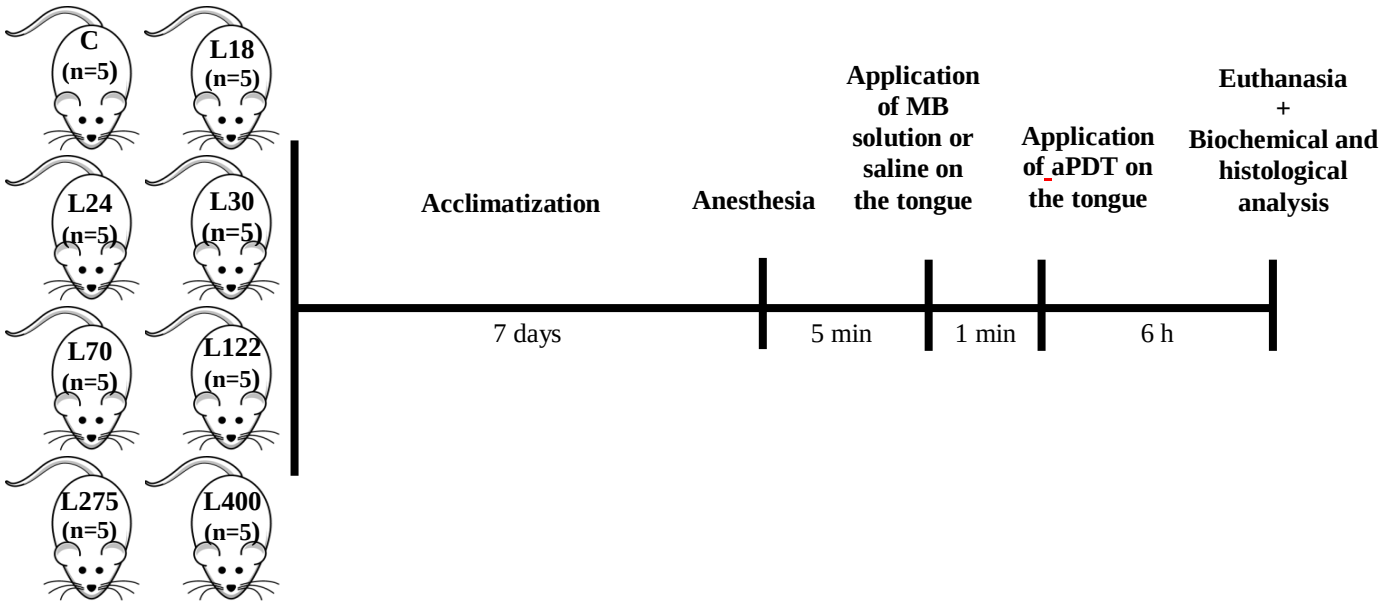
Fig 1 Experimental design

Fig 2 ROS generation, lipid peroxidation and protein carbonyls levels in healthy tongue of rats (a, b and c, respectively) after different doses of the aPDT employing MB as photosensitizer. Data are expressed as mean±S.E.M (n=5). Different lowercases letters (a-c) indicate significant difference among experimental groups ($P<0.05$). *Indicates significant difference of control group ($P<0.05$).

Fig 3 Influence of aPDT in different doses on catalase (CAT) activity (a) and reduced glutathione (GSH) levels (b) in healthy tongue of rats. Data are expressed as mean±S.E.M (n=5). Different lowercases letters (a-c) indicate significant difference among experimental groups ($P<0.05$). *Indicates significant difference of control group ($P<0.05$).

Fig 4 Sagittal sections of the rat tongue. No inflammatory infiltrate was observed in the control (no aPDT) (a), L18 (b) and L24 (c) groups. After 6h from aPDT exposure, L30 (d) L70 (e), L122 (g), L275 (g) and L400 (h) groups showed inflammatory cells in the connective tissue underneath the intact epithelium. Sections of tongue specimens (5μm) stained with hematoxylin and eosin, x100, bar: 100μm.

Figure 1


C
(n=5)
L18
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L122
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L275
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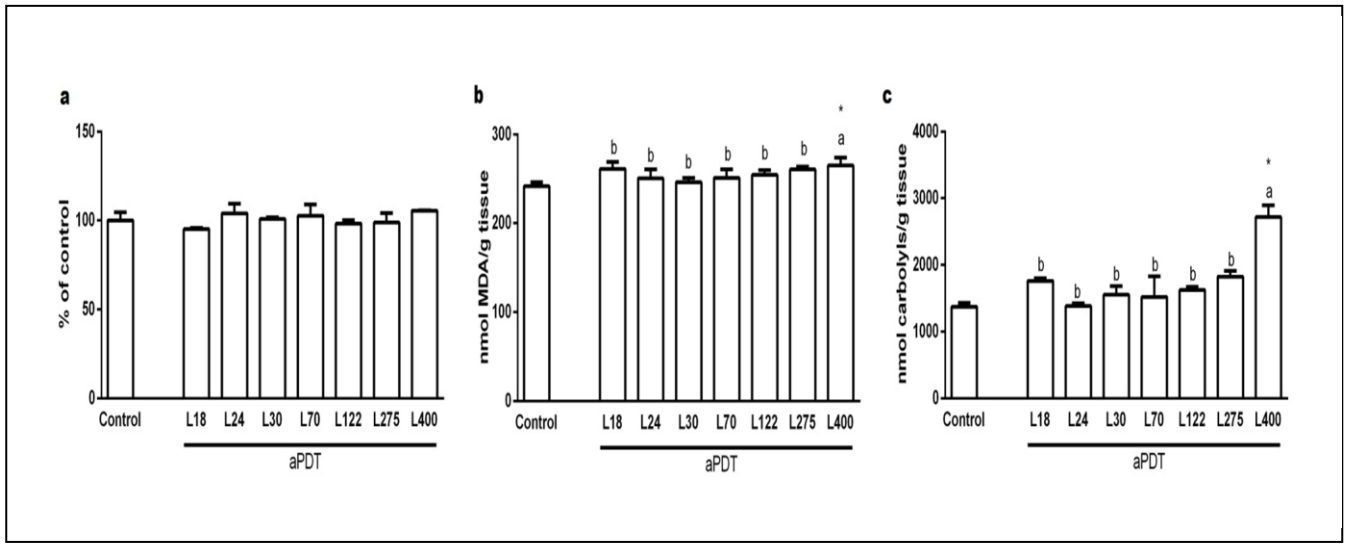
L400
(n=5)
Acclimatization
Anesthesia
Application of MB solution or saline on the tongue
Application of aPDT on the tongue
Euthanasia + Biochemical and histological analysis
7 days
5 min
1 min
6 h

Figure 2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*
c
nmol carbolyls/g tissue
4000
3000
2000
1000
0

Figure 3

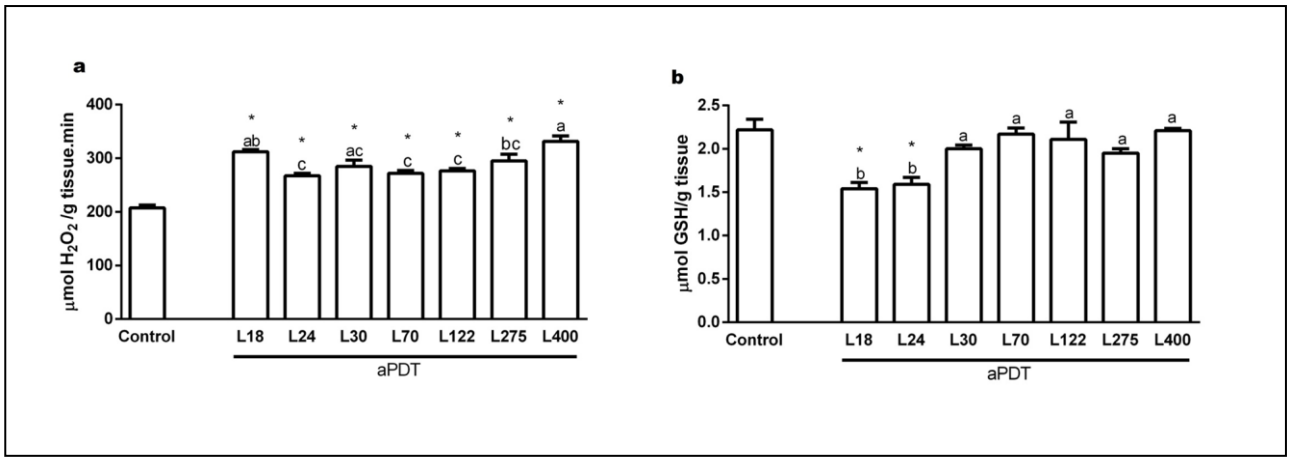
a
μmol H_2O_2 /g tissue.min
400
300
200
100
0
ab
c
ac
c
c
bc
a
Control
L18
L24
L30
L70
L122
L275
L400
aPDT
b
μmol GSH/g tissue
2.5
2.0
1.5
1.0
0.5
0.0
b
b
a
a
a
a
a
Control
L18
L24
L30
L70
L122
L275
L400
aPDT

Figure 4

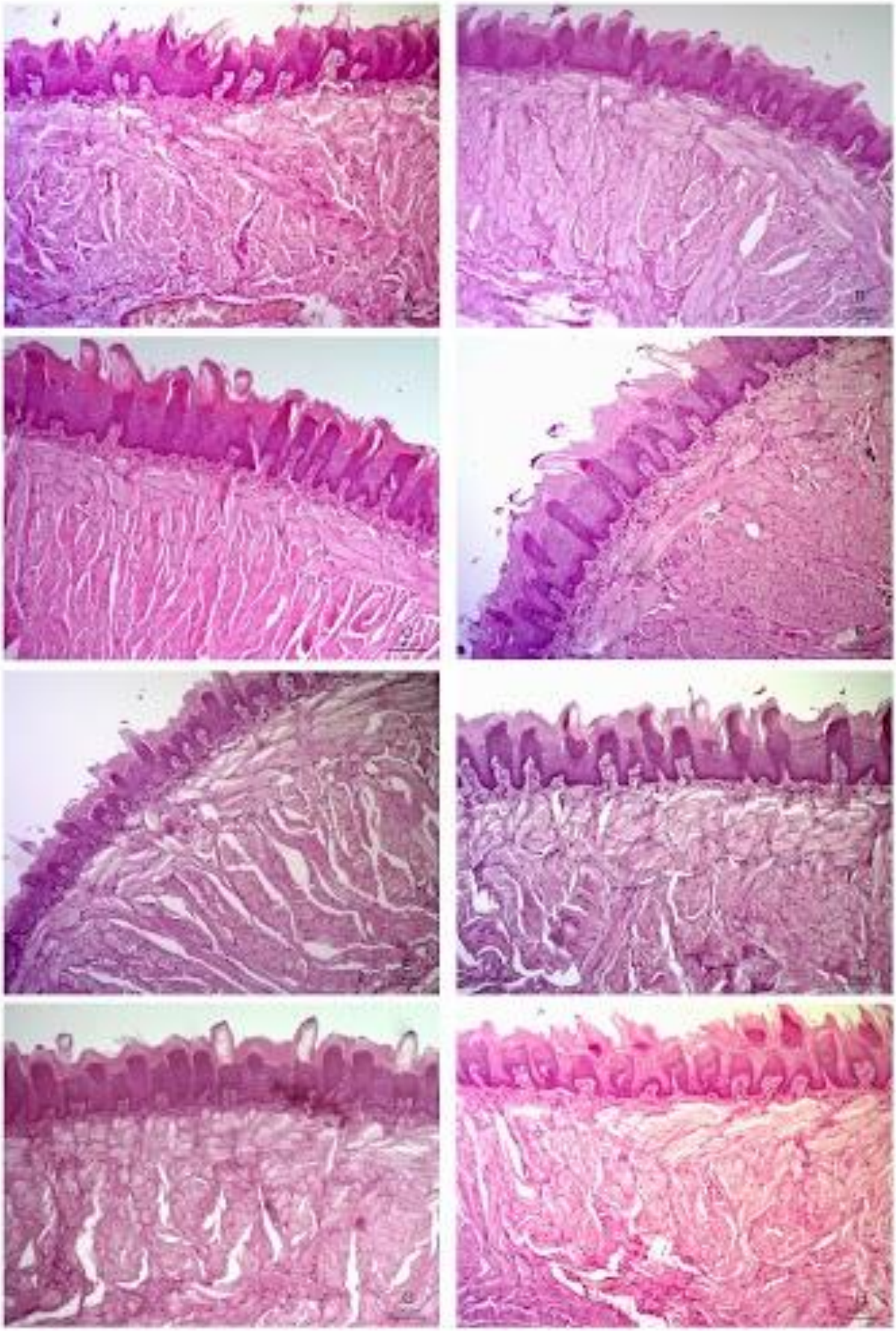

Table 1 Dose-dependent effects of antimicrobial photodynamic therapy (aPDT) on histopathological evaluation on the rat healthy tongue mucosa.

Group	Inflammation scores
Control	0.80±0.02
L18	0.87 ± 0.05^{b}
L24	0.79 ± 0.06^{b}
L30	$1.00\pm0.01^{a*}$
L70	$0.98\pm0.01^{a*}$
L122	$1.00\pm0.01^{a*}$
L275	$1.00\pm0.01^{a*}$
L400	$0.95\pm0.03^{a*}$

Data are expressed as mean±SEM. Different lowercase letters (a-c) indicate significant differences among experimental groups ($P<0.05$). *Indicates significant difference of control group ($P<0.05$).

3. CONSIDERAÇÕES FINAIS

Com base nos resultados deste estudo, podemos concluir que a TFDa é um tratamento seguro e bem tolerado quando utilizado nas doses testadas e de forma aguda, pois ativou mecanismos de defesa antioxidante e estes puderam evitar que ocorresse um dano oxidativo induzido pelas EROs provenientes da utilização da terapia. Porém, este estudo ainda é um passo inicial, para a confirmação da total segurança desta terapia, necessitando estudos adicionais sobre a TFDa principalmente com relação a seu uso com doses repetidas, com diferentes fotossensibilizantes, além de outros protocolos diferentes dos testados neste estudo.

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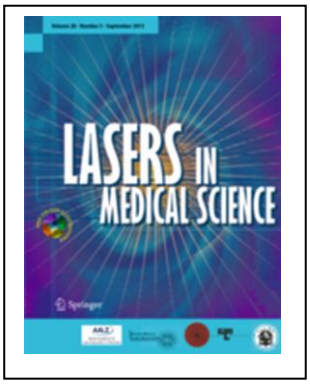

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Please provide 4 to 6 keywords which can be used for indexing purposes.

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- LaTeX macro package (zip, 182 kB)

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Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

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Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should be given at first mention.

Units and abbreviations

- Please adhere to internationally agreed standards such as those adopted by the commission of the International Union of Pure and Applied Physics (IUPAP) or defined by the International Organization of Standardization (ISO). Metric SI units should be used throughout except where non-SI units are more common [e.g. litre (l) for volume].
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- When drugs are mentioned, the international (generic) name should be used. The proprietary name, chemical composition, and manufacturer should be stated in full in Materials and methods.

References

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

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2. This result was later contradicted by Becker and Seligman [5].

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

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

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- Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

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Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

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- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

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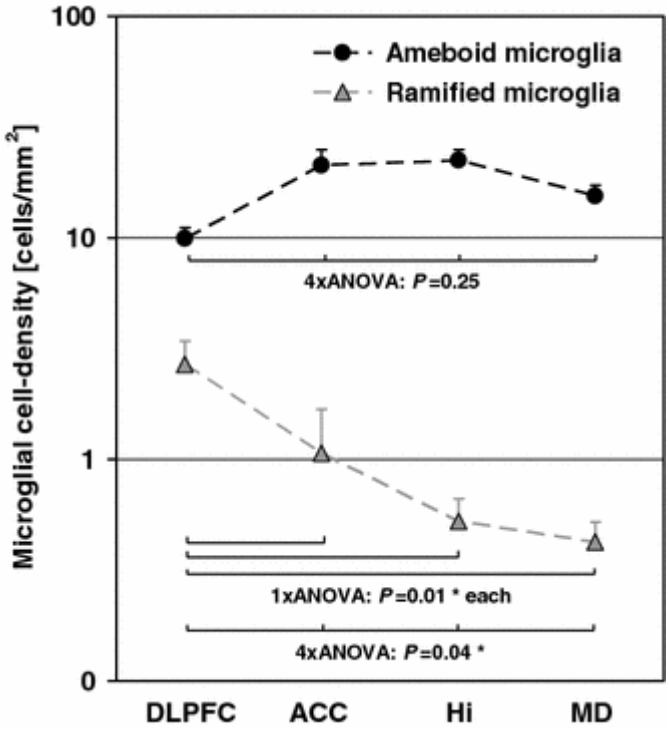

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- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.

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- Vector graphics containing fonts must have the fonts embedded in the files.

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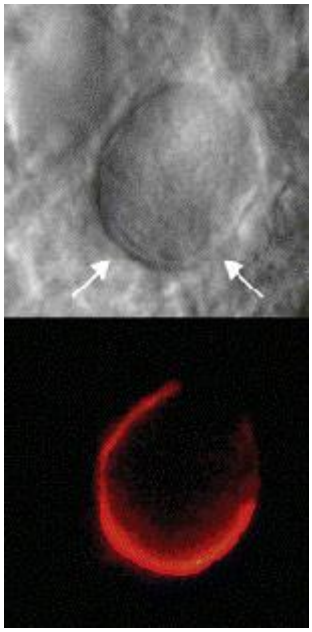

- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.

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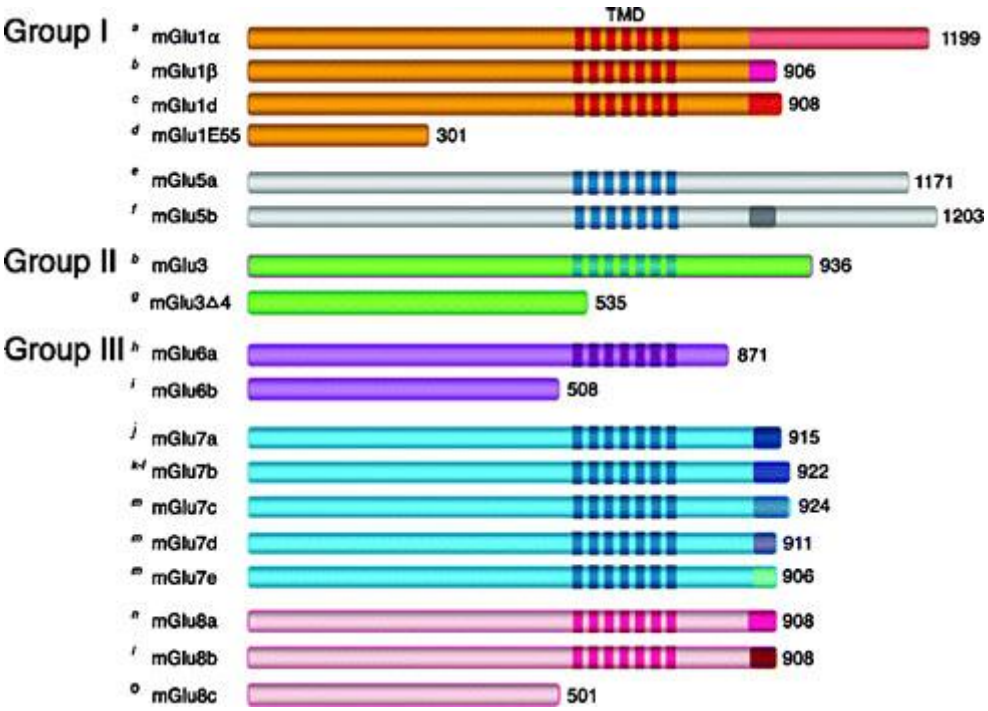

- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
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- Color art is free of charge for online publication.
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UNIVERSIDADE 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

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