Antimicrobial Photodynamic Therapy with Photosensitizer Improved the Gingival Collagen, Oxidative Status and Protect the Bone in Short Term Experimental Periodontitis

Victor de Mello Palma[1,*], Raquel Cristine Silva Barcelos[2,4], Marcela Mozzaquatro Pasini[1], Fernanda Maia Pillusky[1], Luisa Machado Barin[1], Luciana Thaschetto Vey[3], Roberto Marinho Maciel[1], Karla Zanini Kantorski[1], Marilise Escobar Bürger[2,3], Kívia Linhares Ferrazzo[4] and Cristiane Cademartori Danesi[1,4]

[1]*Programa de Pós-Graduação em Ciências Odontológicas, Rua Marechal Floriano Peixoto, 1184, Centro, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil*

[2]*Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria, Av. Roraima, 1000, Cidade Universitária, Camobi, Santa Maria, RS, Brazil*

[3]*Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria, Av. Roraima, 1000, Cidade Universitária, Camobi, Santa Maria, RS, Brazil*

[4]*Departamento de Patologia, Universidade Federal de Santa Maria, Av. Roraima, 1000, Cidade Universitária, Camobi, Santa Maria, RS, Brazil*

Abstract: *Objectives:* To evaluate the influence of antimicrobial photodynamic therapy (aPDT) with methylene blue (MB) dissolved in ethanol used as an adjunct to scaling and root planing (SRP) in periodontitis treatment on bone loss, collagen fibers and gingival oxidative status.

Methods: Wistar rats were randomly randomized in five experimental groups according to the periodontal treatment: NC (negative control; no periodontitis); PC (positive control; periodontitis without any treatment); SRP (periodontitis and SRP), aPDT I (periodontitis and SRP+aPDT+MB solubilized in water), and aPDT II (periodontitis and SRP+aPDT+MB solubilized in ethanol 20%). To induce the experimental periodontitis, one mandibular right first molar of each animal received the cotton ligature in a submarginal position. The periodontal treatments were performed after 7 days of ligature removal.

Results: PC group had more bone loss compared to the other groups. SRP and aPDT I groups showed equivalent bone losses and the aPDT II group showed bone loss similar to the aPDT I group, smaller than the PC and SRP groups on the 7th day. PC and SRP groups showed higher gingival lipoperoxidation levels on the 7th day, but only the PC group exhibited higher gingival lipoperoxidation on the 15th day. Besides, it indicated an aPDT II protective action on the gingiva on the 7th day observed by the maintenance of the gingival glutathione (GSH) levels similar to NC group. The aPDT treatments were able to prevent gingival collagen degradation with 7 days of treatment.

Conclusions: aPDT can act as a beneficial adjuvant tool to minimize bone loss, collagen degradation and periodontitis-induced oxidative damages. Therefore, it is possible to suggest that the short-term aPDT causes beneficial responses accelerating periodontal healing.

Keywords: Periodontal disease, Gingiva, Oxidative stress, Root planning, Phototherapy.

INTRODUCTION

Periodontal disease, the most prevalent oral infection in humans, stands as a leading cause of tooth loss in adults (Hyvärinen *et al*., 2015). This condition not only poses a significant public health challenge but also detrimentally affects people's quality of life (Albandar *et al*., 1999; Susin *et al*., 2004; Al-Harthi *et al*., 2013). Periodontitis, resulting from the interplay between bacterial biofilm on dental surfaces and the host response, is characterized by immuno-inflammatory and oxidative reactions, leading to the loss of periodontal tissue support (Akalin *et al*., 2005; Akalin *et al*., 2007). Excessive production of reactive oxygen species (ROS) causes oxidative damage, while intensified inflammation contributes to the degradation of the connective tissue collagen matrix (Akalin *et al*., 2007).

Mechanical removal of biofilm and mineralized deposits adhered to dental surfaces by scaling and root planing (SRP) is the gold standard treatment for periodontitis (Cobb *et al*., 2002). However, the success of SRP may be compromised in diabetic patients, smokers, or those with specific dental features like root concavities (Tervonen & Oliver, 1993; Bergström *et al*.,

*Address correspondence to this author at the Dental Sciences Post Graduation Program, Federal University of Santa Maria (UFSM), 97105-900 Santa Maria (RS) Brazil; E-mail: victorpalmaod@hotmail.com

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2000; Wennström *et al*., 2011). Adjuvant therapies, such as antibiotics, are considered in these cases, but bacterial resistance is a growing concern (Slots, 2004; Arweiler *et al*., 2014; Yoshikawa, 2002).

As an alternative, antimicrobial photodynamic therapy (aPDT) has shown effectiveness in eliminating periodontal pathogens in biofilms. aPDT is non-invasive, has a broad spectrum, low risk of resistance development, and lacks a limited dose level (Fontana *et al*., 2009; Hamblin & Hasan, 2004; Maisch, 2007). The aPDT involves a local application of a photosensitive drug (photosensitizer) and its photo-activation by light with an appropriate wavelength. After the absorption of light energy, the photosensitizer (PS) is activated to an excited state, performing electronic transfers with neighboring molecules to form ROS, or energy transfers with molecular oxygen, generating singlet oxygen molecules (Soukos & Goodson, 2000).

These aPDT-induced ROS are toxic to microbial cells, damaging their cell membrane or DNA (Wainwright, 2000). There is a wide variety of dyes with photosensitizing properties used in aPDT, and methylene blue (MB) has been a popular choice for clinical trials, usually solubilized in water (Betsy *et al*., 2014). However, solubilization in solvents containing ethanol has been shown to improve photophysical and photochemical properties, resulting in an increased singlet oxygen half-life and better antimicrobial effect (George & Kishen, 2007).

Previous studies have indicated that aPDT, particularly when MB is dissolved in ethanol, can induce a systemic protective response against periodontitis-induced oxidative stress, recover gingival collagen, and promote greater tissue repair, besides inducing the recovery of systemic antioxidant defenses (Pillusky *et al*., 2017; Barin *et al*., 2017). The term hormesis, defined in the literature as stimulatory effects caused by low levels of potentially toxic agents (Stebbing, 1982), is relevant here. Hormesis is a physiological response that helps the organism react to the continuous presence of a small stimulus, such as low ROS levels, inducing an increase in antioxidant defenses and promoting compensatory processes following an initial disruption in homeostasis (hormesis hypothesis) (Calabrese *et al*., 2002). In this study, in addition to investigating the influence of this aPDT protocol on bone loss and collagen content, we evaluated the local gingival oxidative status of rats with periodontitis, unlike previous studies where systemic oxidative status was assessed.

MATERIALS AND METHODS

Animals

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

Experimental Periodontal Disease Protocol

The animals were randomly assigned in five experimental groups: NC (negative control; no periodontitis; n=11); PC (positive control; periodontitis without any treatment; n=11); SRP (periodontitis and scaling and root planning; n=16), aPDT I (periodontitis and SRP+aPDT+MB solubilized in water; n=16), and aPDT II (periodontitis and SRP+aPDT+MB solubilized in ethanol 20%; n=16). To induce the experimental periodontitis, under general anesthesia (ketamine/xylazine, 70 and 6 mg/kg, intramuscular injection, respectively), one mandibular right first molar of each animal received the cotton ligature in a submarginal position. The ligature was removed after 7 days of periodontal disease induction (Garcia *et al*., 2013) and periodontal treatments were carried out immediately after the ligature removal. On days 7 and 15 after the periodontal treatments, half of each group was anesthetized with isoflurane (2-3%) (Isothane®, Baxter Healthcare®, Guayama, Puerto Rico) (Taylor *et al*., 2013) and euthanized by exsanguination (Figure **1**).

Scaling and Root Planning Treatment

The SRP was carried out manually with mini five GRACEY Curettes (Hu-Friedy®, Chigaco, IL, USA) through 10 distal-mesial traction movements in the buccal and lingual aspects. The interproximal areas and furcation were instrumented with the same curettes using cervico-occlusal traction movements (Garcia *et al*., 2013). One blind operator performed all SRP procedures.

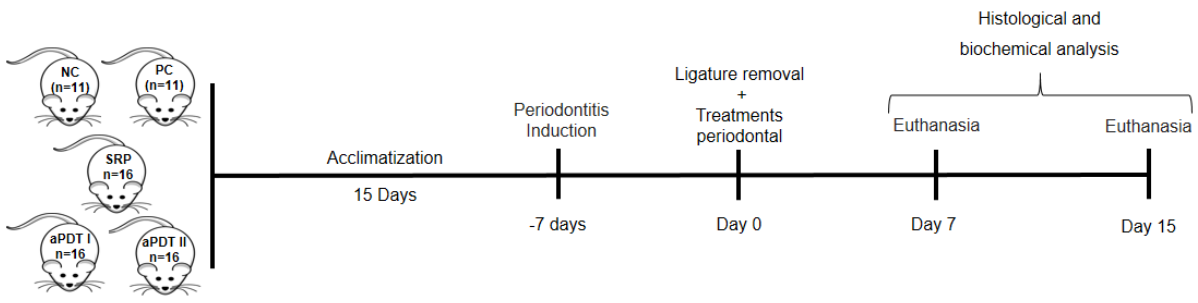

Figure 1: Experimental procedures.

Antimicrobial Photodynamic Therapy Protocols

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

The MB formulation was slowly poured in the periodontal pocket around the mandibular right first molar using a syringe (1mL) and an insulin needle (BD® UltrafineTM, U-100, 0.5mL, 8mm x 0.3mm) without a bevel. After 1 minute, low-level laser therapy (LLLT) was applied in three points of each buccal and lingual aspect of the mandibular right first molar. The laser used was an indium-gallium-aluminum-phosphorous (TheraLase®, DMC Equipments, São Carlos, SP, Brazil). The laser parameters were: wavelength 660nm, continuous emission mode, power output of 30mW transmitter, with spot size of 0.0283cm^2. The laser was activated for 4s in each point (4.94J/cm^2). The tooth received a total energy density of 29.64 J/cm^2 (Garcia *et al*., 2013). One blind operator performed the aPDT procedures.

Histological Analysis

Linear Bone Loss Measurements

For the histomorphometric analyses, histological sections from each specimen, which was hematoxylin and eosin (HE) stained were selected. Histological sections were carefully obtained, the interproximal alveolar bone crest and the coronal and root pulp chambers of the mandibular right first molar were clearly identified. The linear distance from the cement to the remaining alveolar crest to center parts of the bifurcation and alveolar bone loss measurements in the furcation region of the mandibular right first molar was measured to determine a histometric bone loss (μm) (De Oliveira *et al*., 2016). The linear bone loss in the furcation region of the mandibular right first molar represents the average value obtained from two measurements for each rat from each experimental group (n=6) after 7 days of ligature removal. A blind examiner evaluated the experimental groups with an interval of one week and mean values were calculated, with modifications. Images were obtained through an image analysis system (Axiovision, Carl Zeiss MicroImagnig, Jena, Germany) in a 10x magnification, captured with a digital camera coupled to the light microscope (AxioStar PluSS, Carl Zeiss) and visualized with the aid of a computer with processor (Pentium 4, with 3.00 GHz, 512Mb of RAM - Operating System Microsoft Windows XP - Monitor LG model FLATRONezT710SH, 64M, 17 inches color), associated with a binocular optical microscope (Olympus, model BX51 / BX52) with video camera (Olympus, model OLY-200) attached.

Influence of aPDT on the Total Ginival Collagen - Masson Trichrome Staining

The Masson Trichrome histological staining provides blue color to the collagen fibers of the connective tissue and was used to quantify the area occupied by such fibers (Masson, 1929). The slides were prepared following the specific protocol of the manufacturer (Masson Trichrome with aniline blue, Easy Path®, Erviegas Surgical instruments Ltda, São Paulo, SP, Brazil). The images were captured by digital camera attached to a light microscope (Axiovision, Carl Zeiss MicroImagnig, Jena, Germany) transferred to a computer in JPEG format and with a resolution of 2560x1920 pixels. Quantification of the area percentage (%) occupied by collagen fibers was performed using an image analysis system (FIJI to ImageJ®, version 1.47i, Wayne Rasband, National Institutes of Health, USA) under 400x magnification in five fields randomly underlying the epithelium of each blade, from left to right, a total of fifteen fields. Collagen area of each blade was given by the average of five

determinations and the corresponding area of each animal was given by the mean of three lamina area.

Collagen Maturation Index (CMI) Measurement

The collagen fibers type I and type III deposition by the upper connective tissue (directly under the epithelial membrane) of the gingiva was evaluated to CMI measurement. The samples around the mandibular right first molar of each animal were dissected at the end of the experiments and fixed in 10% formaldehyde in phosphate buffer (pH 7.2) for 24h. The gingiva specimens embedded in paraffin were sectioned (5μm), deparaffinized and stained with Picrosirius red commercial kit (Picrosirius Red Staining, Easy Path®, Erviegas Instrumental Cirúrgico Ltda., São Paulo, SP, Brazil) for polarized light microscopic evaluation. The staining with Picrosirius red allows an analysis of collagen fibers in the gingiva by different interference colors, intensity and birefringence of stained tissue. Thus, the staining differentiates the type I and type III collagen fibers.

Type I collagen (mature) show colors from yellow to red fibers strongly birefringent, and type III collagen (immature) appears green and has low birefringence (Boyne & Ellman, 1972). The stained tissue sections were examined using a microscope (Leica, model DM2000, Germany) with a 20x objective equipped coupled to digital image capture camera (Leica, model DFC295, Germany). The images generated by the camera were transferred to a microcomputer and converted into 256 different grey levels (Ejeil *et al*., 2003). For all the gingival samples, all extension of the histological slide was analyzed.

The percentages of type I and III collagen deposition were calculated for each rat and used to estimate the ratio among the percentages of type I over type III collagen (% collagen I / % collagen III), which is defined as CMI. Results >1 were considered as predominance of mature collagen (Coelho-Lemos *et al*., 2004), namely, higher percentage of type I collagen on the percentage of collagen type III and demonstrate the healing state maturity. For the morphometric determination of collagen types I and III (Rich & Whittaker, 2005), the examiners underwent training and completed double measurements of 60 specimens, with a 10-day interval between each measurement. The interexaminer and intraexaminer reproducibility revealed a high correlation (Kappa>0.81).

The results were expressed as area fraction (AA%) occupied by gingival collagen.

Preparation of Gingival Sample for Biochemical Analysis

On the 7 and 15 days of periodontitis experimental induction, animals were euthanized and gingival tissue of the mandibular right first molar region was excised from each rat, it was homogenized in TrisHCl buffer (10mM; pH 7.4) (Sigma-Aldrich®, São Paulo, SP, Brazil), and centrifuged at 3640g for 15min. The supernatants were used to determine oxidative damage parameters.

Gingival Lipid Peroxidation Estimation

Gingival lipid peroxidation was assessed by quantifying thiobarbituric acid reactive species (TBARS) levels as described by Ohkawa *et al*. (1979). The TBARS occurring because of excessive ROS generation was determined by the pink chromogen produced by the reaction of the thiobarbituric acid (TBA) to malondialdehyde (MDA) at 100°C and spectrophotometrically measured at 532nm. Results were expressed as nmol MDA/g gingiva.

Estimation of Non-enzymatic GSH Gingival Antioxidant Defense

GSH levels were determined after the samples reaction with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, Proquímios®, Rio de Janeiro, RJ, Brazil). The yellow formed was read at 412nm (Boyne & Ellman, 1972). A standard curve was plotted using GSH to calculate the GSH levels, which were expressed in GSH μmol/g gingiva.

Statistical Analysis

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

RESULTS

Morphometric Analysis

Linear Bone Loss Measurements

7 days after ligature removal, the PC group had more bone loss compared to the NC, SRP, and aPDT I and II. The SRP and aPDT I groups showed equivalent and greater bone losses than the NC group. The aPDT II group presented similar bone loss to the aPDT I

Table 1: Influence of aPDT with Methylene Blue Diluted in Water (aPDT I) and in Water/Ethanol 20% (aPDT II), used as an Adjunct to SRP, in the Periodontal Treatment on the Bone Loss, Collagen Degradation and Deposition of Rats Gingiva with Periodontitis

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

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

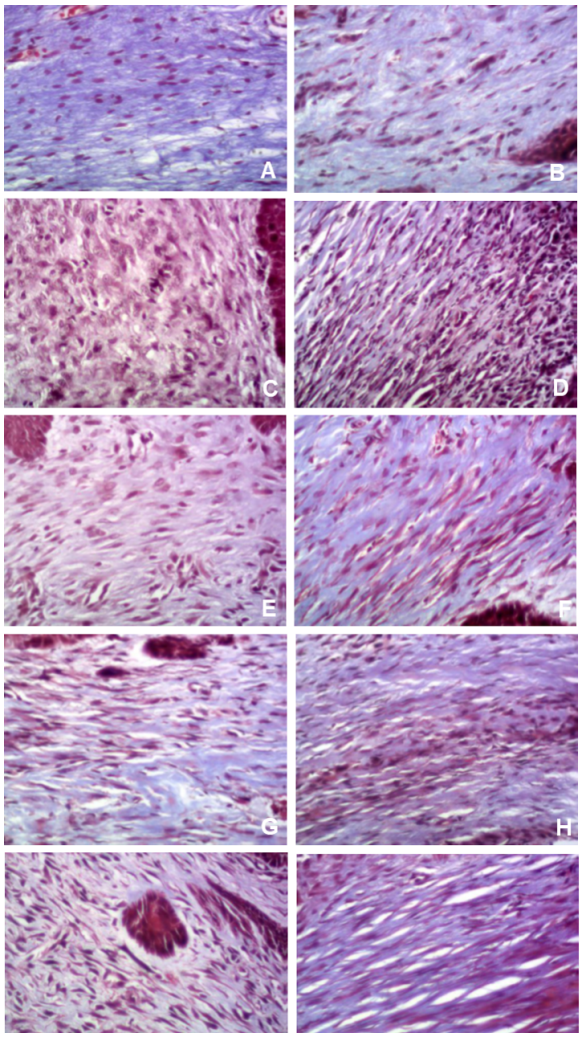

Figure 2: Influence of aPDT on the total gingival collagen area. Histological sections were stained with Masson trichrome and examined under microscope. The first column corresponds to the 7th day of the evaluations. The second column corresponds to 15th day of evaluation. **A/B**: NC group; **C/D**: PC group; **E/F**: SRP group; **G/H**: aPDT I group; **I/J**: aPDT II group. Magnification: 400x.

group, smaller than the PC and SRP groups, and larger than the NC group [*P*<0,05] (Table **1**).

Influence of aPDT on the Gingival Total Collagen Area

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

On the 7th and 15th evaluation days, the groups NC, aPDT I and aPDT II presented the highest gingival total collagen area. Regarding the same experimental times, the PC group showed the smallest gingival total collagen area. The SRP group had a gingival total collagen area higher than the PC group and smaller than the I and II aPDT groups. In the intra-group comparison, after 15 days of periodontal treatment, PC

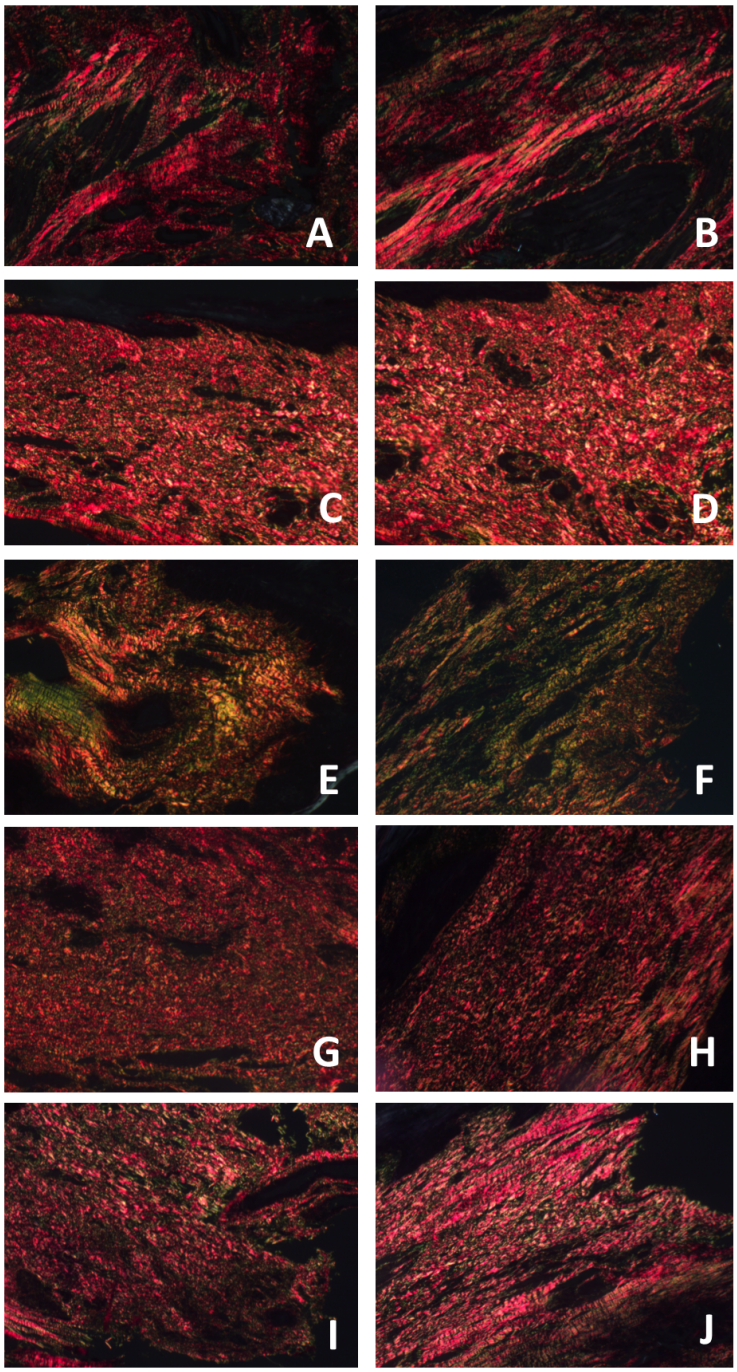

Figure 3: Influence of aPDT on gingival collagen maturation index. Histological sections were stained with Picrosirius red trichrome and examined under polarizing microscope. The first column corresponds to the 7th day of evaluations. The second column corresponds to 15th day of evaluation. **A/B**: NC group; **C/D**: PC group; **E/F**: SRP group; **G/H**: aPDT I group; **I/J**: aPDT II group. Magnification: 200x.

and SRP groups had a higher gingival total collagen area compared to day 7 (Table **1**; Figure **2**).

CMI Measurement

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

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

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

Biochemical Measurements

Influence of aPDT on Gingival Lipid Peroxidation Levels

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

The *post-hoc* test showed that PC and SRP groups increased gingival lipid peroxidation levels 7 days after the ligature removal in relation to NC, aPDT I and aPDT II groups, which showed similar values. The evaluations on the 15th day showed that the gingival lipid peroxidation levels remained higher in PC group compared to other groups, whose values were similar. Additionally, the SRP group showed smaller gingival lipid peroxidation levels on the 15th day than on 7th day of evaluation (Figure **4**).

Influence of aPDT on Gingival Antioxidant Defense Levels

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

The *post-hoc* test showed gingival GSH levels of PC and treated groups were similar in NC group 7 days after the ligature removal. On the 15th day of evaluation, gingival GSH levels were similar among all experimental groups, except for the aPDT I group, which was smaller than all the other groups. Additionally, SRP group showed higher gingival GSH levels on the 15th day than on 7th day of evaluation (Figure **4**).

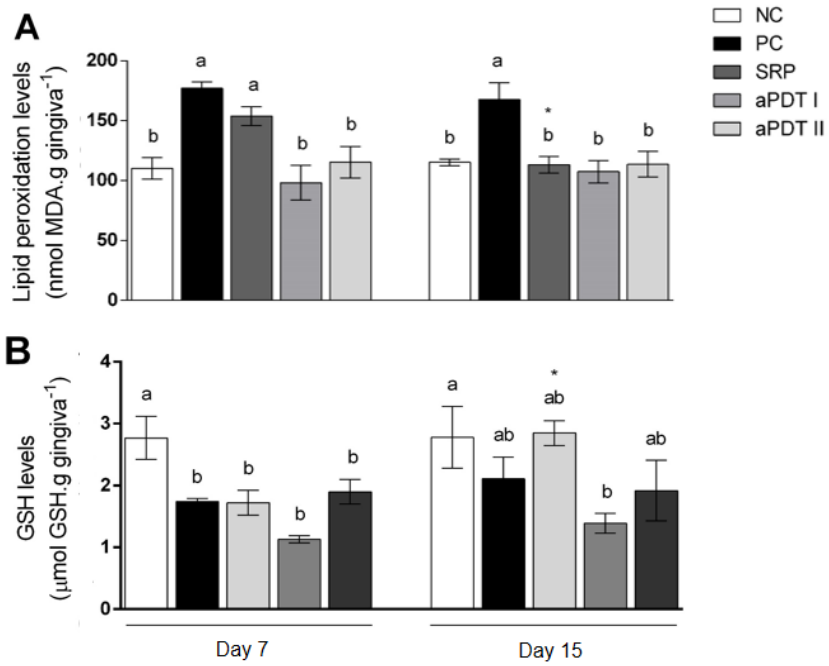

Figure 4: Influence of aPDT with MB solubilized in water (aPDT I) and in ethanol 20% (aPDT II) used as an adjuvant to SRP on the periodontal treatment on lipid peroxidation and GSH levels in the gingiva. Data are expressed as mean ± SEM. Different lowercase letters (a-b) indicate significant difference among periodontal treatment in the same evaluation. *Indicates significant difference from 7th day of evaluation in the same periodontal treatment (*P*<0.05).

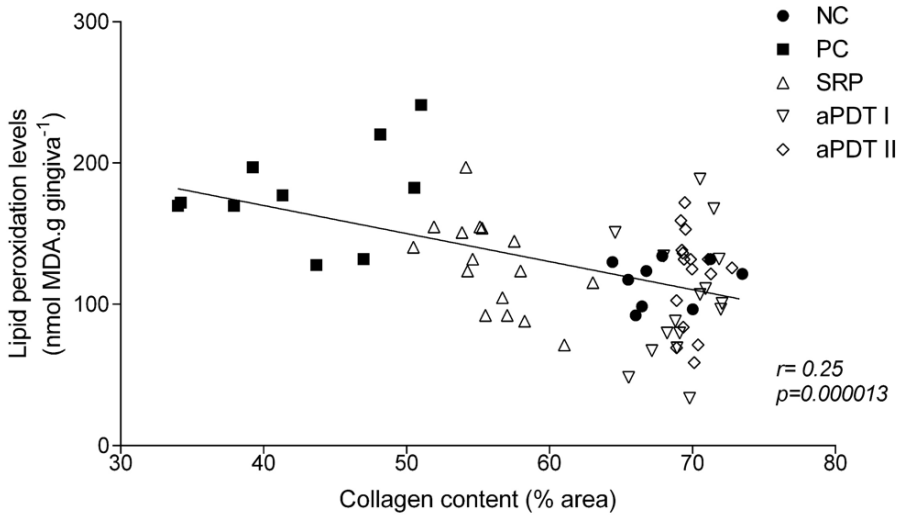

Figure 5: Linear regression analysis of the lipid peroxidation and collagen degradation in the gingiva. Statistical analysis revealed the following significance levels of *P* for the values of r -0.25 (*P*=0.00013). Groups: NC (negative control; no periodontitis); PC (positive control; periodontitis without any treatment); SRP (periodontitis and scaling and root planing), aPDT I (periodontitis and SRP+aPDT+MB solubilized in water), and aPDT II (periodontitis and SRP+aPDT+MB solubilized in ethanol 20%).

Linear Regression Analysis of the Gingival Lipid Peroxidation and Collagen Degradation

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

DISCUSSION

Previous studies of our group showed that aPDT as an adjunct to the SRP can induce the systemic protective response against periodontitis-induced oxidative stress, recover the gingival collagen (Pillusky *et al*., 2017) and increase the number of blood vessels in a short term thus promoting periodontal healing (Barin, *et al*., 2017), particularly when the MB is dissolved in ethanol 20%. Our findings showed: (i) aPDT groups had smaller bone loss; (ii) aPDT accelerates and facilitates the gingival collagen repair in short-term; (iii) MB solubilized in ethanol 20% (aPDT II group) protected gingival lipoperoxidation and antioxidant defense (GSH). In this study, the periodontitis was demonstrated by the greater bone loss observed in the PC group, which is in agreement with previous studies (Chang *et al*., 2013). The ligature was maintained for 7 days and then removed but the removal did not result in bone repair, since the PC group presented greater bone loss in relation to other groups in the same period, the same result was found by Chang *et al*. (2013). The three treatment groups of our study (SRP / aPDT I / aPDT II) presented differences among the control groups (NC and PC), demonstrating that the therapies result in bone repair in a short term.

The oxidative stress occurs when ROS generation exceeds the tissue antioxidant capacity, that is, it results in an increase production or endogenous antioxidant defenses decrease, or both (Trachootham *et al*., 2009). The ROS-mediated tissue damage during the oxidative stress can be measured by the end-product levels of lipid peroxidation, such as MDA (Tsai *et al*., 2005) in the TBARS assay (Ohkawa *et al*., 1979). In this sense, the cellular redox system plays a key role in homeostatic imbalance and damage in the gingiva, which can result from an imbalance among pro-oxidant and antioxidant stimuli. In addition, the periodontitis induced an increase of the MDA levels and decreased the antioxidant parameter, represented by GSH levels in the gingiva of the PC group when compared to the NC group, suggesting an oxidative damage in this oral tissue. The MDA levels are one of the most frequently used indicator of lipid peroxidation, they can be a potential biomarker indicating oxidative stress (Nielsen *et al*., 1997) and it has been reported that higher MDA levels are connected to chronic periodontitis (Aziz *et al*., 2013). Besides, recent studies have shown a reduction in the antioxidant capacity and an increase in oxidative stress biomarkers in periodontitis (Akalin *et al*., 2007), corroborating with our results.

The SRP, associated to adherence to maintenance schedules, can promote stability in periodontal levels

and promote teeth maintenance over the years (Axelsson *et al*., 2004). This procedure employs manual instruments to remove supra and subgingival bacterial deposits and is considered the gold standard in periodontal therapy (Balata *et al*., 2013), and new therapies are compared to it (Betsy *et al*., 2014). In this study, the SRP treatment alone was able to decrease lipid peroxidation levels in the gingiva in time-dependent manner, confirming the effectiveness of this classic procedure for periodontal treatment (Mlachkova & Popova, 2014). The mechanical instrumentation (SRP) cannot completely remove subgingival biofilm (Petelin *et al*., 2015), and the aPDT has shown to be effective as adjunctive therapy to the SRP in the periodontitis treatment in both animal (Pillusky *et al*., 2017; Barin, *et al*., 2017; De Almeida *et al*., 2008) and human studies (Theodoro *et al*., 2012). Furthermore, the aPDT as an adjuvant tool associated to SRP may result in ROS generation and the lipid peroxidation decreasing in the gingiva, improving periodontal disease treatment when compared to the standard treatment (SRP) alone, regardless of the solvent used for solubilization of the photosensitizer, as shown in our results. Besides this, the solubilization of the MB in water (aPDT I group) or ethanol 20% (aPDT II group) solution used as photosensitizer in aPDT did not differ observing the lipid peroxidation levels in all analyzed times and smaller than the PC group.

The glutathione is an oxidized (GSSG) or reduced (GSH) tripeptide considered one of most important endogenous antioxidant defenses, which has nucleophilic and reduced properties (Arrigo, 1999), and its reduced form can be decreased by oxidative stress (Barcelos *et al*., 2015). In this study, the gingival GSH evaluation of groups with periodontitis demonstrated that this non-enzymatic antioxidant defense decreased in all experimental groups compared to the NC group levels after 7 days of ligature removal.

This result suggests a consumption of gingival GSH due to an oxidative stress-induced periodontitis. Our results are in accordance with Azuma *et al*. (2011), which reported the gingival GSH/GSSG ratio reduction in the oxidative stress. In the evaluation of the 15th day, gingival GSH levels were completely restored in all groups, except in the aPDT I group; while the standard treatment (SRP) alone (SRP group) was able to increase the antioxidant defense compared to the evaluation of the 7th day. In this context, the bacterial biofilm reduction from the tooth surface using an association of non-surgical methods, as SPR and aPDT, reduced gingival lipid peroxidation levels, which contributed to the restoration of GSH levels in this tissue. Previous studies have showed that antioxidants can be beneficial tools as adjuncts to improve treatment outcome in periodontitis patients (Tomofuji *et al*., 2009; Kudva *et al*., 2011), and the application of the Er:YAG laser (erbium-doped: yttrium, aluminum, and garnet) in chronic periodontitis treatment showed promising experimental and clinical results (Domínguez *et al*., 2010; Ishikawa *et al*., 2004), showing the importance of this type of light therapy.

The oxidative stress has been associated to collagen degradation (Alge-Priglinger *et al*., 2009), which is a major component of the soft tissues and bones (Sorsa *et al*., 2006). Moreover, the periodontitis induces alterations in the gingival connective tissue composition (Pillusky *et al*., 2017; Page & Schroeder, 1976). In the present study, the gingival collagen total area significantly decreased on the 7th day after ligature removal in the PC and SRP groups compared to the NC group. The SRP group showed lipid peroxidation levels similar to PC group in a 7-day period and both were higher than the NC group, which can be related to a reduced collagen area in this group observed on the 7th and 15th days of evaluation. In this sense, we may infer that periodontitis-induced oxidative stress degraded the gingival collagen, as demonstrated by the area reduction of these fibers in the gingiva. Our findings are in accordance with previous studies reporting oxidative stress and lower collagen content in tissues (Pillusky *et al*., 2017; Galli *et al*., 2011). The collagen degradation has been considered the main marker of periodontal disease progression (Waddington *et al*., 2000). In this study, the collagen area in the gingival tissue in the aPDT I and aPDT II groups were not affected by periodontitis, which was demonstrated by the preservation of the gingival collagen area that was equal to the NC group. The aPDT could protect the lipid peroxidation in the gingiva, reducing the oxidative stress-related collagen degradation in these groups, independent of the solvent used to solubilized the photosensitizer in all evaluated times.

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

The collagen deposition has been reported to show the healing process (Ehrlich & Krummel, 1996) and was included in the present study as CMI. The periodontitis affected the gingival collagen maturation in the SRP group, which showed smaller CMI than the NC group on the 7th day of evaluation, indicating the

predominance of immature versus mature collagen, but this difference did not show statistical significance. On the 15th day, the PC and SRP groups exhibited equivalent and lower CMI than the NC group, indicating collagen deposition to restore the contents of gingival collagen reduced by periodontitis. In contrast, the CMI on both aPDT groups, on the 7th and 15th days, was not significantly different from the NC group, suggesting that both aPDT protocols preserved deposition and consequent collagenous maturation, which was similar to NC group. Based on these results, we suggest that the periodontitis repairing phase is related to type III collagen because the fibroblasts synthesize high levels of type III collagen and because of the reduced levels of type I collagen (Larjava *et al*., 1990). Type I collagen is essential for the reestablishment of functional dynamic conditions and type III collagen is essential for the type I collagen formation (Liu *et al*., 1997). Of particular importance, aPDT protocols showed ability to induce collagen maturation in a short term compared to standard periodontal treatment (SRP), which may be related to the protective action against oxidative stress-induced periodontitis observed in aPDT groups (Pillusky *et al*., 2017).

According to Mendez *et al*. (2004), Meirelles *et al*. (2008) and Gonçalves *et al*. (2013), enhanced collagen maturation may be observed in different protocols employing GaAlAs laser (λ 830nm) at 50J/cm^2, low level laser therapy (LLLT) at an energy density of 20J/cm^2 and laser GaAsAl 30 and 90J/cm^2, respectively.

The negative correlation among collagen area and lipid peroxidation levels, both observed in the gingiva, indicates the close relation between collagen degradation and oxidative damage development in this supporting oral tissue in rats. According to our findings, Gonçalves *et al*. (2013) found a moderately negative correlation among lipid peroxidation levels and CMI in all groups. Considering that periodontitis and the consequent oxidative status in gingiva were modified by aPDT, it is possible that these physiological changes are related to adaptation and/or hormesis.

The photosensitizer excitation results in ROS generation, which mediate cellular effects such as lipid peroxidation and vascular effects, resulting in direct or indirect cytotoxic effects on the treated cells (Brackett & Gollnick, 2011). In this sense, one hypothesis can be proposed to explain the lower oxidative toxicity of aPDT to periodontal tissue in the presence of periodontitis: development of hormesis due to a stressor, as periodontitis and the consequent biofilm-induced bacterial ROS. This hypothesis suggests that organisms exposed to stressors trigger defense mechanisms, which would act much more efficiently than in those not previously having stressors. This hypothesis has been used to explain the increase in the antioxidant defenses in different animal species (Dolci *et al*., 2013; Laughlin *et al*., 1981). Our data also point towards the development of hormesis, gingival oxidative damage, as lipid peroxidation and collagen degradation, both ROS generators, were not observed in the groups treated with aPDT; however, it was only observed in the group treated with SRP (SRP group). Our findings are consistent with the development of hormesis, exposing the gingiva to periodontitis following aPDT, allowing physiological changes to counter-act the damages caused by ROS aPDT-induced. According to our findings, a low dose of irradiation also showed to be effective on the treatment against greater stress, as the gingivitis (Luckey, 2006). In this context, authors conceptualized hormesis, which is a useful way to think about the effect of stressors on the gingiva, *i.e*., periodontitis and aPDT. In our study, the lower levels of lipid peroxidation and collagen degradation observed in the gingiva of aPDT treated groups may indicate tolerance to a hostile environment and adaptation mechanisms development, even as an increment of the defense mechanisms (Hamdoun & Epel, 2007), resulting from hormesis.

Here, we did not observe an increase in the measured defense mechanism because all groups showed a depletion of GSH gingival levels on the 7th day of evaluation. We believe that another antioxidant or defense mechanism may be involved in the gingival tissue protection, since the GSH is the first line of antioxidant defense that organism have against ROS in favor of homeostase and therefore, the first to be consumed during oxidative stress (Thompson & Franklin, 2010). In this context, more studies are necessary to better understand the hormesis mechanisms at the molecular and cellular level in different approaches in search of prevention and treatment of periodontal diseases.

The present study has some limitations: (i) periodontitis was induced in a short period and the acute nature of these models may not fully reflect the chronic pathological conditions in humans; and (ii) bone loss analyzed in a short period (7 days).

Our results employing aPDT showed that the AM solubilization in ethanol 20% prevented the bone loss,

collagen degradation and periodontitis-induced lipid peroxidation in a short term. From this, it is possible to propose that the short-term aPDT causes beneficial physiological responses, which may be related to hormesis, that culminated in periodontal healing acceleration. This reinforces previous studies of our group regarding the use of aPDT, especially AM, solubilized in ethanol 20%, which was used as an adjuvant therapy to the mechanical treatment for periodontitis.

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CONFLICTS OF INTEREST

The authors deny any conflicts of interest related to this study.

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