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ORIGINAL ARTICLE

Adjunctive antimicrobial photodynamic therapy using methylene blue/ethanol formulation in experimental periodontitis in diabetic rats: short-term results

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Abstract

The aim of this study was to evaluate the effect of an MB experimental formulation (ethanol 20%) in aPDT used as an adjuvant to scaling and root planing (SRP) in the periodontal treatment of diabetic rats. Forty male Wistar rats received streptozotocin-intraperitonial injections to induce diabetes. After 14 days, 5 animals were allocated in the non-ligate group (NLG), and 35 animals received ligature at the first right mandibular molar to induce periodontitis. After 7 days, the ligature was removed and the animals were randomized into 4 groups: LG (without treatment, $n = 5$), SRPG (SRP, $n = 10$), aPDTW (SRP+aPDT-MB/water, $n = 10$), and aPDTEt (SRP + aPDT-MB/water/ethanol/carboxymethylcellulose, $n = 10$). Animals were euthanized after 7 days. Data of bone loss (BL) area, degree of inflammatory cell response, and collagen fibers percentages were statistically analyzed ($p < 0.05$). Percentage of animals that presented mild and severe inflammatory infiltrate was 10% and 40% for SRPG, 20% and 30% for aPDTW, and 50% and 0% for aPDTEt, respectively. BL area (mm^2) was statistically higher in the LG (0.39 ± 0.15) than NLG (0.05 ± 0.02). aPDTEt showed the lowest value of BL (0.08 ± 0.03), followed by aPDTW (0.21 ± 0.15) and SRPG (0.31 ± 0.18). Statistical differences were verified between aPDTEt and SRPG. In relation to the LG, aPDTEt, aPDTW, and SRPG recovered the equivalent 80%, 46%, and 20% of the BL. aPDTEt showed collagen content statistically higher than SRPG and LG, and presented higher mean values than NLG ($p > 0.05$). Our findings showed aPDTEt presented promising results. aPDT using MB/ethanol can have potential as an adjunctive periodontal treatment in diabetics.

Keywords Alveolar bone loss · Periodontal disease · Root planing · Phototherapy

Introduction

Periodontitis is a chronic inflammatory disease caused by bacterial infection affecting teeth-supporting tissues. Severe periodontitis was ranked as the 6th most prevalent condition within the Global Burden of Disease 2012 Study, with an estimated prevalence of 10.8% [1]. Periodontitis is a major cause of tooth loss, which affects the quality of life of people in terms of reduced functional capacity, self-esteem, and social relationships [2]. Risk factors for periodontitis include poorly controlled diabetes [3]. In 2015, approximately 415 million people worldwide have diabetes mellitus, and this number is predicted to reach 642 million by 2040 [4].

Scaling and root planning (SRP) is the *gold standard* treatment for periodontitis [5], but in diabetic patients, SRP alone may be ineffective to control the periodontal disease [6, 7]. Adjunctive therapies have been sought, as the systemic antibiotics; however, this alternative has showed limited benefits in diabetic patients who have periodontitis [8, 9].

Antimicrobial photodynamic therapy (aPDT) has been investigated as adjunctive therapy to the SRP. Past animal studies observed that aPDT had successfully controlled the progression of experimental periodontitis in rats [10–14]. However, the outcomes of clinical trials are inconsistent.

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Systematic reviews suggested the adjunctive aPDT provides modest benefits when comparing to SRP alone [15–17]. Clinical trials showed the benefits from adjunctive aPDT are doubtful in systemically healthy, smokers, and diabetic patients [18–21]. In this context, strategies to optimize the aPDT effect on periodontal outcomes are necessary.

In vitro studies found the aPDT effect could be modulated by a solvent in which the PS is dispersed [22]. The formulations commonly employed water as solvent for methylene blue (MB) resulting in lower reactive oxygen species and singlet oxygen production [22], reduced half-life (4 μs) [23], and low diffusion potential [24] when compared to experimental formulations containing ethanol. Evidences showed the 20% ethanol inclusion in the MB formulations reducing the viability of *Pseudomonas aeruginosa* [25], *Actinomycetes actinomycetemcomitans*, and *Entercoccus faecalis* biofilms [22].

To the best of knowledge, there is no evidence of the aPDT adjunctive effect using formulations with MB and ethanol on periodontal therapy. Then, we proposed to evaluate this possibility in a ligature model to induce experimental periodontitis in diabetic rats. We hypothesized that aPDT with an experimental formulation of MB/ethanol would result in lower bone loss (BL), minor inflammatory response, and higher collagen content when compared to SRP alone and SRP plus aPDT using MB/water.

Material and methods

The present study was a controlled, blinded, and randomized animal experiment. Figure 1 presents the flowchart of the study. The study protocol was approved by Committee on Animal Research of the Federal University of Santa Maria, Brazil (027/2013).

Forty (males, *Rattus norvegicus albinus*, Wistar) 90-day-old rats (250–300 g) were housed with food and distilled water ad libitum. The room presented controlled temperature (23 ± 1 °C), relative humidity of the air about to 60% and exhaust air, noise control (maximum 85 dB), and standard light-dark cycle (12 h). The animals were allowed to acclimatize to the laboratory for a period of 2 weeks.

Diabetes induction

Diabetes was induced, after 6 h fasting, by intraperitonial injection of 50 mg/kg streptozotocin (STZ) (Sigma Chemical Company, St. Louis, MO, EUA) diluted in citrate buffer under 0.01 M and pH 4.5 [26]. Fasting blood was drawn from the tail veins of all animals on the same day prior to STZ injection, after 7 days, and on the days of periodontitis induction, periodontitis treatment, and euthanasia (Fig. 1). Blood glucose levels were measured using a glucose monitor (Accu-check Active, Roche Diagnosis, Sandhofer, Germany). Animals with glucose levels greater than 250 mg/dL were considered diabetic [26].

Periodontitis induction

After 14 days of diabetes induction [27], 5 animals, which did not receive any intervention, were allocated to non-ligate group (NLG). Thirty-five animals were anesthetized (ketamine 70 mg/Kg/10% and xylazine 6 mg/Kg/2%, by intramuscular via) and a 4–0 silk ligature (Ethicon 4–0, Johnson & Johnson, São Paulo, SP, Brazil) was placed on the gingival margin level of the right mandibular first molar for 7 days [13, 14]. After, the ligatures were removed and the animals were randomized into the 4 experimental groups (Table 1). The

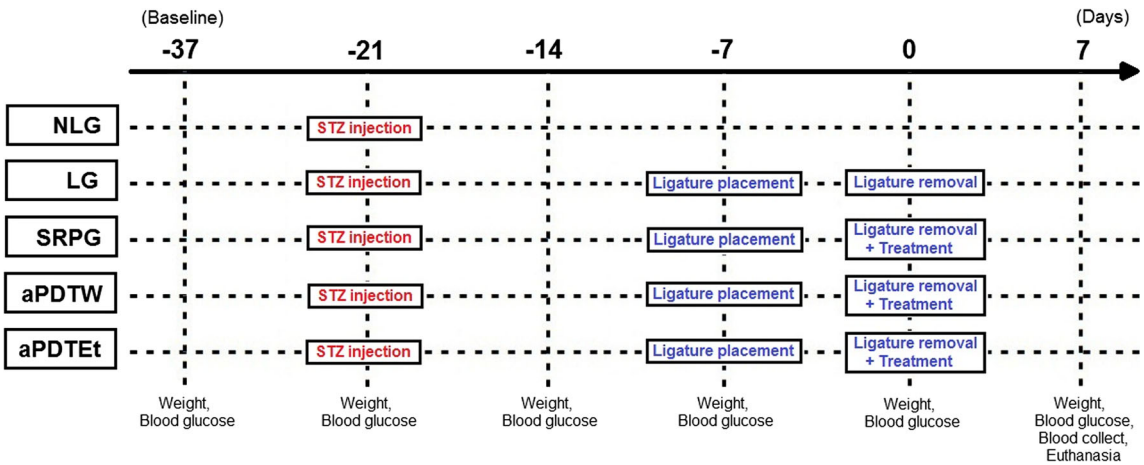

Fig. 1 Study design. NLG, non-ligate animals; LG, ligate animals without treatment; SRPG, SRP; aPDTW, SRP + aPDT with MB in water; aPDTEt, SRP + aPDT with MB in water/ethanol/carboxymethylcellulose

Table 1 Description of experimental groups

Groups	Experimental groups description	Rats number
NLG	Non-ligate animals.	5
LG	Ligate animals, without treatment.	5
SRPG	Ligate animals, treated with SRP.	10
aPDTW	Ligate animals, SRP plus aPDT-MB (0.01%) solubilized in ultra-pure water.	10
aPDTEt	Ligate animals, SRP plus aPDT-MB (0.01%) solubilized in ultra-pure water, ethanol, and carboxymethylcellulose (77:20:3).	10

For ethical reasons, 5 animals were used for groups that not received treatment

SRP scaling and root planing, *aPDT* antimicrobial photodynamic therapy, *MB* methylene blue

animals of the control group were manipulated in the same fashion as those of the other groups.

Randomization and blinding process

A computer software (Random Allocation Software, version 1.0, May 2004) was used to randomly allocate the ligate animals to the experimental groups. One study team member (RMM) performed the randomization process. A single operator (DAMD) performed both the SRP and aPDT procedures. The operator was informed if the animal should receive an additional aPDT only after the SRP procedure. The formulations used for aPDT presented the same coloration and equal syringes. The animals were treated following the sequence generated by the randomization process.

Scaling and root planing and aPDT

The right molar was subjected to SRP with manual 1–2 mini five curettes (Hu-Friedy Co. Inc., Chicago, IL, USA) through 10 distal-mesial traction movements in both buccal and lingual aspects. The furcation and interproximal areas were scaled through cervico-occlusal traction movements [12].

aPDT employed the MB (0.01%) ($C_{16}H_{18}ClN_3S \cdot 3H_2O$) (Sigma Aldrich) solubilized in ultra-pure water (Milli-Q) for aPDTW group, and in ultra-pure water, ethanol (Sigma Aldrich), and carboxymethylcellulose (Sigma Aldrich) in the proportion of 77:20:3, respectively, for the aPDTEt group. 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 formulation (1 mL) was poured into the periodontal pocket around the right mandibular first molar using a syringe (1 mL) and an insulin needle (8 mm × 0.3 mm). After 1 min, the laser was applied to three equidistant points at each buccal and lingual aspect perpendicularly and in contact with the gingivae. The laser was activated at a power of 30 mW for 4 s per point (energy of 0.14J/point, and energy density of 4.94J/cm^2). The tooth received 0.84 J of energy and a total energy density of 29.64 J/cm^2 [12].

Seven days after treatments, the animals were anesthetized with isoflurane 2–3%, inhaled. Euthanasia was performed by total exsanguinations.

The right mandibular specimens were fixed with 10% neutral buffered formalin (pH 7.2) for 48 h and then demineralized with 10% EDTA (pH 7.4) for 13 weeks. The decalcified mandible tissues were neutralized, after which they were dehydrated, embedded in paraffin, and serially sectioned using a microtome. Serial paraffin sections (4 μm) were obtained in the mesial-distal direction from buccal surface. After excluded the first section in which the furcation region was evident, three serial sections were obtained and then dyed with hematoxylin and eosin (HE), Masson's trichrome (MT), and picrosirius red (PRed).

Histometric analysis

All histometric evaluations were performed in the furcation region defined as the non-occupied area by bone tissue between the top of the interradicular bone septum and the cementum surface [28, 29].

One HE dyed section was viewed using × 100 magnification, and then graded by a 4-point scoring system (0, absent; 1, mild or discrete; 2, moderate; 3, intense or severe) [30] to evaluate the presence and degree of inflammatory cell response.

The area (mm^2) of BL in the furcation region was evaluated as a previously reported method [29], using a × 50 magnification view of one MT stained section [12] (Fig. 2). The area was histometrically determined using an image

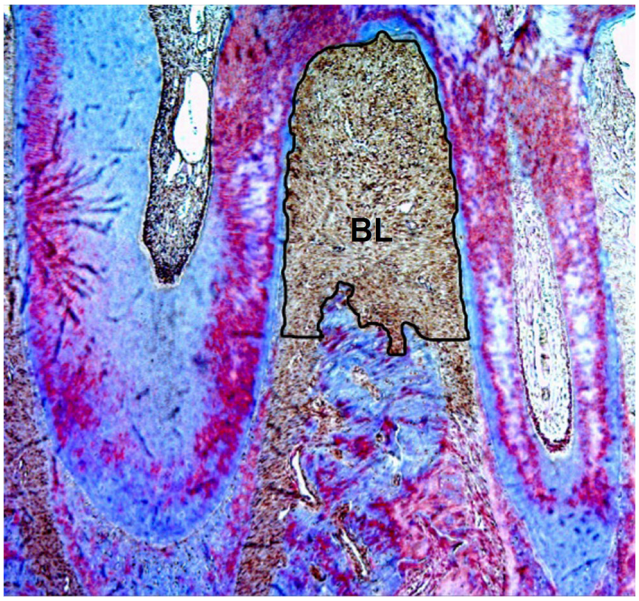

Fig. 2 Measurement of bone loss in the furcation region of first molar of ligate group after 7 days of periodontal disease induction (Masson Thricome staining, magnification × 50)

analysis system (Axiovision; Carl Zeiss MicroImaging, Jena, Germany).

Collagen fibers percentage in the furcation region was evaluated using two different methodologies. Firstly, from one MT stained section, an image into the region of interest (ROI, rectangle with size defined for each section) was selected using a × 50 magnification by an image-processing software (Zen lite 2012 blue edition, Carl Zeiss MicroImagnig, Jena, Germany). Then, a second image software (ImageJ v1.50i, National Institutes of Health, USA) was used to binarized the selected images, and the percentage of collagen fibers within the ROI was calculated [28, 29]. Secondly, from one PRed dyed section, the furcation region image was digitalized using × 400 magnification [28, 29] by a polarization microscopy (Leica, model DM2000, v. 4.0, Germany). Images of collagen fibers that displayed red hue inside the microscopic field of view were selected by an image-processing software (Leica, model DFC295, Germany). The selected images were then binarized and the percentage of area filled by collagen fibers was calculated using software (ImageJ v1.50i) [29].

A single examiner (SMR) who was blinded to the experimental groups performed all the histometric analyses. The evaluations of the degree of inflammatory cell response and BL area were measured twice, in different days, in order to reduce the data variations. The mean average values were compared statistically. The examiner was calibrated by duplicate measurements of 10 specimens with an interval of 1-week. The concordance of BL area measurements was verified by intraclass correlation coefficient (ICC = 0.86), and the degree of inflammatory cell response was verifying using Kappa coefficient (kappa = 0.95).

Statistical analysis

The animal was the analysis unit. Normality of the data was confirmed by Shapiro-Wilk test. Data were presented as mean and standard deviation (SD). The intergroup analyses were performed by one-way ANOVA and post hoc Bonferroni's test. Intra- and inter-group differences to blood glucose level were determined by repeated measures ANOVA and mixed models ANOVA, respectively, and Tukey's test. Differences were considered significant when $p < 0.05$. The distribution frequencies of the scores (0 to 3) of the degree of inflammatory cell response were calculated. The BL means and the collagen fibers mean percentages of the animals with periodontitis and without treatment (LG) were considered as reference to evaluate the size effect of the treatments. The percentage of BL and the percentage of collagen increase, considering the treatment groups (SRPG, aPDTW, aPDTEt) in relation to the LG, were calculated.

Results

Two rats were excluded from the study due diabetes complications (aPDTW, aPDTEt). Diabetes was successfully induced in the animals via STZ injection (Table 2).

Mild inflammatory infiltrate was observed in 10%, 20%, and 50% of the SRPG, aPDTW, and aPDTEt, respectively. Intense or severe infiltrate was not verified in aPDTEt, and was observed in 30% and 40% in the aPDTW and SRPG, respectively (Fig. 3). LG showed the highest value of inflammatory cell response 2.4 (0.5), followed by SRPG 2.3 (0.6), NLG 2 (0.7), aPDTW 1.66 (1), and aPDTEt 1.1 (0.7). Statistical difference was verified among aPDTEt and SRPG ($p = 0.03$).

The ligature model was effective in causing BL, since the LG (0.39 ± 0.15 mm) showed significantly higher BL than NLG (0.05 ± 0.02). None statistical difference was observed among aPDTEt (0.08 ± 0.03) and aPDTW (0.21 ± 0.15), and among aPDTW and SRPG (0.31 ± 0.18). aPDTEt displayed a statistically lower BL than group submitted to the SRP alone (Fig. 4).

All treatment groups increased the collagen fibers mean percentages when compared to the LG, which represent the maximum value of collagen destruction in this study. Significant difference ($p < 0.05$) was observed only between LG and aPDTEt. In MT stain, aPDTEt presented

Table 2 Mean (standard deviation) of the blood glucose levels (mg/dl)

	NLG ($n = 5$)	LG ($n = 5$)	SRPG ($n = 10$)	aPDTW ($n = 9$)	aPDTEt ($n = 9$)	All groups
Previous diabetes	112 (16)*	94 (18)*	101 (16)*	102 (16)*	110 (22)*	104 (6)*
Periodontitis induction day	568 (43)	589 (22)	549 (48)	569 (41)	584 (43)	572 (15)
Periodontal treatment day	549 (63)	449 (80)	496 (46)	560 (50)	560 (53)	524 (50)
Euthanasia day	565 (39)	594 (12)	585 (35)	593 (21)	578 (40)	583 (12)

Intra- and inter-group differences by repeated measures ANOVA and mixed models ANOVA and Tukey's test, respectively

No difference was observed between groups ($p < 0.05$)

*Independently of the experimental groups, the animals presented a significant statistically increase in the blood glucose level after STZ injection ($p < 0.05$)

significantly higher collagen fibers percentage than SRPG. aPDTEt presented higher mean values of collagen fibers than the group without periodontitis, even so the differences were not statistically significant (Table 3).

Figure 5a shows the reduction percentages of BL area of the treatment groups in relation to the LG. aPDTET, aPDTW, and SRPG recovered the equivalent to 80%, 46%, and 20% of the BL verified in the LG. aPDTEt was approximately four and two times more effective to protect the bone reabsorption than SRPG and aPDTW, respectively. aPDTEt was the treatment that provided the highest increase in the collagen content when comparing to the LG (Fig. 5b, c).

Discussion

This study examined the effect of the aPDT using an experimental formulation of MB with ethanol as an adjunctive therapy to SRP in diabetic rats with periodontitis. Our findings indicated that animals which received aPDTEt presented less BL, lower local inflammatory infiltrate, and higher collagen fibers percentage when compared to those that received SRP alone. Animals treated with aPDTW showed no statistical differences when compared to the animals treated with SRP alone. aPDTEt showed no statistical benefits when compared to aPDTW.

Collectively, our findings showed aPDTEt presented most promising results for all the outcomes evaluated, followed by the aPDTW and SRP. The reduced number of animals impaired the statistical power. Efforts to use high number of animals or repeated histological section to reach high precision should be considered unnecessary, and promotes no additional information besides that obtained from randomized and representative evaluations.

Beneficial effect of the aPDTEt could be explained by the strongest local antimicrobial effect, related to the highest production of singlet oxygen. Ethanol promotes lower MB molecular aggregation, i.e., a higher proportion of monomers in relation to dimmers [22]. Dimmers are less effective in capturing energy [31], acting predominantly in reactions involving electronic exchanges with the substrate (type I), resulting on less production of

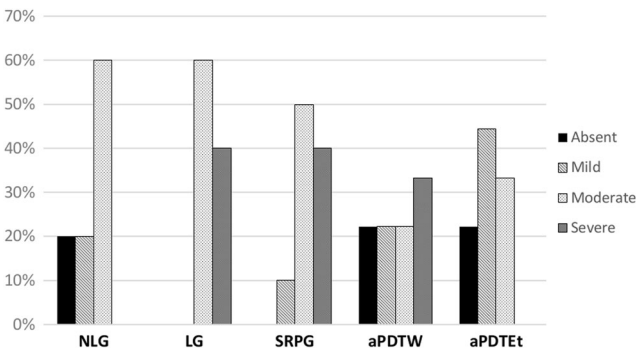

Fig. 3 Score distribution frequency of the degree of inflammatory cell response. NLG, non-ligate animals; LG, ligate animals without treatment; SRPG, SRP; aPDTW, SRP + aPDT with MB in water; aPDTEt, SRP + aPDT with MB in water/ethanol/carboxymethylcellulose

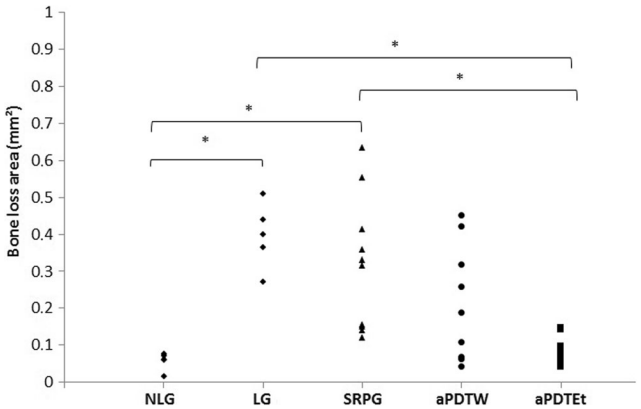

Fig. 4 Graph of plotted values for BL area. NLG, non-ligate animals; LG, ligate animals without treatment; SRPG, SRP; aPDTW, SRP plus aPDT with MB in water; aPDTEt, SRP plus aPDT with MB in water/ethanol/carboxymethylcellulose. * Significant statistically differences between groups ($p < 0.05$). One-way ANOVA and post hoc Bonferroni's test

Table 3 Mean (standard deviation) of the collagen fibers percentages

	NLG ($n = 5$)	LG ($n = 5$)	SRPG ($n = 10$)	aPDTW ($n = 9$)	aPDTEt ($n = 9$)
MT	14.9 (8.8)^{AB}	2.2 (1.9)^{B}	9.0 (2.4)^{B}	15.3 (9.9)^{AB}	24.2 (10)^{A}
PRed	18.7 (8.2)^{ab}	9.6 (2.9)^{b}	16.2 (6.8)^{ab}	18.2 (8.1)^{ab}	24.9 (8.3)^{a}

Line: one-way ANOVA and Bonferroni's test

Different capital letters represent statistical difference between groups in MT stain ($p < 0.05$)

Different lower case letters represent statistical difference between groups in PRed stain ($p < 0.05$)

MT, Masson's trichrome; *PRed*, picrosirius red

singlet oxygen (type II reaction). Singlet oxygen has been considered the main responsible for bacterial photo-destruction. Then, a formulation that stabilizes the monomers is preferable for therapeutic purposes [22].

Studies showed low-level laser therapy (LLLT) accelerates tissue repair [32], increases collagen deposition, stimulates the proliferation of cultured gingival fibroblast [33, 34], inhibits the production of inflammatory mediators by gingival fibroblast [35], favors cellular chemotaxis, and promotes local vasodilatation and angiogenesis [36, 37]. The higher blood vessel counts in animals treated with LLLT could promote increased oxygen diffusion through the tissue favoring the healing process, once collagen secretion by fibroblasts occurs in the presence of high rates of oxygen [38]. Such observations probably explain the differences in the local degree of inflammatory cell response and in the collagen behaviors between adjunctive aPDT, regardless of the PS formulations, and SRP alone verified in the present study.

In diabetic rats, one previous study [10] evaluated the aPDT effect as an adjuvant treatment to the SRP on induced periodontitis. Authors observed lower BL in animals treated with aPDT than SRP alone, for all experimental periods (7, 15 and 30 days after treatment). We did not find statistical differences between SRP alone and conventional aPDT with the water-based PS formulation. The differences between our study and the one of Almeida et al. may be related to differences in the PS type. While we use MB, Almeida et al. utilized Toluidine blue (TB).

Some strengths of this study should be emphasized. First, we look for to follow research principles, such as sample randomization, blinding of the operator and examiner, and calibrated examiner to minimize potential biases and to produce valid estimates. Second, two control groups were considered in the present study. There is an increase in the BL associated with animals aging [39] that are not biofilm-related [40]. BL observed in the NLG is more associated with physiological than pathological process. Then, NLG was necessary to estimate the portion of physiological BL on entire BL observed into ligate animals. The second control group included animals that received the ligature and no treatment (LG). Rats show a fast metabolism and healing process. From LG, we can observe if the data of BL in the treatment groups were only associated to the treatment effect or if were basically from physiological healing of the rats after the ligature removal. Therefore, the lower BL observed in the treatment groups than LG indicates that the protective effect on BL was associated with the treatments.

One disadvantage of the ligature model, which submerging totally a silk ligature into the gingival sulcus for 7 days [10–14], is the mechanical trauma. We placed the ligature at the gingival level partially into gingival sulcus in order to minimize the trauma. The BL magnitude verified in the present study was lower than the above-mentioned studies

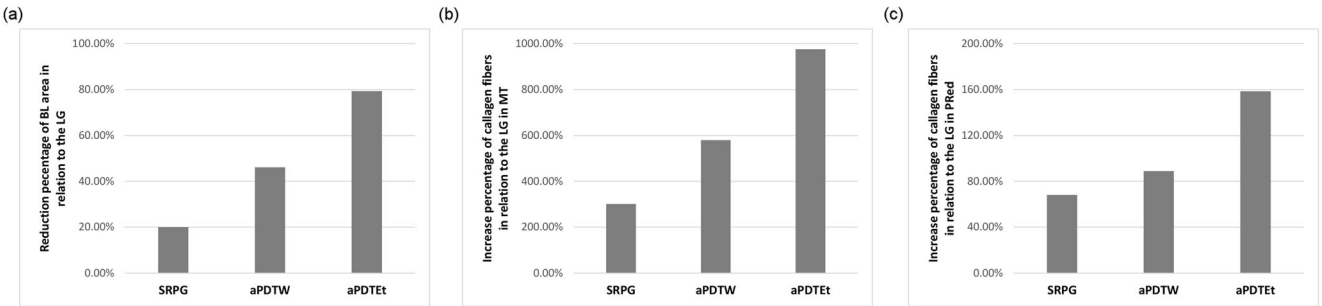

Fig. 5 Reduction percentage of BL area (**a**), increase percentage of collagen fibers in MT (**b**), and in PRed (**c**), considering the treatment groups (SRPG, aPDTW, aPDTEt) in relation to the LG (ligate animals without treatment). SRPG, SRP; aPDTW, SRP plus aPDT with MB in water; aPDTEt, SRP plus aPDT with MB in water/ethanol/carboxymethylcellulose

[10–14]. However, we can clearly discriminate the BL of the animals with no ligature (NLG) of those with ligature and no treatment (LG).

The current study has some limitations: (i) diabetes and periodontitis were induced in a short period and the acute nature of these models might not fully reflect the chronic pathological conditions in humans; and (ii) short period (7 days) to analyze the periodontal healing. Considering the promising effect of the aPDTEt when comparing to SRP and/or aPDTW, we suggest future research with more complex animal models, and with higher follow-up periods.

We concluded that adjunctive aPDT with 0.01% MB solubilized in ultra-pure water/ethanol/carboxymethylcellulose (77:20:3) can have beneficial potential to periodontal treatment of diabetics.

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Compliance with ethical standards

The study protocol was approved by Committee on Animal Research of the Federal University of Santa Maria, Brazil (027/2013).

Conflict of interest The authors declare that they have no conflict of interest.

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