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Research Article

Effects of Antimicrobial Photodynamic Therapy on the Periodontal Tissues: Histomorphometric and Immunohistochemical Analysis

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Abstract

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

Keywords: Ethanol; Inflammation; Periodontal Diseases; Photosensitizing Agents; Therapeutics; Vascular Endothelial Growth Factor

Introduction

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

The inflammatory process caused by persistent bacteria leads to periodontal tissue damage. The reduction or elimination of these etiological factors is the main purpose of periodontal treatment. Scaling and root planing (SRP) is the golden standard treat-

ment for periodontitis [6]; however, this conventional mechanical treatment can fail to eliminate periodontopathogenic bacteria in hard-to-reach areas, such as furcation regions, bone fissures and concavities [7]. In this sense, the antimicrobial photodynamic therapy (aPDT) is a promising adjuvant modality that can suppress periodontal pathogens and increase the effectiveness of manual mechanical treatment [8,9]. The advantages of this therapy is low probability of developing bacterial resistance, local action, non-invasive behavior, large spectrum of action and no limit dose. aPDT consists of combining three elements: a photosensitizer (Ps), light and oxygen. Thus, the photochemical reaction generated releases cytotoxic products to microorganisms causing cell death [10].

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

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

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

Material and Methods

Animals

One hundred and twenty male Wistar rats (275 ± 25g and 3 months of age) were used. They were housed at five animals per Plexiglas® cages with free access to food (standard chow; Supralab®, Alisul Alimentos LTDA, São Leopoldo, RS, Brazil) and water in a room with controlled temperature (23 ± 1°C), a 12:12 light:dark cycle and had free access to water. Before starting the experimental protocols, animals underwent an acclimatization period of 15 days. The experimental protocols followed ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines and were approved by Committee on Animal Research of the Federal University of Santa Maria, Brazil (027/2013).

Experimental period

The ligature remained for a period of 7 days, then it was removed and treatments were applied. At 7, 15 and 30 days after baseline [21], all animals were anesthetized with isoflurane (2-3%, inhaled) (Isothane®, Baxter Healthcare® of Puerto Rico, Guayama, Porto Rico, USA) and euthanized by exsanguinations (Figure 1).

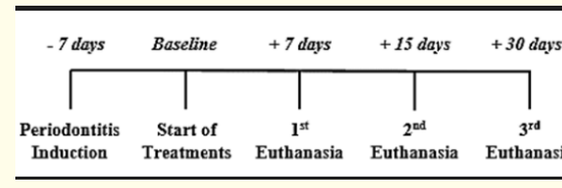

Figure 1: Experimental procedures.

Randomization and blinding operator

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

Periodontitis Induction Protocol

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

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

Table 1: Description of experimental groups.

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

Scaling and root planning protocol

The SRP protocol was manual performed using the micro Gracey curettes Mini-Five 1-2 (Hu-Friedy®, Chigaco, IL, USA) through 10 distal-mesial traction movements in the buccal and lingual sites. The furcation and interproximal areas were instrumented with the same curettes using cervico-occlusal traction movements [21].

Antimicrobial photodynamic therapy protocol

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

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

Histomorphometric analysis

Bone Loss

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

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

Inflammatory infiltrate

The gingival connective tissue from buccal region of periodontitis induction was removed and soaked in 10% neutral buffered formalin (pH 7.2) for 24 hours and then embedded in paraffin. Serial sections were cut with a thickness of 6 μm in a mesial-distal direction and staining with H&E. The intensity of inflammatory infiltrate was graded as absent, mild, moderate and severe at an objective magnification of 100x [24]. The specimens were evaluated by a single blinded examiner and characterized as absent (0) when it is characterized by absence of inflammatory cells in the connective tissue; mild (1) when presenting distribution sparse inflammatory cells in connective tissue; moderate (2) when found dense accumulation of inflammatory cells in isolated areas, sparse distribution in other areas of connective tissue and severe (3), when it presents dense aggregation of inflammatory cells throughout the connective tissue.

Immunohistochemical analysis

The blocks containing the subsequent sections of gingival connective tissue were used for immunohistochemical. VEGF immu-

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

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

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

Intra-examiner reproducibility

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

Results

Histomorphometric and immunohistochemical analysis

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

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

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

The intensity of inflammatory infiltrate was graded as absent (0); mild (1); moderate (2) and severe (3).

The immunoreactivity was graded in absence of staining (0); light to moderate staining (1) and intense staining (2).

NC: Negative Control; PC: Positive Control; SRP: Scaling and Root Planing; aPDT**:** Antimicrobial Photodynamic Therapy.

Histomorphometric analysis

- **NC group:** At 7 days a very discrete bone loss was observed in the furcation region [17] (Figure 2A). In gingival connective tissue most of the inflammatory intensity was mild (Figure 3). At 15 days and 30 days, it was observed gradual increase of absence cases of inflammatory infiltrate (Table 2).
- **PC group:** At 7 days, periodontal breakdown, characterized by alveolar bone loss was evidenced at furcation region [17] (Figure 2B). The gingival connective tissue showed inflammatory infiltrate of moderate characteristics (Figure 3) and also some

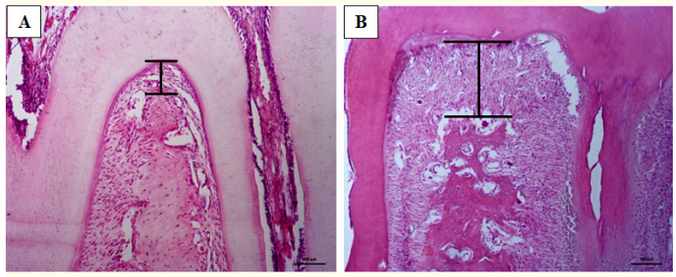

Figure 2: Linear measure of bone loss in the furcation region of the mandibular right first molar in 7 days, at an objective magnification of 100x. A) NC; B) PC. H&E staining.

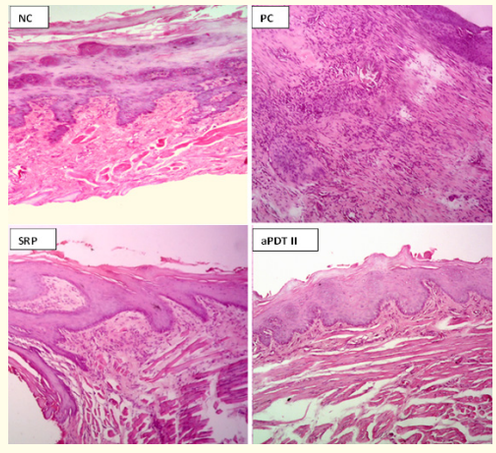

Figure 3: In the gingival connective tissue at 7 days, the intensity of inflammatory infiltrate was observed under an objective magnification of 100x NC (1); PC (2); SRP (1); aPDT II (1). H&E staining.

severe cases. At 15 days, it was not observed severe cases, most of cases still remained in moderate grade and there was inflammation absence. At 30 days, the number of mild grade and absence increased. No moderate cases was found (Table 2).

- **SRP group:** At 7 days, in all cases, mild inflammatory intensity (Figure 3) was observed. At 15 and 30 days, there was a gradual increase of cases with absence of inflammatory infiltrate (Table 2).
- **aPDT I group:** At 7 days, the inflammatory infiltrate was mild in most cases and cases with absence inflammatory infiltrate were observed. At 15 and 30 days, we observed the similar results for SRP group (Table 2).
- **aPDT II group:** At 7 days, very similar responses were observed to aPDT I (Figure 3), as well as at 15 days. At 30 days, aPDT II group presented almost all cases with total absence of inflammatory infiltrate, surpassing even the NC group (Table 2/Figure 4).

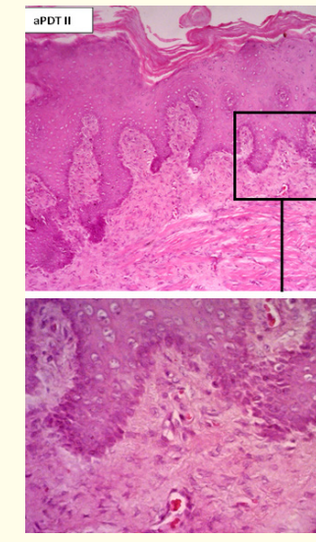

Figure 4: In the gingival connective tissue at 30 days, the intensity of inflammatory infiltrate was observed under an objective magnification of 100x, followed by 400x magnification of the highlighted area, aPDT II (0). H&E staining.

Immunohistochemical analysis

- **NC group:** At 7 (Figure 5), 15 and 30 days was observed the absence of immunostaining by VEGF, with very few exceptions (Table 2).
- **PC group:** At 7 days, the highest VEGF intensity of this study was observed, most cases presented light to moderate amounts of staining and some cases with severe staining (Figure 5). At 15 days, the behavior of this factor was similar to 7 days. However, at 30 days, was observed a decreased of this immunostaining (Table 2). Interestingly, only in this group and scattered by the experimental time there was in isolated cases the coloration of gingival epithelial tissue.
- **SRP group:** At 7 days, most cases showed absence of immunostaining. At 15 and 30 days, almost all the cases reached total absence staining (Table 2).
- **aPDT I group:** At 7 days, similar to SRP group and some light staining cases, 15 and 30 days this growth factor behavior was similar to SRP group (Table 2).
- **aPDT II group:** At 7 days, more light staining cases (Figure 5), 15 and 30 days, the immunostaining was similar to SRP and aPDT I group (Table 2).

Discussion

In the present study, we reproduced a previously reported model of experimental periodontitis in rats caused by the ligature presence in subgingival position in mandibular right first molar [16,17]. This induction model is characterized by accumulation of

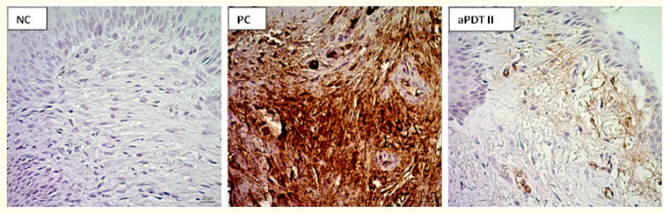

Figure 5: Immunoreactivity for VEGF in the gingival connective tissue at 7 days under magnification of 400x. NC (0); PC (2); aPDT II (1). Immunohistochemical staining.

plaque, flattening and displacement of the gingival crest, increased proliferation of epithelium into underlying connective tissue and infiltration of mononuclear inflammatory cells [27]. In our study, PC group, showed moderate inflammatory infiltration and significant alveolar bone loss in furcation region. On the other hand, NC presented mild inflammation and discrete bone alveolar loss. This statement is in agreement with previous histopathological reports that showing progressive bone alveolar loss and presence of mononuclear inflammatory intense in ligature-induced periodontitis in rats [21,28,29].

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

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

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

The Ps is a pivotal component of aPDT, which act at the surface of several types of bacterial to increase their permeability and allow a significant amount of Ps to accumulate at the level of the cytoplasmic membrane [37]. The changing possibility in Ps formulations can be a key point to increase the effectiveness for therapy. In short-time, the parameters evaluated in this study were very similar to the solutions of MB in water or in ethanol. Although, at 30 days we observed that, interestingly, in the aPDT II almost all the cases presented abstention of leukocyte infiltrate, surpassing the NC group. These findings, reinforced by Barin., *et al*. [17], showed that this same group at 30 days presented total vitamin C recovery, an important antioxidant in organism defense, reaching the same levels of the NC group, as also recovered the GSH, confirming that in the long-term there was a better local and systemic response of this therapy. On regarding VEGF, there was no immunostaining at all periods, with very few exceptions. A very similar behavior to other treated groups and NC group was observed.

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

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

However, there was a notable regional variation in the intensity of immunostaining for VEGF only in the PC group, with staining in gingival epithelial tissue, like some studies also point out [30,39], reinforcing the staining in non-vascular sources [43]. The changes in the vascularity of the periodontal connective tissues in untreated advanced periodontitis may be, in part, a consequence of altered expression of angiogenic activity by the epithelium. In turn, this may reflect to the epithelial response to microbial flora [34], which may prove our observations because the PC group is the most affected and represents the mechanism for biological defense epithelium. The discrepancy in the location of VEGF may also be justified by the difference in antibody protocols. In our study, we applied anti-human monoclonal antibodies for the parallel detection of three VEGF isoforms: VEGF-A (VEGF-121), VEGF-B (VEGF-165) and VEGF (clone VG1) diluted 1:100 and we obtained epithelial staining. Artese., *et al*. [30], provided no source of the antibodies applied to localize VEGF and even used different dilution than 1:50, also finding staining in epithelial tissue. In divergence [44,45], studies with the same dilution and VEGF, failed to detect this immunostaining in epithelial tissue.

Conclusions

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

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

Conflicts of Interest

The authors declare that they have no conflict of interest.

Statement of Human Rights

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

Statement on the Welfare of Animals

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

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