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Multiple aPDT sessions on periodontitis in rats treated with chemotherapy: Histomorphometrical, Immunohistochemical, Immunological and Microbiological Analyses.

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Abstract

Background: The aim of this study was to evaluate the effect of multiple sessions of antimicrobial photodynamic therapy (aPDT) on the treatment of experimental periodontitis (EP) in rats treated with chemotherapy.

Methods: Chemotherapy using 5-fluorouracil (5-FU) consisted of intraperitoneal administration of 60 and 40 mg/kg of 5-FU. 120 rats were subjected to chemotherapy with 5-FU and divided into groups: PT (periodontal treatment); PT+1aPDT (PT and single aPDT session); PT+4aPDT (PT and 4 sessions of aPDT); 1aPDT (single aPDT session); 4aPDT (4 sessions of aPDT). EP was induced in the mandibular molars via ligature placement. The alveolar bone loss (ABL) area in the furcation region was analysed histometrically. Proliferating cell nuclear antigen (PCNA), tartrate-resistant acid phosphatase (TRAP), receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG) and cleaved caspase-3 (CC3) were analysed by immunohistochemistry. Prostaglandin E2 was quantified using an ELISA, tumor necrosis factor (TNF)- α and interleukin (IL)-6 were assessed using a multiplex method. The prevalence of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella nigrescens*, *Prevotella intermedia* and *Fusobacterium nucleatum* was assessed using PCR. The data were statistically analysed ($P < 0.05$).

Results: The PT+4aPDT group showed lower ABL than the PT or 1aPDT groups on day 7. Rats treated with aPDT showed a higher number of PCNA-positive cells with reduced immunolabeling of RANKL. Significant reductions in *Prevotella nigrescens* were observed in the PT+4aPDT group and in *Aggregatibacter actinomycetemcomitans* for the 1aPDT and 4aPDT groups.

Conclusion: Repeated sessions of aPDT as an adjunct or alternative therapy were effective at reducing ABL, regulating bone metabolism, and reducing *Prevotella nigrescens* and *Aggregatibacter actinomycetemcomitans*.

Keywords: periodontitis; alveolar bone loss; chemotherapy; photochemotherapy; rats

1. Introduction

After surgery, chemotherapy and radiation are the interventions most frequently applied in the treatment of cancer [1]. During such treatment, it is common for changes in the oral cavity to occur: such changes include mucositis, ulceration, gingival bleeding [2], degeneration in oral keratinocytes [3] and altered collagen synthesis [4], as well as changes in connective tissue and the oral microbial environment [5]. Furthermore there is increased destruction of periodontal tissues [6,7] with progressive periodontal attachment loss [8], increased production of proinflammatory cytokines [9] and the remodelling ability of bone and soft tissue is compromised [10].

Immunosuppressive therapy therefore alters both periodontitis severity and progression with gingival overgrowth or ulceration and the migration of junctional epithelium [11, 12].

Recent studies shown a high prevalence of periodontal diseases in patients receiving life-long immunosuppressive therapy [13-15] along with increases in plaque, bleeding, and gingival indices [16, 17] and probing depth [18]. Indeed, studies in animals treated with chemotherapy have reported destruction and disorganization of all periodontal tissues, with the presence of large spicules of necrotic bone and intense inflammatory infiltrate, with a significant increase of alveolar bone loss in the furcation region [6, 7, 19-21].

5-Fluorouracil (5-FU) is a drug widely used in the treatment of carcinomas [22], is an analogue of the pyrimidine base uracil. It is converted intracellularly to active nucleotides

that interfere with the synthesis of RNA and DNA. Several studies have demonstrated that 5-FU causes cell death through apoptosis in tissue such as epithelial cell lines [23].

Non-surgical periodontal treatment is considered the gold standard for the treatment of periodontitis; scaling and root planing (SRP) is often not able to completely eliminate the infection and associated inflammation, especially in individuals with systemic involvement and those prone to more severe cases of periodontitis [24, 25]. Therapeutic approaches to the suppression of potential pathogens in the oral microflora with antibiotics to decrease the risk of infections in patients undergoing chemotherapy have been proposed. These approaches have sometimes been successful [26], but they can also promote increased bacterial resistance [27].

Antimicrobial photodynamic therapy (aPDT) is based on the inactivation of target microorganisms by the use of a photosensitizer and a light source (laser or LED), which can form cytotoxic singlet oxygen and free radicals locally. These are detrimental to components of microorganisms [28] and can damage proteins, lipids, nucleic acids and other cellular components such as lysosomes and cell membranes [29]. aPDT can reduce periodontal pathogens such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Pseudomonas aeruginosa* *in vitro* and *in vivo* [30, 31]. Another study demonstrated that the mechanism of action of aPDT is not limited to its bactericidal properties; it can contribute to periodontal healing processes through the inactivation of inflammatory cytokines interleukin (IL)-1 β and tumor necrosis factor (TNF)- α through a mechanism independent of bacterial lysis [32]. Therefore, aPDT may contribute to restoring periodontal homeostasis and promote rapid repair after treatment [33]. This can be a beneficial therapy for patients who present with systemic alterations, who tend to have an altered biological response in the periodontal tissues during the repair process after SRP procedures [34].

The use of aPDT has the advantage of being a minimally invasive therapy, easy to apply, inexpensive, causing no discomfort. In addition, it can be performed in the hospital environment for patients undergoing chemotherapy as an alternative approach to periodontal treatment.

Recent studies have shown satisfactory results in the use of aPDT in the treatment of experimental periodontitis (EP) in animals [30, 35-45] and humans [46-54] and mainly the modified systemically as nicotine, diabetes and immunosuppression by chemotherapeutics [55]. aPDT as monotherapy can also be considered a non-invasive approach compared to conventional treatment methods and to be advantageous in terms of modulation of cytokines [56]. With respect to multiple aPDT sessions in periodontal treatment there are studies demonstrating a reduction in depth of probing and reduction of bleeding [54, 55, 57,58]. However, the study by Petelin et al. [59] who performed 3 applications of aPDT (1,3 and 7 days after RAR) showed no reduction in depth of probing and gain of clinical attachment level, but there was a reduction in bleeding at 3 and 12 months.

These controversial results are due to the variation of different treatment protocols used in aPDT, regarding the use of lasers (pre irradiation time, mode, number and frequency of laser irradiation), the number of applications of aPDT, the type of photosensitizer, the concentration and the residence time of the phototosensitizer in the periodontal pocket [60].

Conversely, there are only rare studies evaluating the effect of aPDT in periodontal treatment in conditions of immunosuppression by chemotherapy [44]. Therefore, this study aimed to assess the effects of multiple sessions of aPDT, as adjunctive or alternative therapy, in EP in animals subjected to 5-FU chemotherapy through histological, histometric, immunohistochemical, immunological and microbiological evaluations.

2. Materials and Methods

This study was carried out with 120 male adult (3-month-old) rats (*Rattus norvegicus*, albinus, Wistar) weighing from 200 to 300 g. The rats were kept in plastic cages with access to food and water *ad libitum*. Prior to the surgical procedures, the rats were allowed to acclimatize to the laboratory environment for a period of 2 weeks. Procedures for experimental manipulation were carried out according to the guidelines established by the "Guide for the Care and Use of Laboratory Animals" (ARRIVE) and the experimental protocol was approved by the Ethics Committee on Animal Use (CEUA) of the School of Dentistry, Araçatuba Campus, São Paulo State University. All procedures for the experimental use of animals were performed under anaesthesia with ketamine hydrochloride (70 mg/kg; Vetaset – Fort Dodge, IA, USA) with xylazine chlorhydrate (6 mg/kg; Coopazine, Coopers, SP, Brazil) via intramuscular injection. Experimental periodontitis was induced through the presence of a cotton thread (Corrente Algodão nº 24, Coats Corrente, SP, Brazil) around the right and left first mandibular molars for a seven-day period [42]. The treatment with 5-FU (5-fluorouracil; 50 mg/ml; Eurofarma Laboratórios, SP, Brazil) consisted of intraperitoneal administration of 60 and 40 mg/kg of 5-FU (30) on the day of ligature placement and 48 h after this procedure, respectively, followed the protocol reported followed the protocol reported by Theodoro et al. [7].

The ligature was removed seven days after installation, and the animals were randomly assigned by a computer-generated table distributed into five groups: PT (periodontal treatment) (n=24) – systemic treatment with 5-FU and scaling and root planing (SRP); PT+1aPDT (n=24) – systemic treatment with 5-FU and SRP followed by a single aPDT session; PT+4aPDT (n=24) – systemic treatment with 5-FU and SRP followed by four aPDT sessions at 0, 24, 48 and 72 h, 1aPDT (n=24) – systemic treatment with 5-FU followed by single aPDT session; and 4aPDT (n=24) – systemic treatment with 5-FU followed by four aPDT sessions at 0, 24, 48 and 72 h (Figure 1).

SRP was performed using mini-five hand curettes (#1-2, Hu-Friedy Co. Inc., Chicago, IL, USA) through 10 distal–mesial traction motions at the buccal and lingual aspects and 10 cervical–occlusal traction motions at the interproximal and furcation regions followed the protocol reported by Garcia et al. [42].

2.1. Antimicrobial Photodynamic Therapy (aPDT)

For the aPDT treatment, SRP of the left mandibular first molar was performed in the groups PT, PT+1aPDT and PT+4aPDT. Methylene blue (MB) solution (Sigma Chemical Co., St. Louis, MO, USA) solution (100 µg/mL) [42] was slowly poured into the periodontal pocket around the left mandibular first molar, using a syringe (1 mL) and an insulin needle without a bevel [42]. After 1 min of MB application, laser light was applied to one point at each buccal and lingual aspect of the left mandibular first molar in contact with the gingivae in groups treated with aPDT. At the time of the aPDT there was no blood at the area. The laser irradiation followed the protocol reported by Theodoro et al. [7]. An Indium-Gallium-Aluminium-Phosphorus (InGaAlP) laser (THERA LASE®, D.M.C. Equipamentos Ltda®, São Carlos, SP, Brazil) with a 660-nm wavelength and a 0.0283-cm² output fibre diameter was used in this study. The irradiation was performed in each session in both first molars (left and right) with 0.035 W power for 60 seconds in the centre of the labial surface and for 60 seconds in the centre of the lingual surface (4.2 J total energy). The tooth received 74.2 J/cm² (2.1 J/point) energy density per point.

2.2. Laboratory processing for histological, histometric and immunohistochemical analysis

Eight animals of each group were subjected to euthanasia and the left hemimandibles were removed and fixed in 4% formaldehyde for 48 hours for histological and immunohistochemical analysis. Five equidistant sections were stained with haematoxylin and eosin (HE) for histological and histometric analysis. For the indirect immunoperoxidase method, five sections were subjected following primary antibodies: anti-proliferating cell nuclear antigen (PCNA) (Vector Laboratories, Burlingame, CA, USA); anti-tartrate-resistant acid phosphatase (TRAP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-receptor activator of nuclear factor kappa-B ligand (RANKL) (Santa Cruz Biotechnology); anti-osteoprotegerin (OPG), (Santa Cruz Biotechnology) and anti-cleaved caspase-3 (CC3) (Cell Signalling Technology, Beverly, MA, USA). Immunohistochemical processing followed the protocol reported by Garcia et al.[40].

2.3. Processing for microbiological and immunological analysis

One sample was collected from the gingival collar bordering the right mandibular first molar of all animals and one sample of subgingival plaque was collected from the buccal region of the left mandibular first molar at baseline and 7, 15 and 30 days after treatment of all animals. Sterile absorbent paper cones (#20 Tanari, Manacapuru, AM, Brazil) were placed inside the gingival sulcus for 30 s [7] and were removed and stored in phosphate-buffered saline (PBS, pH 7.0) at -80°C [7]. The subgingival samples were thawed, vortexed and centrifuged (10,000 rpm/10 min). After removal of the paper points and supernatant, samples were submitted to DNA extraction as described by Sardi et al. [61] . Briefly, samples were lysed with extraction buffer and treated with proteinase K. They were then purified using chloroform and isoamyl

alcohol, followed by DNA precipitation with isopropanol and washing with 70% ethanol. The DNA was re-suspended in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5), with 10 µg/mL RNase and stored at -20 °C for qPCR analysis. DNA concentration and quality were analyzed using a Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at 260 nm. For samples less than 10 ng/µl, DNA was amplified using Ready-to-go GenomiPhi V3 DNA Amplification Kit (GE Healthcare, Piscataway, NJ, USA). Samples were analyzed by Polymerase Chain Reaction using specific primers for: *A. actinomycetemcomitans* [62], *P. gingivalis* [63], *P. nigrescens*, *P. intermedia* [64] and *F. nucleatum* [65]. PCR amplifications were performed using 200µM dnTPs, 2.5 mM of MgCl₂, 0.3µM of each primer, 1.25U Taq DNA Polymerase and 10ng of genomic DNA to obtain a volume of 25µl. Thermal conditions were: DNA denaturation at 95° C for 5 min, 35 cycles at 95° C for 30s, primer hybridization at 55° -62° C (depending on the primer) for 30s and extension at 72° C for 1 min and finalizing the reaction at 72° C for 7 min. The PCR products were separated by electrophoresis in 2% agarose gels containing Syber Safe staining on Tris-Borate-EDTA running buffer. Gels were visualized under UV illumination and molecular bands compared with a 100 pB DNA Ladder. Specificity of primers was tested using *in silico* PCR tools such as NCBI BLAST page (https://blast.ncbi.nlm.nih.gov/Blast.cgiPAGE_TYPE=BlastSearch) and NetPrimer launch (<https://www.premierbiosoft.com/netprimer/>).

One sample was collected from the gingival collar bordering the right mandibular first molar of all animals, frozen in liquid nitrogen and stored at -80°C [66]. Total proteins were extracted from gingival tissue samples using extraction buffer (Tissue Protein Extraction Reagent [T-PER], Pierce Biotechnology; Thermo Fischer Scientific, Rockford, IL, USA) containing protease inhibitor cocktail (Protein Stabilising

Cocktail; Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). Tissue samples were ground in buffer (50 μ L/mg tissue) and centrifuged for five minutes at 16,000 g at 4°C, and the supernatant was discarded [66].

2.4.Histological and histometric analysis

Histological analysis of the periodontal tissues was performed in light microscopy by a single blinder and calibrated examiner that evaluated the following parameters: 1) intensity of local inflammatory response; 2) extension of inflammatory process; 3) external root resorption; 4) pattern of structuration of the connective tissue; and 5) pattern of structuration of the bone alveolar (Table 1).

The area of alveolar bone loss (ABL) was measured in each animal in five sections equidistant from the furcation region using an image analysis system (Axiovision 4.8.2, Carl Zeiss MicroImaging GmbH, 07740 Jena, Germany) according to the method of Garcia et al. [42] by a single blinder and calibrated examiner.

2.5.Immunohistochemical analysis

A treatment-blinded, trained examiner selected the sections and another treatment-blinded examiner conducted the data analysis. Quantitative analyses of the immunolabeling cells were performed for TRAP, PCNA and CC3, and semi-quantitative analyses of RANKL and OPG immunolabeling were performed followed the protocol reported by Theodoro et al. [7].

Before the histometric and immunohistochemical analyses were performed, the examiner was trained by double measurements of 30 specimens with a 1-week interval. Paired t-test statistics were calculated, and no differences were observed between the mean values of the two sets of measurements ($P < 0.05$). Additionally, the Pearson correlation coefficient revealed a very high correlation (0.95) between the two sets of measurements for both the histometric and immunohistochemical analyses.

2.6. Analysis of inflammatory mediators

The quantification of proteins extracted from gingival tissues was performed using the Lowry method, and the sample proteins were analysed by enzyme-linked immunosorbent assay (ELISA) to assess prostaglandin E_2 (PGE_2) concentrations (ELISA Kit for Prostaglandin E_2 , CEA538Ge, Life Science Inc., USCNK, Cologne, Germany). These tests were performed according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA), and the spectrophotometer results were normalised to the total protein concentrations in the samples. Multiplex kits (Rat Cytokine/Chemokine MAGNETIC Bead Panel - 2Plex [IL-6/TNF- α], Millipore, RCYTOMAG-80K-02, Billerica, MA, USA) were used to assess IL-6 and TNF- α , and the analysis was performed using a Luminex II xMap 100/200 (Millipore, LX10009314401, Austin, TX, USA) [7].

2.7. Microbiological analysis

To confirm primer specificity or universality, the samples were analysed in agarose gel (1.5%) stained with SYBR Safe DNA gel stain (Invitrogen, São Paulo, Brazil, S33102) in a horizontal electrophoretic system (Major Science, Hoefer, Inc., Richmond, CA, USA) connected to an electrophoresis power source (Smart Rosa Source, Major Science, 50-60 Hz, 144 VA Hoefer, Inc., San Francisco, CA, USA) at 96 V for 40 minutes and immersed in Tris-

boric acid-EDTA (TBE) buffer solution. At the end of each run, the gel bands were immediately observed with an ultraviolet light transilluminator and subsequently photographed using a digital imaging system (UVP, mod. GelDoc-It B 2310, Upland, CA, USA) [7].

2.8. Statistical analysis

Calculation of sample size $n=8$ showed a 85% study power ($p<0.05$). Software (Bioestat – version 5.3, Instituto Mamirauá, Manaus, Brazil) was used to conduct the statistical analyses ($\alpha=5\%$). The Shapiro-Wilk test was used for normality analysis, and analysis of variance (ANOVA) followed by Tukey's post-test was used for the intra- and inter-group analyses of body weight, ABL, PCNA, TRAP and CC3. The nonparametric Kruskal-Wallis test followed by the Student-Newman-Keuls post-test was used for RANKL, OPG, PGE₂, TNF- α and IL-6 dosages. The Chi-squared test was used for the intra-group analysis of the prevalence of microorganisms, and the Mann-Whitney test was used for the inter-group analysis.

3. Results

3.2. Clinical analysis

The body weight data are shown in Table 2. The animals of all groups showed progressive weight gain, when compared with the baseline period in which the animals were treated with the chemotherapeutic (5-FU). In the intragroup analysis, rats from PT, PT + 4aPDT and 4aPDT groups showed higher body weight at 15 days (374.00 ± 45.24 , 329.50 ± 50.49 ; 389.25 ± 22.39) and 30 days (421.75 ± 27.47 , 409.00 ± 39.09 , 419.12 ± 36.21) than rats from the baseline (314.16 ± 36.5 , 276.33 ± 35.58 , 347.87 ± 32.95). Rats from PT + 1aPDT group showed higher body weight at 30 days (392.62 ± 36.81) and rats from 1aPDT group showed

higher body weight at 15 days (388.12 ± 28.18) than rats from the baseline (281.37 ± 36.96 , 338.58 ± 30.39).

In the intergroup analysis when the different treatments were compared, rats from PT + 1aPDT (281.37 ± 36.96) and PT + 4aPDT (276.33 ± 35.58) groups showed lower body weight than rats from the PT group (314.16 ± 36.5) at the baseline. Rats from PT + 4aPDT (273.00 ± 37.98) and 1aPDT (318.25 ± 41.16) groups showed lower body weight than rats from the PT group (341.75 ± 33.04) at 7 days. Rats from PT + 1aPDT group (304.50 ± 39.31) showed lower body weight than rats from the PT group (374.00 ± 45.24) at 15 days. Rats from 1aPDT group (367.87 ± 22.87) showed lower body weight than rats from the PT group (421.75 ± 27.47) at 30 days. Rats from 4aPDT (347.87 ± 32.95 ; 389.25 ± 22.39) showed higher body weight than rats from the PT group (314.16 ± 36.5 ; 374.00 ± 45.24) at baseline and 15 days.

3.3.Histological and histometric analyses

The histological analysis results are shown in Table 1. PT+4aPDT and 4aPDT groups show lower magnitude of local inflammation and more favourable progression in the periodontal repair process (Figure 2).

The histometric analysis results are shown in Figure 2. In the intergroup analysis, when the different treatments were compared, rats from the PT+4aPDT ($1.27 \pm 0.39 \text{ mm}^2$) group showed lower ABL than rats from the PT ($2.31 \pm 0.30 \text{ mm}^2$) and 1aPDT ($2.16 \pm 0.30 \text{ mm}^2$; $p < 0.05$) groups on day 7. Rats from the PT+4aPDT ($1.41 \pm 0.59 \text{ mm}^2$) and 4aPDT ($1.47 \pm 0.68 \text{ mm}^2$) groups showed lower ABL compared with rats from the PT ($2.45 \pm 0.53 \text{ mm}^2$; $p < 0.05$) group on day 30 (Figure 2).

3.4.Immunohistochemical analysis

A higher number of TRAP-positive cells was detected on day 7 in the PT and 1aPDT groups compared to the PT+4aPDT group, and rats from the PT group showed higher numbers of TRAP-positive cells than the PT-1aPDT group ($p < 0.05$). A lower number of TRAP-positive cells was detected on day 15 in the PT+4aPDT group than in the PT or 1aPDT groups (Figures 3a, 3b, 3c, 3d, 3e, 3f).

Immunolabeling of PCNA was found predominantly in connective tissue and in bone surface cells. Rats of the PT+1aPDT, PT+4aPDT, 1aPDT, and 4aPDT groups showed a higher number of PCNA-positive cells than rats of the PT group on day 7; rats of the PT+1aPDT group showed a higher number of PCNA-positive cells than rats of the PT group on day 15. The intragroup comparison revealed significant reductions in PCNA immunolabeling in the 1aPDT group on days 15 and 30 when compared with day 7 ($p < 0.05$; Figures 3g, 3h, 3i, 3j, 3k, 3l).

The intergroup comparison revealed significant reductions in RANKL immunolabeling on day 7 in the PT+4aPDT and 4aPDT groups when compared with PT ($p = 0.0227$; $p = 0.0068$), and it revealed significant reductions in RANKL immunolabeling in the 4aPDT group when compared with the PT+1aPDT ($p = 0.0493$) and 1aPDT ($p = 0.0493$) groups. Rats of the PT+4aPDT and 4aPDT groups revealed significant reductions in RANKL immunolabeling on days 15 ($p = 0.0012$) and 30 ($p = 0.004$) when compared with the PT group. Rats of the PT+4aPDT and 4aPDT groups had significant reductions in RANKL immunolabeling on day 15 when compared with the PT+1aPDT ($p = 0.0323$) and 1aPDT ($p = 0.0323$) groups and on day 30 when compared with the 1aPDT ($p = 0.0323$) group. The intragroup comparison revealed significant reductions in RANKL immunolabeling in the PT+4aPDT group on days 15 and 30 when compared with day 7 ($p = 0.0339$) (Figures 4a, 4c, 4d, 4e, 4f, 4g).

No significant differences were observed in OPG (Figures 4b, 4h, 4i, 4j, 4k, 4l) immunolabeling. For CC3 in all experimental groups there was no statistically significant difference intra-group or inter-group for any of the evaluated periods (Figures 4m, 4n).

3.5. Analysis of biomarkers

Analysis of the presence of the proinflammatory biomarkers TNF- α and IL-6 revealed no significant differences between groups ($p > 0.05$). The PT+4aPDT group exhibited lower concentrations of PGE₂ at 30 days when compared to 7 days. The PT+1aPDT group exhibited lower concentrations of TNF- α on days 15 and 30 than on day 7; the PT+4aPDT group exhibited lower concentrations on day 30 than on days 7 and 15; and groups 1aPDT and 4aPDT exhibited lower concentrations on day 30 than on day 7 ($p < 0.05$). The PT+1aPDT group exhibited lower concentrations of IL-6 on day 15 than on day 7; the PT+4aPDT group exhibited lower concentrations of IL-6 on day 30 than on days 7 and 15; and the 1aPDT and 4aPDT groups exhibited lower concentrations on day 30 than on day 7 ($p < 0.05$).

3.6. Microbiological analysis

P. intermedia was not detected in any group at any study time point. Significant reductions in *P. nigrescens* were observed on days 7 and 30 in the PT+4aPDT group compared with baseline ($p < 0.05$). Significant reductions in *A. actinomycetemcomitans* were observed on day 30 when compared with baseline and on day 7 for the 1aPDT group ($p < 0.05$). The 4aPDT group showed significant reductions in *A. actinomycetemcomitans* on day 30 when compared with baseline on days 7 and 15, and significant reductions in *P. nigrescens* on days 15 and 30 when compared with baseline on day 7 ($p < 0.05$).

4. Discussion

In the analysis of the results in this study, it was observed that periodontitis in animals treated with 5-FU presents a significant ABL in the furcation region. These results indicate that 5-FU worsened the periodontitis and are in agreement with the results of other studies by our team showing that 5-FU reduced the percentage of bone in the furcation region, with large areas of necrotic bone involving almost the entire bone in the furcation [6, 7, 44]. 5-FU is an antineoplastic drug widely used in the treatment of solid neoplastic lesions and promotes the alteration of basal cells, reduces osteoblasts, increases osteoclasts and promotes increase of bone-resorption activity [3, 18, 67,68].

The results of this study demonstrated that multiple sessions of aPDT as adjunct or alternative therapy promotes the healing processes, minimizing the magnitude of the local inflammatory response and accelerating the process of tissue restructuring in these animals.

The PT+4aPDT group showed lower ABL than rats from the PT or 1aPDT groups on day 7. On day 30, in animals treated with multiple sessions of aPDT as adjunctive and alternative treatment, there was a decrease in ABL in the area of furcation compared to animals that were treated with PT only. In PT+4aPDT group there was less ABL at 7 and 30 days when compared to day 15. These findings corroborate previous studies' results demonstrating a reduction in ABL in EP after adjuvant treatment with one episode of aPDT [37,40-42].

In previous study it was showed that one session of aPDT as adjunctive treatment is more effective than the use of photobiomodulation by using a low-level laser in rats subjected to other protocol of chemotherapy [44]. However, in this study it was observed that the number of applications of aPDT also can influence the ABL in animals subjected to chemotherapy by 5-FU. The protocol of aPDT used in the present study was based also on another experimental study [44]. The use of one session of aPDT as an adjunctive treatment

of experimental periodontitis under immunosuppression by high doses of corticoid has been also been reported elsewhere [39]. In the previous study toluidine blue-O was used as the photosensitizer, with a low-level laser (660 nm; 24J). The results showed also that aPDT was effective in reducing bone loss in normal and immunosuppressed animals. Beneficial effects of aPDT could be explained also by increased angiogenesis supplying more oxygenation, as well as the anti-microbial effect [39]. Vasodilation and angiogenesis may also have influenced the periodontal repair, because collagen secretion by fibroblasts in the extracellular space occurs only in the presence of high levels of oxygen [34]. The histological analysis of PT+4aPDT and 4aPDT groups showed a lower magnitude of local inflammation and a more favourable periodontal repair process. Analysis of PCNA was performed to analyse the effects of aPDT on fibroblasts and osteoblasts. Animals treated with aPDT showed greater numbers of PCNA-positive cells at 7 days, so had increased cell proliferation in early periods, corroborating the findings of another study in rats [40], probably due to the effect of aPDT on reducing periodontopathogens and the possible effect of photobiomodulation of the low-level laser used [7].

Furthermore, PGE₂ levels were reduced at 30 days in animals treated with 4 episodes of aPDT as adjuvant. The high level of PGE₂ can be related to the occurrence and development of the periodontal disease process. The increase of PGE₂ secretion that occurs during the process of ABL induced by inflammation plays a crucial role in promoting the expression of TNF- α activity [69]. In this study this reduction occurred while there was a decrease in ABL.

The results also showed that there was a significant reduction of TNF- α and IL-6 in animals treated with aPDT adjuvant PT. In animals that were only treated with aPDT, there was a significant reduction of TNF- α and IL-6 at 30 days and a reduction in the tissue destruction process. TNF- α is a proinflammatory mediator that may control the destruction of

bone and connective tissue and IL-6 is a cytokine that plays a key role in the immune and inflammatory responses in periodontitis [70, 71].

The reduction of these biomarkers in animals treated with aPDT may have affected the reduction of the bone tissue destruction process, which is worsened by chemotherapy. Corroborating the results of this study, it has been demonstrated that aPDT has the potential to inactivate inflammatory cytokines such as IL-1 β and TNF- α [32]. Other authors have demonstrated in humans that there was a reduction of TNF- α in areas treated with aPDT as adjuvant to PT [72].

In this study, those animals treated with aPDT presented a reduction of pro-inflammatory biomarkers, which can be related to the reduction in the expression of RANKL, which binds directly to RANK on the surface of pre-osteoclasts and osteoclasts, observed at all evaluation times. These facts were also confirmed by the significant reduction of TRAP-positive cells, which demonstrate osteoclast activity, in the animals treated with several episodes of aPDT. These results are corroborated by previous studies that demonstrated greater RANKL immunolabeling in animals treated with 5-FU [6] and lower numbers of TRAP-positive cells and RANKL in animals treated with aPDT [40, 42]. These inflammatory and immunomodulation effects during therapy may accelerate wound healing and bone repair which may not occur during a mechanical treatment during a period of chemotherapy [6].

On the other hand the results of this study did not demonstrate high levels of CC3 in the animals treated with 5-FU, and no effects of the treatments on this marker were observed.

There are limitations of the animal model in representing all aspects of human periodontal disease [73]. As observed in human periodontitis, alveolar bone loss in the ligature model is dependent upon bacteria [74]. *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia* and *F. nucleatum* are the main periodontopathogenic microorganisms [7]. In the present study, after 7 days of EP, the presence of *A. actinomycetemcomitans*, *P. gingivalis*, *P.*

nigrescens and *F. nucleatum* was observed; however, *P. intermedia* was not detected, a fact corroborated by another study of ligature induction in rats [75]. This showed that in animals treated with 5-FU no reduction of any species of bacteria tested were found at 30 days while in animals not treated with 5-FU the detection of *P. nigrescens* and *F. nucleatum* was decreased at 30 days in experimental periodontitis.

Several sessions of aPDT were able to promote reduction of the detection of some species of periodontopathogenic bacteria such as *A. actinomycetemcomitans* and *P. nigrescens* during the evaluation period. A previous study in animals confirmed the reduction of *A. actinomycetemcomitans* after one application of aPDT in sockets previously contaminated by periodontal disease [76].

aPDT is based on the principle of a photosensitizer that binds to target cells and is activated by light of an appropriate wavelength. Subsequent electronic promotion and interaction with *in situ* oxygen leads to the production of free radicals (Reaction Type I) and singlet oxygen (Reaction Type II), which are toxic to bacteria, typically due to the destruction of membrane, cell wall or DNA [77]. The use of a low level red laser can also change host cell behaviour by affecting the mitochondrial respiratory chain or membrane calcium channels, facilitating collagen synthesis, angiogenesis and the release of growth factors that accelerate healing [78].

One possible limitation of this study is lack of a methylene blue-alone treatment group. This fact is justified because several studies of our group showed that adjunctive use of aPDT causes a significant improve of periodontitis, when compared with use of phenothiazinium photosensitizers or SRP alone in rats [37, 34, 42, 39].

5. Conclusion

Based on the results of this study, it was concluded that multiple sessions of aPDT as an adjunct or alternative therapy promote a reduction of periodontopathogenic bacteria and minimize the deleterious effects of 5-FU on periodontal tissues in rats.

Conflict of interest: The authors declare that they have no conflict of interest. Mariéllen Longo declares that he has no conflicts of interest. Valdir Gouveia Garcia declares that he has no conflicts of interest. Edilson Ervolino declares that he has no conflicts of interest. Márcio Luiz Ferro Alves declares that he has no conflicts of interest. Cristiane Duque declares that she has no conflicts of interest. Mark Wainwright declares that he has no conflicts of interest. Letícia Helena Theodoro declares that she has no conflicts of interest.

Research involving animals – ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Table 1. Parameters, scores and distribution of specimens according to histopathological analysis in PT, PT+1aPDT, PT+4aPDT, 1aPDT and 4aPDT groups at different study time points.

PARAMETERS AND RESPECTIVE SCORES	PERCENTAGE OF THE ANIMALS														
	Experimental groups and time points														
	PT			PT+1aPDT			PT+4aPDT			1aPDT			4aPDT		
	7d	15d	30d	7d	15d	30d	7d	15d	30d	7d	15d	30d	7d	15d	30d
INTENSITY OF LOCAL INFLAMMATORY INFILTRATE															
(0) Absence of inflammation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(1) Small amount of inflammatory cells	0	0	0	0	0	25	0	25	62	0	0	25	0	25	38
(2) Moderate amount of inflammatory cells	0	75	100	38	100	75	62	75	38	75	100	75	75	75	62
(3) Large amount of inflammatory cells	100	25	0	62	0	0	38	0	0	25	0	0	25	0	0
EXTENSION OF INFLAMMATORY INFILTRATE															
(0) Absence of inflammation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(1) Extending to part of the connective tissue of the furcation area	0	0	0	0	0	25	0	25	62	0	0	25	0	25	38
(2) Extending to the whole connective tissue of the furcation area	0	75	75	38	100	75	62	75	38	75	100	75	75	75	62
(3) Extending to the whole connective tissue and to the bone tissue of the furcation area	100	25	25	62	0	0	38	0	0	25	0	0	25	0	0
EXTERNAL ROOT RESORPTION (CEMENTUM AND DENTIN)															
(0) Absent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(1) Only inactive resorption areas	0	25	25	0	25	25	25	38	38	38	62	62	38	62	62
(2) Few active resorption areas	100	75	75	100	75	75	75	62	62	62	38	38	62	38	38
(3) Many active resorption areas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ALVEOLAR BONE RESORPTION															
(0) Within normality patterns	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0
(1) Small amount of resorption bone areas	0	0	0	38	62	62	62	62	62	25	25	38	25	25	38
(2) Moderate amount of resorption bone areas	0	25	38	62	38	38	38	38	25	75	75	62	75	75	62
(3) Large amount of resorption bone areas	100	75	62	0	0	0	0	0	0	0	0	0	0	0	0
PATTERN OF STRUCTURATION OF THE CONNECTIVE TISSUE															
(0) Moderate amount of fibroblasts and large amount of collagen fibers (dense connective tissue)	0	0	0	0	0	0	0	25	25	0	0	25	0	0	25
(1) Moderate amount of both fibroblasts and collagen fibers	0	0	0	0	25	25	0	50	50	0	62	50	0	62	50
(2) Small amount of both fibroblasts and collagen fibers	0	25	38	75	50	50	100	25	25	100	38	25	100	38	25
(3) Severe tissue breakdown with necrotic areas	100	75	62	25	25	25	0	0	0	0	0	0	0	0	0
PATTERN OF STRUCTURATION OF THE BONE ALVEOLAR															
(0) Bone trabeculae with regular contour coated with active osteoblasts, including areas of new bone formation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(1) Bone trabeculae with irregular contour coated with active osteoblasts and osteoclasts	0	0	0	0	0	38	0	38	38	0	0	25	0	0	25
(2) Bone trabeculae with irregular contour coated with active osteoclasts	0	25	25	75	100	62	75	62	62	75	100	75	75	100	75
(3) Areas of necrotic bone and bone trabeculae with irregular contour coated with active osteoclasts	100	75	75	25	0	0	25	0	0	25	0	0	25	0	0

Table 2. Mean and standard deviation (M±SD) of body weight (g) in each group and period.

Groups	Periods			
	Baseline	7 days	15 days	30 days
PT	314.16 ± 36.5	341.75 ± 33.04	374.00 ± 45.24*	421.75 ± 27.47*
PT+1aPDT	281.37 ± 36.96 [§]	289.62 ± 37.47 [§]	304.50 ± 39.31 [§]	392.62 ± 36.81*
PT+4aPDT	276.33 ± 35.58 [§]	273.00 ± 37.98 [§]	329.50 ± 50.49*	409.00 ± 39.09*
1aPDT	338.58 ± 30.39	318.25 ± 41.16 [§]	388.12 ± 28.18*	367.87 ± 22.87 [§]
4aPDT	347.87 ± 32.95 [§]	340.25 ± 34.77	389.25 ± 22.39 ^{*§}	419.12 ± 36.21*
N	120	40	40	40

* Statistically significant difference with baseline in the same group (p<0.05; ANOVA and Tukey test).

[§] Statistically significant difference with PT Group in the same period (p<0.05; ANOVA and Tukey test).

Figure 1. Timeline of study.

Figure 2. Histological and histometric analysis of the periodontal tissue of the left mandibular first molar. (a) Graph showing the mean and SD of the area of alveolar bone loss (mm²) in the furcation regions of the mandibular first molars in different experimental groups and at different study time points. (b-u) Photomicrographs of the mandibular first molar with experimental periodontitis showing the magnitude of the local inflammatory response, level of alveolar bone loss and periodontal repair process in the PT (b, g, l, q), PT+1aPDT (c, h, m, r), PT+4aPDT (d, i, n, s), 1aPDT (e, j, o, t) and 4aPDT (f, k, p, u) on days 7 (b-k) and 30 (l-u). Abbreviation and Symbols. ab, alveolar bone; nb, necrotic bone remains; *, infiltrate inflammatory; †, significant difference compared with PT group at the same study time point; ‡, significant difference compared with 1aPDT at the same study time point. Magnification: (b–f) and (l–p): 40X; (g–k) and (q–u): 160X. Scale bars: (b–f) and (l–p): 500 µm; (g–k) and (q–u): 100 µm. Stain: haematoxylin and eosin.

Figure 3. TRAP and PCNA immunolabeling in the furcation region of the left mandibular first molar. (a) Graph showing the mean and SD of the numbers of TRAP-positive cells/mm² in different experimental groups and at different study time points. (b-f) Photomicrographs showing the TRAP-positive cells (arrows) on day 7 in the PT (b), PT+1aPDT (c), PT+4aPDT (d), 1aPDT (e) and 4aPDT (f). (g) Graph showing the mean and SD of the numbers of PCNA-positive cells/mm² in the different experimental groups and at different study time points. (h-l) Photomicrographs showing the PCNA-positive cells (arrows) on day 7 in the PT (h), PT+1aPDT (i), PT+4aPDT (j), 1aPDT (k) and 4aPDT (l). Abbreviation and Symbols: ab, alveolar bone; †, significant difference compared with PT group at the same study time point; ‡, significant difference compared with 1aPDT at the same study time point; ¶, significant difference compared with day 7 at the same experimental group. Magnification: (b–f) and (h–l): 1000X. Scale bars: (b–f) and (h–l): 25 µm. Counterstaining: (b–f): Harris's haematoxylin; (h–l): Fast Green.

Figure 4. Immunolabeling of RANKL, OPG and CC3 in the furcation region of the left mandibular first molar. (a-b) Graph showing the distribution of scores regarding the immunolabeling patterns of RANKL (a) and OPG (b). (c–l) Photomicrographs showing the

immunolabeling patterns of RANKL (blue arrows) on day 7 and of OPG (blue arrows) on day 15 in the PT (c, h), PT+1aPDT (d, i), PT+4aPDT (e, j), 1aPDT (f, k) and 4aPDT (g, l). (m) Graph showing the mean and SD of the number of CC3-positive osteocytes/mm² of alveolar bone in different experimental groups and at different study time points. (n) Photomicrographs showing a CC3-negative osteocyte (red arrows) and a CC3-positive osteocyte (blue arrows) in the alveolar bone. Abbreviation and Symbols: ab, alveolar bone; §, significant difference compared with day 15 and 30 at the same experimental group; †, significant difference compared with PT group at the same study time point; ‡, significant difference compared with 1aPDT at the same study time point; ¶, significant difference compared with PT+1aPDT at the same study time point. Magnification: (c-l) 1000X; (n): 5000X. Scale bars: (c-l) 25 µm; (n): 5 µm. Counterstain: Harris's haematoxylin.