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ORIGINAL ARTICLE

The effect of a single episode of antimicrobial photodynamic therapy in the treatment of experimental periodontitis. Microbiological profile and cytokine pattern in the dog mandible

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Abstract The purpose of this study was to evaluate the effect of a single application of antimicrobial photodynamic therapy (aPDT) on microbiological profile and cytokine pattern in dogs. Periodontal disease was induced by placing 3.0 silk ligatures around the mandibular pre-molars bilaterally during 8 weeks. The dogs were randomly treated with aPDT using a dye/laser system, scaling and root planning (SRP), or with the association of treatments (SRP+aPDT). Plaque samples were collected at baseline, 1, 3, and 4 weeks, and the mean counts of 40 species were determined using DNA-DNA hybridization. Gingival biopsies were removed and the expression of tumor necrosis factor alpha (TNF-α), receptor activator of NF-kB ligand (RANKL), osteoprotegerin (OPG), matrix metalloproteinase (MMP-1), interleukin (IL) 6, IL-10 and total bacterial load by analysis of 16 S rRNA gene were evaluated through real-time PCR. The results shows that the levels of the majority of the species were reduced 1 week post-therapy for all treatments, however, an increase in counts of *Prevotella intermedia* (p=0.00), *Prevotella. nigrescens* (p=0.00) and *Tannerella forsythia* (p=0.00) was observed for aPDT and SRP+aPDT. After 4 weeks, a regrowth of *Porphyromonas gingivalis* (p=0.00) and *Treponema denticola* (p=0.00), was observed for all treatments. Also, a strikingly reduction of counts on counts of *Aggregatibacter actinomycetemcomitans* was observed for the aPDT (p=0.00). For the cytokine pattern, the results were similar for all treatments, and a reduction in the expression of cytokines and bacterial load was observed throughout the study. Our results suggest that SRP, aPDT in a single application, and SRP+aPDT affects different bacterial species and have similar effects on the expression of cytokines evaluated during the treatment of ligature-induced periodontitis.

Keywords Photodynamic therapy · Photosensitizing agents · Cytokines · Animal studies

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Introduction

Periodontitis is an oral disease that leads to the collapse of the supporting structures of the teeth. It is triggered by

periodontopathic bacterial species, and tissue destruction is greatly influenced by the local host immune response [1]. It is well known that certain bacterial species are responsible for periodontal tissue breakdown and that the treatment of periodontitis is based on the suppression of these periodontal pathogens [2].

It is generally accepted that mechanical removal of periodontopathic pathogens comprises the conventional treatment for periodontitis and numerous studies reported significant improvements in clinical and microbiological parameters following non-surgical periodontal therapy [3–6]. To further enhance the effectiveness of scaling and root planing (SRP), power-driven instruments, such as sonic and ultrasonic devices, have been introduced; however, studies demonstrated comparable clinical results following sonic or ultrasonic and manual instrumentation [7, 8]. Despite the fact that non-surgical periodontal treatment may result in significant clinical improvements in the great majority of cases, none of the currently available instrumentation techniques are predictable in the complete elimination of subgingival bacteria and calculus [9–11]. These limitations could be attributed to several factors, such as the complex anatomy of teeth, invasion of periodontal pathogens into the surrounding soft tissues, or possible recolonization of periodontal pockets from other diseased sites or intraoral niches [12, 13].

More recently, alternatives that might offer the possibility of efficient removal of periodontal bacteria from the hard tissue surfaces with minimum damage to the systemic health are being sought. In this scenario, the antimicrobial photodynamic therapy (aPDT) has been suggested as a promising new approach for periodontal treatment. aPDT was introduced in the medical field in 1904, as a process in which light, after been absorbed by dyes, sensitizes organisms for visible light-inducing cell damage [14]. aPDT involves the combination of visible light, usually through the use of a diode laser and a photosensitizer. Each factor is harmless by itself, but when combined they can produce lethal cytotoxic agents that can selectively destroy target cells [15].

The action mechanism of this treatment has been described [16]. In brief, upon illumination, the photosensitizer is excited from the ground state to the triplet state. The longer lifetime of the triplet state enables the interaction of the excited photosensitizer with the surrounding molecules, and it is generally accepted that the generation of the cytotoxic species produced during aPDT occurs while in this state [15]. The cytotoxic product, generally singlet O_2, cannot migrate >0.02 μm after this formation, making it ideal for the local application of aPDT without endangering distant molecules, cells, or organs [16].

The objective of our study was to investigate the microbiological changes and cytokine patterns in dogs after treatment with antimicrobial photodynamic therapy in a single application (aPDT), scaling and root planing (SRP), or scaling and root planing associated with antimicrobial photodynamic therapy (SRP+aPDT) in different periods of observation.

Material and methods

The study protocol was reviewed and approved by the institution's Animal Research Committee of the School of Dentistry of Ribeirão Preto, University of São Paulo on December 5, 2005 (protocol 05.1.1038.53.9). Eight young adult male mongrel dogs (30 kg) were used. They had intact maxillae and mandibles, were in good general health, and had no viral or fungal oral lesions. All procedures were performed under anesthesia consisting of an intravenous injection of sodium pentobarbital (30 mg/kg, 500 mg of pentobarbital diluted in 20 ml sodium chloride, resulting in a 25% solution).

Periodontal disease induction phase

A total flap was raised in the region of the four mandibular premolars and a shallow notch was placed on the mesial and distal surfaces of each tooth with a ½ round bur to act as a retentive groove for the ligature. Doubled ligatures of 3.0 silk sutures were tied around the premolars bilaterally of each dog and then the flaps were repositioned and sutured with absorbable 4–0 sutures. The dogs were fed with a plaque-promoting diet of water-moistened dog chow [17]. The ligatures were checked weekly and any missing ligatures were replaced immediately. The experimental periodontitis was induced for 8 weeks and confirmed by clinical and radiographic examination. At the end of 8 weeks when approximately 25–30% of the initial bone support was lost, the ligatures were removed. For the next 12 weeks, natural bacterial plaque was allowed to accumulate and the periodontal disease was established. During this period, the progression of the disease was observed by clinical status and radiographic examination.

Non-surgical treatments

At the beginning of the treatment phase, the six experimental teeth (three of each side) were randomly divided into three treatment groups. The mechanical subgingival instrumentation (SRP group) was performed using hand instruments (Gracey curettes, No. 3/4, 5/6, 7/8, 11/12, and 13/14, Hu-Friedy, Chicago, IL, USA). For the aPDT group, a dye/laser system was applied. The system consisted of a hand-held battery-operated diode laser (HELBO® minilaser 2075 F dent, HELBO Photodynamic Systems GmbH & Co

KG, Grieskirchen, Austria). The laser wavelength was 660 nm with a power of 0.06 W/cm^2 for 10 s and fluency of 212.23 J/cm^2. The dye used was a commercial solution based on a phenothiazine chloride (HELBO Blue Photosensitizer®, HELBO Photodynamic Systems). The photosensitizer was applied placing the applicator at the bottom of the periodontal pocket and was continuously deposited in a coronal direction for 1 min followed by copious irrigation with distilled water to remove the excess. Afterwards, the diode laser unit was used with an 8.5-cm-long flexible fiber optic tip curved at an angle of 60° with a spot size 0.06 cm in diameter. The treatment was done in six sites per tooth totaling 1 min of treatment per tooth. For the SRP+aPDT group, the subgingival instrumentation was performed and then the same protocol used in the aPDT group was employed. During the course of the study, the animals received weekly supragingival ultrasound prophylaxis.

Collection of plaque samples

Subgingival plaque samples were taken at 0 h (before treatment), 1, 3, and 4 weeks post-therapy from the proximal pockets (mesial and distal) of the selected teeth. Counts of 40 subgingival species were determined in each plaque sample using the checkerboard DNA-DNA hybridization technique [18, 19]. In brief, after removal of supragingival plaque, subgingival biofilm samples were taken using individual sterile curettes from the proximal surface of each selected tooth and placed into separate microtubes containing 0.15 ml Tris EDTA buffer (10 mm Tris-HCl, 1 mm ethylenediaminetetraacetic acid, pH 7.6). Immediately afterwards, 0.10 ml of 0.5 m NaOH was added to each sample. The samples were boiled for 10 min and neutralized using 0.8 ml of 5 M ammonium acetate. The released DNA was placed into the extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA, USA) concentrated onto a 15 x 15-cm positively charged nylon membrane (Boehringer-Mannheim, Indianapolis, IN, USA) and fixed to the membrane by baking at 120°C for 20 min. The membrane was placed in a Miniblotter 45 (Immunetics, Cambridge, MA, USA) with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes to 40 subgingival species were hybridized in individual lanes of the Miniblotter. Following hybridization, the membranes were washed at high stringency and the DNA probes detected using antibody to digoxigenin conjugated with alkaline phosphatase and chemiluminescence detection and converted to absolute counts by comparison with the regression line determined from data from the standards on the same membrane. Failure to detect a signal was recorded as zero. A total of 384 subgingival samples were evaluated for the eight dogs. Two lanes in each run contained standards at concentrations of 10^5 and 10^6 cells of each species. The sensitivity of the assay was adjusted to permit detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe.

Gingival biopsies

In the same experimental times (0 h, 1, 3, and 4 weeks post-therapy), biopsies of gingival tissue measuring approximately 3 x 5 mm (comprising junctional epithelium, gingival crevicular epithelium, and connective gingival tissue) were obtained from the middle bucal site of each tooth, and afterwards, the wound was sutured with absorbable 5–0 sutures. The samples were then divided in a half in the cervico-apical direction, and a fragment was transferred to a microtube containing Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA), and stored at−70°C for analysis of cytokines expression. The remaining fragment was transferred to a microtube containing milli-Q water and stored at−70°C for subsequent analysis of bacterial load in periodontal tissues.

Real-time PCR reactions, mRNA analysis, and bacterial quantification through 16 S rRNA gene expression

The extraction of total RNA from periodontal tissues samples and the cDNA synthesis were accomplished as previously described [20]. Real-time PCR mRNA analysis was performed in a MiniOpticon system (Bio-Rad, Hercules, CA, USA) using specific TaqMan primers and probes (Applied Biosystems, Foster City, CA, USA) with 2.5 ng of cDNA in each reaction. Negative controls without cDNA and without the primer/probe sets were also performed. Calculations for determining the relative levels of gene expression were made from duplicate measurements of the target gene, with normalization to β-actin in the sample, using the cycle threshold (Ct) method and the 2-ΔCt equation, as previously described [21].

In order to allow the quantification of total bacterial load, the bacterial DNAs were extracted from periodontal tissue samples with Promega's DNA Purification System (Promega, Madison, WI, USA). Real-time PCR DNA analyses were performed in a MiniOpticon system (Bio-Rad, Hercules, CA, USA) using specific primers and probes and 5 ng of DNA in each reaction. The positivity to bacteria detection in each sample was determined based on the comparison to positive and negative controls, as previously described [22] and the values obtained with the Ct were subsequently normalized by tissue weight. The sensibility range of bacteria detection of real-time PCR assay was of 10^1 bacteria.

Statistical analysis

For the microbiological analysis, 41 variables (40 bacterial species and pool of bacteria) and two factors of variation (treatment and time) were studied. Through descriptive analysis, mean and standard deviations for each variable depending on factors were presented. The influence of treatment and time on the results was investigated by analysis using the method of generalized estimating equations (GEE).

For the cytokine analysis, seven variables and two factors of variation (treatment and time) were studied. Through descriptive analysis, mean and standard deviations for each variable depending on factors were presented. This analysis was complemented by graphs containing the confidence intervals for the levels of variation of the factor "time". The influence of treatment and time on the results, and their interaction was investigated by analysis of variance (ANOVA) mixed to two factors. Where relevant, the test of Tukey HSD was used for comparison between pairs. All tests followed a significance level of 0.05, and were performed using a software package for all calculations (SPSS version 16.0.0, SPSS, Chicago, IL, USA).

Results

Clinical results

The induction of periodontitis with ligatures proceeded without complications and resulted in the accumulation of plaque and increases in gingival inflammation, bleeding upon probing, and loss of alveolar bone through radiographic analysis. After the treatment, no complication was observed, such as the development of abscesses or signs of infection. During the experimental period, the dogs gained weight and did not develop any sign or symptom of illness.

Microbiological analysis

All 40 bacterial species evaluated were detected at baseline at different levels, as shown in Fig. 1. Levels of the majority of the species evaluated were reduced 1 week post-therapy for all treatments performed; however, an increase in mean counts of *Prevotella intermedia* ($p=0.00$), *Prevotella. nigrescens* ($p=0.00$), and *Tannerella forsythia* ($p=0.00$) was observed in this period of observation for aPDT and SRP+aPDT groups. The time period from 1 to 3 weeks was characterized by an oscillation in the mean counts of several bacterial species analyzed, notably, the *Actinomyces* species and the members of the "purple" and "yellow" complexes remained at low levels during this time phase. After 4 weeks, the mean counts of the majority of the bacterial species remained low. Additionally, a reduction in mean counts of *P. intermedia* ($p=0.00$) and *P. nigrescens* ($p=0.00$) was observed when compared with the values found after 1 week post-therapy. In particular, after 4 weeks, a recolonization or regrowth of *Porphyromonas gingivalis* ($p=0.00$) and *Treponema denticola* ($p=0.00$) was observed for all modalities of treatments tested; however, more pronounced for the aPDT group (*P. gingivalis*) and for the SRP+aPDT group (*T. denticola*). The mean counts of *T. forsythia* remained high during the course of the study, especially for the aPDT and the SRP+aPDT groups. In addition, a marked reduction of mean counts of *Aggregatibacter actinomycetemcomitans* was observed for the aPDT group ($p=0.00$). When the pool of bacteria analysis was performed (Fig. 2), no statistical significant difference among the treatments was observed ($p=0.11$). However, regarding the time periods, a statistical significance difference was noted when comparing baseline values with the values of 1 week and with the values of 4 weeks. For the other pairs of comparisons performed, no statistically significant differences were found.

Quantitative analysis of cytokines mRNA expression

The complete results for the intensity of the expression of mRNA of the following cytokines TNF-α, MMP-1, IL-6, IL-10, RANKL, and OPG are presented in Fig. 3. The results were similar for the treatments performed and a reduction of the expression of cytokines through the periods of evaluation was observed. The time factor was considered statistically significant regardless the treatment performed ($p=0.00$). For the TNF-α, MMP-1, IL-6, RANKL, and OPG, a decrease of the expression in the periods of 1 to 3 weeks was observed. Additionally, there was no influence of treatments on the results; this empirical evidence confirmed that there was no interaction between time and treatments. The IL-10 showed a different pattern of reduction, no statistically significant difference was observed between the initial time and 1 week ($p=0.13$) and between the later periods of observation (3 and 4 weeks) ($p=0.73$), evaluated by HSD Tukey test.

Quantification of total bacterial load in periodontal tissues

Figure 4 shows the results for the quantification of total bacterial load regarding to time and treatments performed. Regarding of the periods of observation, a progressive reduction of the total bacterial load was observed, following the same pattern of the reduction of the cytokines evaluated ($p=0.00$) and after a period of 3 weeks, a stabilization of the values occurred ($p=0.20$), evaluated by the Tukey HSD test. There was no influence of treatments on the results ($p=0.77$).

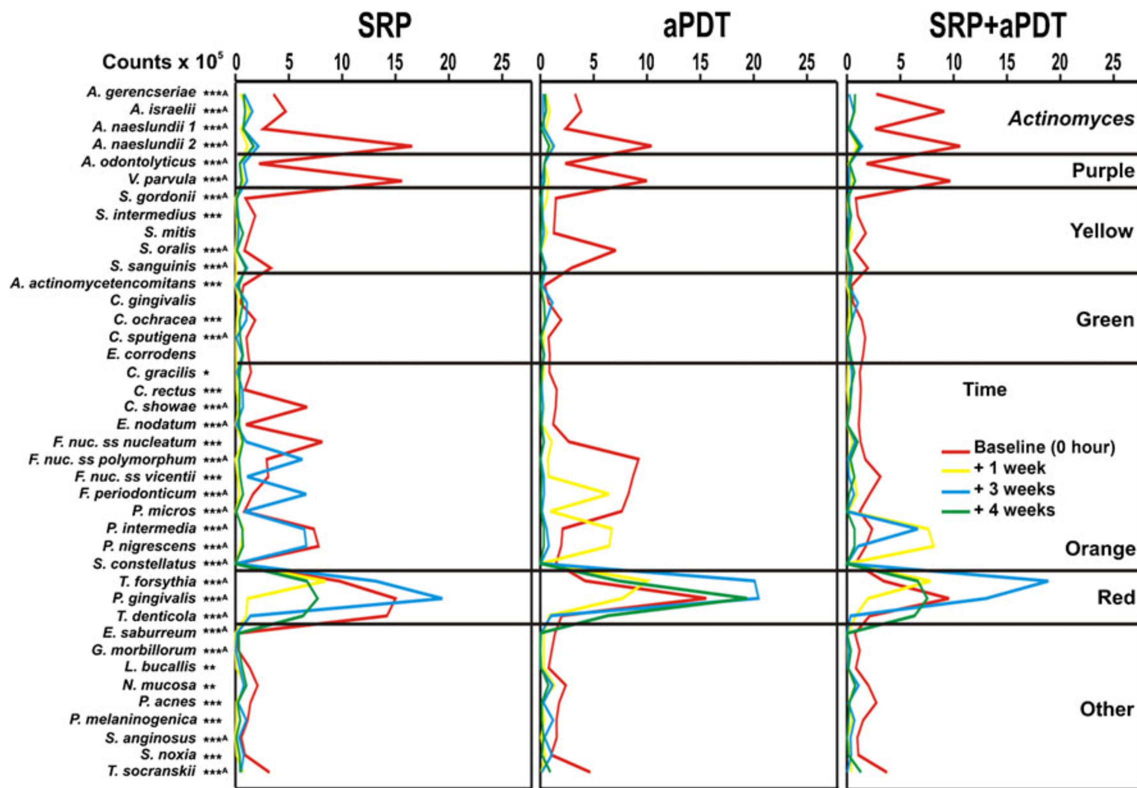

Fig. 1 Mean counts (x 10^5) of 40 bacterial species at baseline, 1, 3, and 4 weeks in the animals in each of the three treatment groups. Significant differences among treatments are marked with the letter "A" ($p<0.05$) and differences over time were marked as $^*p<0.05$. $^{**}p<0.01$ and $^{***}p<0.001$

Discussion

The present study was designed to test the applicability of aPDT in a single episode as an alternative for the treatment of ligature-induced periodontitis, through the evaluation of microbiologial parameters and cytokine expression. In order to investigate the in vivo effect of aPDT, a periodontal disease model was performed in dogs; this induced a more natural infection rather than infection by a single specific species of bacteria. Treatment under in vivo conditions, simulating as much as possible a real clinical situation. The results of our study showed that all

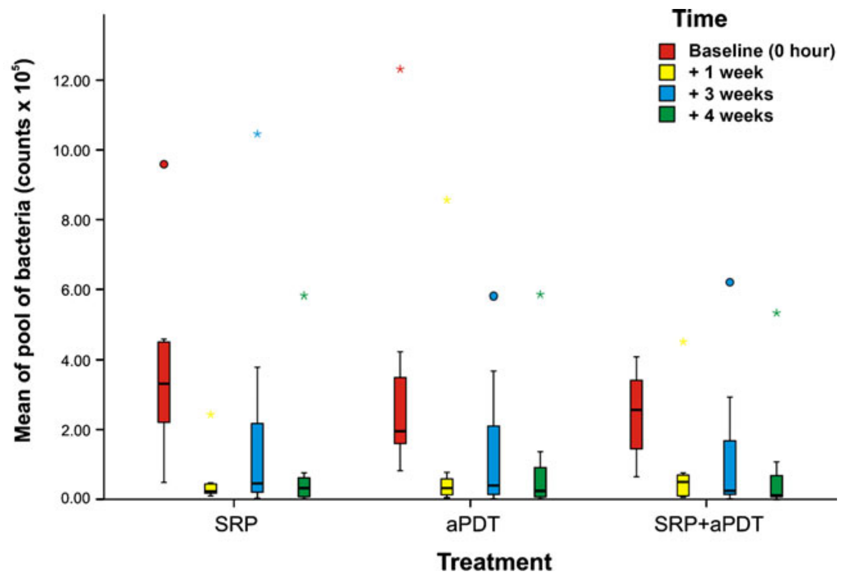

Fig. 2 Mean counts (x 10^5) of the pool of bacteria at baseline, 1, 3, and 4 weeks in the animals in each of the three treatment groups

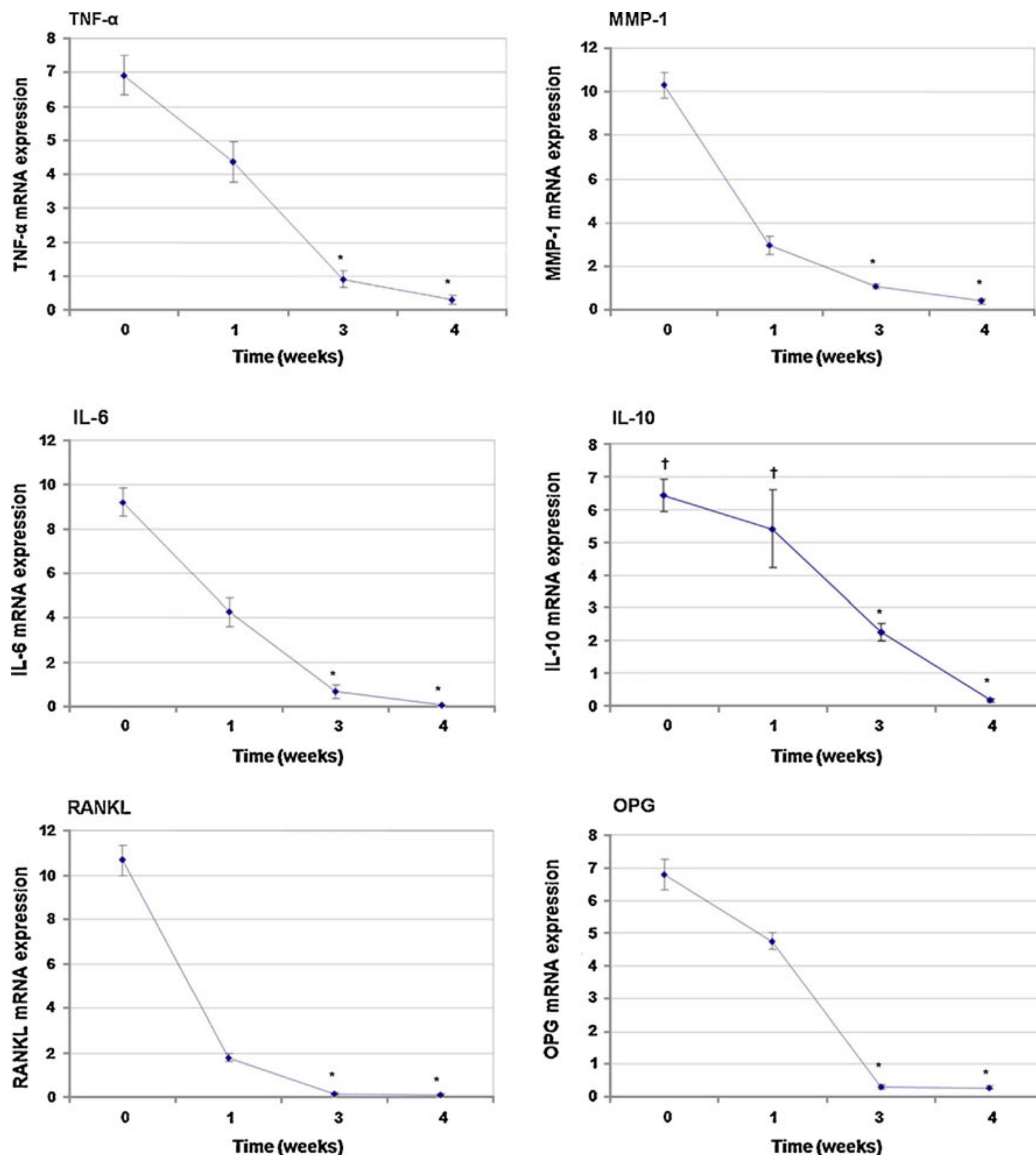

Fig. 3 Quantitative expression of TNF-α, MMP-1, IL-6, IL-10, RANKL, and OPG as a function of time regardless of the treatment. The results are presented as mean±SD of expression of the individual mRNAs, with normalization to β-actin, when compared to the target-internal control using the cycle threshold (Ct) method. The *error bars* represent the confidence intervals at 95%. Values marked with * or † have no significant difference

modalities of treatments tested lead to statistically significant reductions in different bacterial species and a constant reduction of the expression of cytokines analyzed at the different time points. Additionally, the observation that the postoperative healing occurred without complications throughout the development of the study may indicate that non-surgical periodontal treatment with aPDT was well tolerated by the animals.

Regarding total bacterial load in periodontal tissues, our results demonstrated a considerable reduction for all treatments tested and after 1 week a great reduction was achieved, more pronounced for the SRP+aPDT group when compared to SRP and to aPDT. In later periods (3 and 4 weeks), the treatments lead to a similar reduction in bacterial load and no statistically significant differences were observed. The results may indicate that after 3 weeks, a stabilization of total bacterial load was achieved and this remained stable throughout the study (Fig. 4).

When the composition of the microbiota was evaluated over the course of the study, the results showed that the

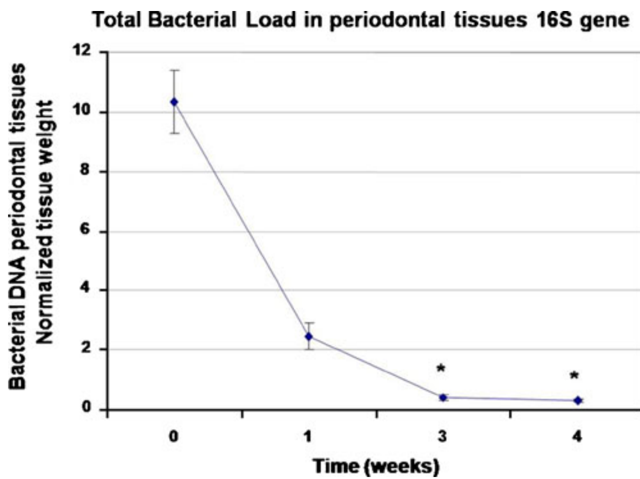

Fig. 4 Total bacterial load bacterial as a function of time regardless the treatment. The results are presented as mean±SD of expression of the total 16 S rRNA gene expression with normalization to periodontal tissue weight, when compared to the target-internal control using the cycle threshold (Ct) method. The sensibility range of bacteria detection of real-time PCR assay was of 10^1 bacteria. Values marked with * have no significant difference

treatments performed affects different bacterial species. For instance, aPDT was more effective in reducing mean counts of *A. actinomycetemcomitans*, a known human periodontal pathogen. This may be due to the ability of the photosensitizer to penetrate through the epithelium and connective tissue [23], since it is known that this particular microorganism can infiltrate through the epithelial barrier into the periodontal tissues. It was also probably able to reach the *A. actinomycetemcomitans* inside the biofilm since the results indicate to have virtually eliminated this microorganism. On the other hand, elevated mean counts of the "red complex" periodontal pathogens (*P. gingivalis*, *T. forsythia*, and *T denticola*) were still observed in all treatment groups after 4 weeks, especially in the aPDT group. This may be due to the short-term evaluation period, which may have not been long enough to achieve the homeostasis of the environment.

These results may indicate that the different periodontal treatments tested present distinct mechanisms of action on the microbiota and thus might have synergistic or even additive effects. SRP would physically lower the biomass of bacteria on the tooth surface and in the periodontal pocket; while aPDT due its non-invasive nature and ability to eliminate microorganisms causing alteration in membranes and/or plasma membrane proteins and DNA damage may present a different spectrum of activity. The beneficial effect of SRP on the subgingival microflora has been investigated as described in recent reviews [3–6]. However, there are conflicting reports about the capacity of SRP to eradicate or suppress important bacteria, such as *A. actinomycetemcomitans*, which have been shown to remain in periodontal pockets after non-surgical therapy [24, 25]. Our results corroborate this information regarding SRP. On the other hand, the capacity of the aPDT to reach the *A. actinomycetemcomitans* inside the biofilm may have valuable clinical applications, since it is well established that *A. actinomycetemcomitans* is an important periodontal pathogen mostly related to the onset and progression of aggressive periodontitis in humans [26].

The presence of invasive bacteria, such as the classic periodontopathogens *P. gingivalis*, *T. denticola*, *T. forsythia, and A. actinomycetemcomitans* have been associated with increased production of pro-inflammatory cytokines [22, 25, 27, 28] classically associated with the recruitment and activation of the monocyte/T lymphocyte axis, and to tissue destruction [29, 30]. Thus, the cytokines evaluated in this study are significant and integral components of the host response to periodontal infection. Additionally, these molecules are important as physiologic mediators in the periodontal environment, serving in both normal processes and as pathogenic mediators. A therapeutic goal in clinical periodontics can be aimed at maintaining a physiological role for cytokines; this may also be the case for aPDT due to its potential to reduce some key virulence factors, such as lipopolysaccharide and proteases in the early phases of the immune cascade [31].

In the present study, aPDT showed a therapeutic effect, regarding the cytokines evaluated, similar to that of scaling and root planning alone. Numerically, the results were slightly superior for the combined therapies. However, no statistical significance was observed. Perhaps the protocol for treating chronic forms of periodontal disease is well established and leads to satisfactory results, however in aggressive forms of periodontal disease [32, 33], or in specific clinical situations, aPDT is expected to have more benefits than conventional therapy, for example, in some sites, such as furcations, deep invaginations, and concavities in the periodontal area, which are difficult to access with hand instruments [34]. The use of aPDT, however, is not affected by this problem, as it is based on photosensitizer and light irradiation and thus it can easily irradiate those inaccessible places. Another problem with conventional therapy is the increase of bacterial resistance to antimicrobial drugs, whereas photodynamic therapy, using reactive oxygen species to kill bacteria in a short time, is highly unlikely to cause bacterial resistance, such as that to antibiotics [31, 35].

Numerous in vitro studies [36–42] demonstrated that aPDT is effective for bacterial reduction, but only some studies [43–46] showed that periodontopathogenic bacteria can be effectively suppressed in vivo by aPDT. A recent experimental in vivo study [47] using Toluidine Blue and a diode laser (635 nm) to treat periodontal disease in rats, concluded that aPDT compared to SRP could cause lethal

photoinactivation of periodontal pathogenic bacteria and eliminate inflammatory reactions in the gingiva with no detectable damage. Recent clinical trials in humans demonstrated the beneficial effects of aPDT in non-surgical periodontal treatment [32, 48]. These clinical studies showed that the use of aPDT alone is as effective as SRP. When interpreting the microbiologic outcomes obtained with aPDT, the possible effects due to the application of the photosensitizer itself should be considered. Furthermore, it is important to emphasize that there are very limited data from controlled studies comparing aPDT used in conjunction with non-surgical periodontal therapy to aPDT alone, SRP alone, or the photosensitizer alone. In different experimental trials that tested the applicability of aPDT, several aspects have been highlighted [47, 49, 50], unfortunately, the animals used in these studies showed metabolic rates that were at least more than twice that of humans. Also, in animal studies, more parameters can be kept constant than ever possible in a clinical trial, so a very cautious approach to transferring these results to a human situation has to be made. However, these data can provide important information for understanding the mechanisms regarding aPDT. Thus, further studies are necessary before any definitive conclusions can be drawn about the possible clinical and microbiological benefits of aPDT used in conjunction with non-surgical therapy. The frequency of the aPDT application is another possible explanation for the results obtained in this study. The manufacturer suggests that aPDT treatment should be performed weekly, for 4 to 6 weeks to enhance the antimicrobial effect. In this study, a single episode of aPDT was performed to avoid an additional confounding factor (i.e., frequency of applied treatment), which could influence the results obtained. Future studies are needed to clarify if and to what extent multiple applications of aPDT might enhance the outcome of therapy.

Conclusions

Under the experimental conditions used in this study, our data suggest that aPDT has an important effect on reducing *A. actinomycetemcomitans*. Furthermore, SRP, aPDT in a single application, and the association of both therapies, have similar effects on the expression of cytokines evaluated in the treatment of ligature-induced periodontitis. Therefore, more detailed, controlled clinical, microbiological, and biochemical research is necessary to determine the effectiveness of aPDT.

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