Antimicrobial Photodynamic Therapy in the Non-Surgical Treatment of Aggressive Periodontitis: Cytokine Profile in Gingival Crevicular Fluid, Preliminary Results

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Background: Aggressive periodontitis is a specific form of periodontal disease that is characterized by rapid attachment loss and bone destruction. Cytokine profiles are of considerable value when studying disease course during treatment. The aim of this trial was to investigate cytokine levels in the gingival crevicular fluid (GCF) of patients with aggressive periodontitis, after treatment with photodynamic therapy (PDT) or scaling and root planing (SRP), in a split-mouth design on −7, 0, +1, +7, +30, and +90 days.

Methods: Ten patients were randomly treated with PDT using a laser source associated with a photosensitizer or SRP with hand instruments. GCF samples were collected, and the concentrations of tumor necrosis factor-alpha (TNF-α) and receptor activator of nuclear factor-kappa B ligand (RANKL) were determined by enzyme-linked immunosorbent assays. The data were analyzed using generalized estimating equations to test the associations among treatments, evaluated parameters, and experimental times (α = 0.05).

Results: Non-surgical periodontal treatment with PDT or SRP led to statistically significant reductions in TNF-α level 30 days following treatment. There were similar levels of TNF-α and RANKL at the different time points in both groups, with no statistically significant differences.

Conclusion: SRP and PDT had similar effects on crevicular TNF-α and RANKL levels in patients with aggressive periodontitis. J Periodontol 2009;80:98-105.

KEY WORDS
Cytokines; periodontal diseases/therapy; photochemotherapy, photosensitizing agents.

The current understanding of the pathogenesis of periodontal disease suggests that tissue may be destroyed following the modulation of host defenses by bacterial and host products that stimulate the host inflammatory process.1 As with other inflammatory diseases, cytokines play an important mediator role by controlling cellular interactions. As documented in clinical and experimental studies,2,3 lipopolysaccharide and other products of bacteria lead to the stimulation of host cells, resulting in the release of cytokines. In turn, target cells are activated to release other cytokines, inflammatory mediators, and, in the case of tissue-destructive diseases, catabolic enzymes. The cytokine network takes control over inflammatory mechanisms to amplify or suppress tissue reactions. Juxtacrine signal transduction mechanisms might play a dominant role in the tissue, allowing interaction of resident cells with cells recruited to the site of destruction and remodeling.4

The acute inflammatory phase involves a reactive and defensive response to the bacterial products. Enhanced neutrophil migration into the sulcus, increased

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flow of serum proteins into the tissues, epithelial cell proliferation, and selective local accumulation of mononuclear cells emphasize these phenomena.\textsuperscript{5}

It is generally accepted that the mechanical removal of contaminants and the adjunctive use of antibiotics and disinfectants are the conventional treatment for aggressive periodontitis. Further, the biofilm structure of dental plaque confers remarkable resistance to species within the biofilm.\textsuperscript{6-8} Also, there is an increasing concern regarding the development of antibiotic resistance.\textsuperscript{9}

The uncertainty about antibiotic usage primarily arises from the lack of knowledge about microbial changes brought about within dental biofilms. Prior to the notion of biofilm-conferred antibiotic resistance, one might have surmised that a systemically administered antibiotic reaching the subgingival microbiota would kill virtually all sensitive species in that region.\textsuperscript{8} After the concept of increased resistance due to biofilm structure, one might entertain the possibility that few, if any, species are affected.\textsuperscript{8-10} In addition, because of the variability in the design of existing studies, we are unable to conclude which antimicrobial agent, dosage, and duration provide the optimal clinical and microbiological effect in aggressive periodontitis patients.

For these reasons, alternatives that might efficiently remove periodontal bacteria from the hard tissue surfaces are being sought. Photodynamic therapy (PDT) could become a new method of antibacterial treatment and may be used as an adjunct to, or as conventional therapy for, the treatment of aggressive periodontitis.

PDT is based on the principle that a photosensitive substance, the photosensitizer, binds to the target cell and can be activated by light of a suitable wavelength. During this process, free radicals of singlet oxygen are formed, which then produce an effect that is toxic to the cell. When reports emerged on the light-absorbing properties and fluorescence of various dyes, it became clear that dye excitation by light exerts a destructive action on biologic systems. This so-called “photodynamic action” was described as a process in which light, after being absorbed by dyes, sensitizes organisms to visible light-inducing cell damage.\textsuperscript{11} Raab\textsuperscript{12} studied this reaction, showing the killing of protozoa in the presence of acridine when irradiated with light in the visible range of the spectrum. Although PDT is more widely known for its application in the treatment of neoplasms,\textsuperscript{13} there is an interest in antimicrobial PDT, because a large number of microorganisms (including oral species) were reported to be killed in vitro by this approach.\textsuperscript{14-17} Furthermore, the potential of some key virulence factors (lipopolysaccharide and proteases) were shown to be reduced by photosensitization.\textsuperscript{18}

Gingival crevicular fluid (GCF) is regarded as a window for the non-invasive analysis of periodontal conditions, including markers of connective tissue and bone destruction.\textsuperscript{19} Tumor necrosis factor-alpha (TNF-\(\alpha\)) upregulates collagenase and prostaglandin E\(_2\) synthesis and is believed to break down attachment via protease-mediated mechanisms.\textsuperscript{20} In acute inflammatory gingivitis lesions, T cells predominate,\textsuperscript{21} whereas in the later-occurring immune response, large numbers of B cells are present.\textsuperscript{22} This has led to speculations on the protective role of cellular immune responses in gingivitis. In aggressive periodontitis, enhanced phagocytosis, lysis of bacteria, and the destructive role of humoral immune responses have been discussed.\textsuperscript{23}

The resorption of bone is regulated by the molecular interplay of receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG), a system of two molecules belonging to the TNF ligand and receptor families, respectively.\textsuperscript{24} RANKL is expressed predominantly as a membrane-bound ligand on osteoblasts, fibroblasts, and activated T and B cells, and its osteoclastogenic action can be blocked by its soluble decoy receptor OPG.\textsuperscript{25,26} The expression of RANKL and OPG is tightly regulated by systemic and local stimuli, including hormones, inflammatory mediators, bacterial products, and immunosuppressive drugs.\textsuperscript{24} Therefore, the aim of this clinical trial was to investigate TNF-\(\alpha\) and RANKL levels in the GCF of patients with aggressive periodontitis at different times before and after treatment with PDT or scaling and root planing (SRP).

**MATERIALS AND METHODS**

**Patient Population**

The research protocol was reviewed and approved by the Human Research Committee of the School of Dentistry of Ribeirão Preto, University of São Paulo, on December 7, 2005 (protocol 05.1.1038.53.9). Ten patients (eight women and two men) aged 18 to 35 years (mean age, 31 years) with a clinical diagnosis of aggressive periodontal disease were selected (Table 1). The selected patients had a minimum of 20 teeth (mean, 26 teeth), with at least one tooth in each posterior sextant and at least one posterior sextant with a minimum of three natural teeth. The subjects also presented with \(\geq 5\) mm of attachment loss around at least seven teeth, excluding first molars and central incisors.\textsuperscript{27,28} Criteria for exclusion from the study were periodontal treatment within the last 6 months, systemic diseases that could influence the outcome of therapy, pregnancy, smoking, and the ingestion of systemic antibiotics within the last 6 months. All participants signed informed consent forms.

**Study Design**

The study was performed using a split-mouth design. Ten pairs of contralateral maxillary single-rooted...
teeth were included (10 lateral incisors, eight canines, and two premolars); each tooth exhibited probing depth ≥5 mm on at least two aspects. One tooth in each contralateral pair was randomly treated, by a coin toss, with subgingival SRP using hand instruments; the other was treated with PDT. All patients were treated by the same experienced operator.

**Oral Hygiene Program**
Fourteen days before treatment all patients were enrolled in a hygiene program according to individual needs and received oral hygiene instructions. Supragingival professional tooth cleaning was performed 7 days before baseline.

**Non-Surgical Treatments**
Mechanical subgingival instrumentation was performed under local anesthesia using hand instruments (Gracey curets,1 numbers 3/4, 5/6, 7/8, 11/12, and 13/14). For the PDT group, a diode laser‡ with a wavelength of 660 nm and a maximum power of 60 mW/cm² was used with a phenothiazine photosensitizer# in a concentration of 10 mg/ml. The photosensitizer was applied with the applicator at the bottom of the periodontal pocket; it was deposited continuously in a coronal direction for 1 minute, followed by copious irrigation with distilled water to remove any excess. The pocket was exposed to the laser light using the fiber optic applicator,** with a diameter of 0.6 mm, for 10 seconds. The treatment was done in six sites per tooth. The mean time needed in the SRP group was 8 minutes, whereas the average time spent for the PDT group was 3 minutes. All tooth surfaces were treated by SRP or PDT, but only the proximal surfaces (mesial and distal) were considered for the GCF analysis (total = 40 sites).

**Collection of GCF Samples**
GCF samples were collected from the proximal sites (mesial and distal) of the experimental teeth at −7 (baseline), 0 (immediately after interventions), +1, +7, +30, and +90 days. Prior to GCF sampling, the supragingival plaque was removed from the interproximal surfaces with a sterile curet; these surfaces were dried gently by an air syringe and were isolated by cotton rolls. Paper strips†† were carefully inserted into the crevice until mild resistance was felt and were left in place for 30 seconds.²⁹ Care was taken to avoid mechanical injury. Strips contaminated with blood were discarded. The absorbed GCF volume of each strip was determined by an electronic gingival fluid measuring device;‡‡ the strips were placed into sterile microtube vials and kept at −70°C until analyzed. The readings from the electronic instrument were converted to an actual volume (microliters) by reference to the standard curve.

**Enzyme-Linked Immunosorbent Assays (ELISAs)**
Measurements of cytokines in GCF were performed as previously described.³⁰ The concentrations of cytokines in periodontal extracts were determined by ELISA using commercially available kits§§ according to the manufacturer’s instructions. Calculation of the TNF-α and RANKL concentration in each GCF sample was performed by dividing the total amount of TNF-α or RANKL by the volume of the sample. The sensitivity of ELISAs were TNF-α: >0.038 pg/μl and RANKL: >5 pg/μl. The results are expressed as picograms of cytokine (±SD) per microliter of GCF, from duplicate measurements.

**Statistical Analysis**
The outcome variables for this trial were the untransformed concentrations of TNF-α and RANKL. Statistical analysis was performed using the method of generalized estimating equations (GEE) with an identity link function. GEE was used in place of traditional analysis of variance because of the lack of independence among measures obtained from sites within each patient’s mouth.³¹,³² Furthermore, an exchangeable working correlation was assumed, and generalized score statistics were used instead of the Wald test. Treatments and time were used as categorical variables, and inserted covariates were “sites” and “concentrations at baseline.” Overall analyses were performed at a 0.05 level of significance, and a Bonferroni correction was applied for multiple comparisons (α = 0.005). A software package¶ was used for all calculations.

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**Table 1.**

**Demographic Data for the Patient Population**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>2/8</td>
</tr>
<tr>
<td>Age (years; mean ± SD)</td>
<td>31.01 ± 4.43</td>
</tr>
<tr>
<td>Teeth (N; mean ± SD)</td>
<td>26.7 ± 5.14</td>
</tr>
<tr>
<td>Lateral incisors (n)</td>
<td>10</td>
</tr>
<tr>
<td>Canines (n)</td>
<td>8</td>
</tr>
<tr>
<td>Premolars (n)</td>
<td>2</td>
</tr>
</tbody>
</table>

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¹ Hu-Friedy, Chicago, IL.
‡ Helbo Therapielaser, Helbo Photodynamic Systems, Grieskirchen, Austria.
§ Helbo Blue, Helbo Photodynamic Systems.
** Helbo 3D Pocket Probe, Helbo Photodynamic Systems.
†† PerioPaper, Oraflow, Amityville, NY.
‡‡ Periotron 8000, Oraflow.
§§ R&D Systems, Minneapolis, MN.
¶ SPSS version 16.0.0, SPSS, Chicago, IL.
RESULTS
The postoperative healing was uneventful in all cases. No complications, such as abscesses or infections, were observed throughout the study.

Figure 1 shows the mean concentrations of TNF-α over time by treatment group. Baseline values were 0.42 ± 0.02 pg/μl and 0.41 ± 0.02 pg/μl for PDT and SRP, respectively. The results seemed to indicate that levels of TNF-α increased after 1 day post-treatment. A progressive decrease was found after that time. No obvious influence from treatment groups could be observed. Estimated concentrations (mean ± SE) of both cytokines were obtained by the GEE method and are shown in Tables 2 and 3. For TNF-α, both treatment groups presented similar results (P = 0.821). Furthermore, time was found to be a significant source of variation, regardless of treatment (P = 0.047). Immediate post-treatment values were the same as at 7 days, whereas a significantly higher value was found at 1 day. Levels at later periods were significantly lower than the others, with the lowest value at 90 days. Results were similar for both treatments at all time periods; this empirical evidence confirmed that there was no interaction between time and group.

Figure 2 shows descriptive statistics for the concentration of RANKL. Baseline values were 394.4 ± 29.5 pg/μl and 378.9 ± 35.6 pg/μl for PDT and SRP, respectively. Although levels of RANKL seemed to undergo a progressive decrease, as for TNF-α, no significant change was observed. The treatment groups showed no apparent difference over time. RANKL levels behaved similarly, i.e., neither treatment significantly influenced outcome (P = 0.515). Additionally, time was not found to be a significant source of variation (P = 0.101). No interaction was observed between treatment and time for RANKL either.

DISCUSSION
The present study was designed to test the applicability of PDT as an alternative for the treatment of aggressive periodontitis through the evaluation of the TNF-α and RANKL levels in GCF samples. Antimicrobial drugs were not used in this trial so their adjunctive effect would not interfere with the outcomes. The results demonstrated that non-surgical periodontal treatment with PDT or SRP using hand instruments led to progressive and significant reductions in TNF-α levels 30 days following treatment. There was a constant reduction in TNF-α over time, although there were no statistically significant differences between the groups. RANKL levels were similar for both groups and were more stable over time. In addition, the observation that the postoperative healing was uneventful in all cases throughout the study period indicated that non-surgical periodontal treatment with PDT was well tolerated by the patients.

It has been clearly demonstrated that periodontitis is an infectious disease, and a current concept for treating periodontitis is based on eliminating the infection. Although several investigators consider mechanical therapy of the root surface the basic prerequisite for long-term treatment success, no definition of a sufficiently planed root surface, from the clinical point of view, could be found in the literature. Additionally, SRP can frequently be difficult and time-consuming because of the complex root morphology when working blindly in deep pockets. Because periodontal debridement requires a certain level of skill, time, and endurance, it seems appropriate to choose a technique that allows the removal of contaminants in a highly efficient and time-effective manner, with less effort on behalf of the clinician.

The effect of SRP on the subgingival microflora has been investigated in several studies, as described in reviews. There is general agreement that this procedure, in addition to improving clinical parameters, reduces the microbial load and results in a shift

Table 2.
Estimated Results for TNF-α

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>PDT Mean (SE)</th>
<th>PDT Pairwise Comparisons</th>
<th>SRP Mean (SE)</th>
<th>SRP Pairwise Comparisons</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0.40 (0.02)</td>
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<td>0.40 (0.02)</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>0.51 (0.02)</td>
<td>B</td>
<td>0.52 (0.01)</td>
<td>B</td>
</tr>
<tr>
<td>7</td>
<td>0.38 (0.01)</td>
<td>A</td>
<td>0.38 (0.02)</td>
<td>A</td>
</tr>
<tr>
<td>30</td>
<td>0.29 (0.01)</td>
<td>C</td>
<td>0.30 (0.02)</td>
<td>C</td>
</tr>
<tr>
<td>90</td>
<td>0.21 (0.01)</td>
<td>D</td>
<td>0.22 (0.02)</td>
<td>D</td>
</tr>
</tbody>
</table>

Values expressed in pg/μl. Vertically, identical uppercase letters denote no significant differences among the time periods.
toward a more health-compatible microflora. However, there are conflicting reports about the ability of SRP to completely eradicate or suppress important periodontal pathogens. *Tannerella forsythia* (previously *T. forsythensis*) and especially *Aggregatibacter actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans*) were shown to remain in periodontal pockets after non-surgical therapy. Bacterial recolonization or regrowth in the subgingival environment is anticipated after SRP, even shortly after treatment; it is suggested that regularly performed periodontal maintenance therapy is essential to prevent a return to pretreatment levels of pathogens.38 This may also be the case with PDT. PDT requires two components: a light source and a photosensitizer capable of binding to the targeted cell. The photosensitization may be important in dealing with aggressive periodontitis because the photosensitizer is capable of penetrating through the epithelium and connective tissue,43 as are the periodontopathogens, particularly *A. actinomycetemcomitans*, which can infiltrate through the epithelial barrier into the periodontal tissues. Considering that PDT is not truly a mechanical therapy, residual calculus is expected to occur. Because of the reduced presence of calculus, aggressive forms of disease and maintenance patients are more prone to the beneficial antimicrobial effect. Additionally, PDT has advantages, such as reduced treatment time, no need for anesthesia, the destruction of bacteria is achieved in a very short period of time (<60 seconds), development of resistance by the target bacteria is unlikely, and damage to the adjacent host tissues can be avoided.44

In a previous study46 we evaluated clinically the applicability of PDT as an alternative for the treatment of aggressive periodontitis. The results demonstrated that non-surgical periodontal treatment with PDT or SRP using hand instruments led to clinically and statistically significant improvements in probing depth and relative clinical attachment level 3 months following treatment. There were no statistically significant differences in any of the evaluated parameters between the groups; however, numerically the results were more pronounced for the PDT group. Also, the results for cytokines in the present study are in accordance with the clinical outcomes in the previous publication.46 These findings reinforce the notion that both cytokines are valuable markers of the periodontal status and are indicators of the response to treatment.47

Cytokine patterns are of considerable value when studying periodontal tissue destruction. It is known that bacterial products stimulate monocytes/macrophages, lymphocytes, and resident cells (fibroblasts and endothelial cells) to secrete proinflammatory and immune-regulatory cytokines. The penetration of bacteria and/or its products into the tissues results in the recruitment and activation of the monocyte/T lymphocyte axis. This, in turn, leads to the enhanced monocytic release of TNF-α and other cytokines associated with periodontal tissue destruction. Interleukin-8 secreted by monocyctic cells, but also from keratinocytes, endothelial cells, and fibroblasts, induces matrix metalloproteinase (MMP)-8 release by neutrophils. This MMP is a potent collagenase and plays a critical role in degrading connective tissue at the site of inflammation during the formation of periodontal pockets. The microflora of aggressive periodontitis consist of an elevated number of periodontal pathogens, as well as species pertaining to the exogenous flora, and cause more severe and widespread periodontal destruction.48 In the diseased sites an imbalance in the cytokine network is locally induced by the bacterial challenge, thus contributing to the development of elevated B-cell responses in the inflamed gingival tissues. One of the cytokines involved in the pathogenesis of periodontal disease is TNF-α, which is present at high levels in the GCF and periodontal tissues of diseased sites. Studies49,50 clearly demonstrated that TNF-α plays a central role in the inflammatory reaction, in alveolar bone resorption,
and in the loss of connective tissue attachment in experimental periodontal diseases.

Aggressive periodontitis is caused by bacteria that colonize the tooth surface and invade the adjacent tissues, resulting in inflammation, connective tissue breakdown, and bone resorption. The fact that antibiotics effectively inhibit experimental periodontitis in several animal models provides evidence for the role of bacteria in triggering the destructive effects. However, the preponderance of evidence indicates that periodontal tissue loss is caused more by the host response than from direct bacterial damage. The cytokines of the TNF family play a critical role in stimulating the innate host response and, in this capacity, prepare the host defense against periodontopathogenic bacteria. However, significant collateral damage can occur if this cytokine expression is inappropriate. Some bacteria, in particular A. actinomyctetemcomitans, have the ability to penetrate into the connective tissue and release lipopolysaccharide that can induce the expression of cytokines. Moreover, the initiation of an inflammatory process through the production of TNF stimulates the production of secondary mediators, including chemokines or cyclooxygenase products, which, in turn, amplify the degree of inflammation. Once this occurs, MMPs are induced and destroy the connective tissues. Simultaneously, cytokines may reduce the damaged tissues’ capacity to repair, through apoptosis of resident cells, such as fibroblasts; finally, the induction of an inflammatory cascade stimulates osteoclastogenic activity by the release of RANKL that results in bone destruction.

Thus, cytokines such as TNF-α and RANKL 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 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 PDT because of its potential to reduce some key virulence factors, such as lipopolysaccharide and proteases, in the early phases of the immune cascade.

The results of the present study also provide evidence that reduction of crevicular TNF-α and RANKL after PDT or SRP corresponds to the clinical improvements seen in the periodontal status, and it can be a potential marker of periodontal disease activity. Knowledge regarding the role of cytokines in the outcome of periodontal diseases may provide the basis for future therapeutic interventions aimed at limiting the inflammatory process and tissue damage while improving the repair of periodontal tissues. However, the precise underlying mechanisms of the inflammatory process and bone destruction in aggressive forms of periodontal disease remain unknown; therefore, further research is required to evaluate related cytokines that may be markers of aggressive periodontitis.

CONCLUSIONS

Our preliminary data suggest that SRP and PDT have similar effects on crevicular TNF-α and RANKL in patients with aggressive periodontitis. Therefore, more detailed, controlled clinical and biochemical research is necessary to determine the effectiveness of PDT.

ACKNOWLEDGMENTS

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REFERENCES


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