Comparative Evaluation of the Effects of Different Photoablative Laser Irradiation Protocols on the Gingiva of Periodontopathic Patients

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Abstract

Objective: We aimed at quantifying the presence of periodontopathogens in gingival biopsies from periodontitis patients treated with different photoablative lasers (diode GaAs, Er:YAG, Nd:YAG, and CO2 lasers) and histologically analyzing their effects on the gingiva. Background data: Substantial evidence indicates that intracellular location of periodontal bacteria in the gingival epithelium may contribute to chronic periodontitis. Methods: Sixteen adult subjects with chronic periodontitis were subjected to conventional scaling/root planing and topical chlorhexidine, and immediately laser-irradiated on the inner and outer free gingiva. Small gingival biopsies were subjected to real-time polymerase chain reaction and cytfluorescence to identify periodontopathogens; tissue damage and endothelial ICAM-1 expression were assessed by histological and immunofluorescence analyses. Results: High DNA levels of Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Treponema denticola, Prevotella intermedia, and Eikenella corrodens, were detected in all samples. Nd:YAG and diode lasers were capable of eradicating periodontopathogenic bacteria endowed within gingival epithelial cells outside periodontal pockets, without causing connective tissue damage and microvessel rupture. They also reduced ICAM-1 immunolabelling by the vascular endothelium. Conversely, Er:YAG lasers induced marked microvessel rupture and bleeding and failed to completely and selectively ablate the infected gingival epithelium, whereas CO2 laser caused heat-induced coagulation of the lamina propria. Conclusions: This study indicates that periodontopathogens can persist within cells outside the pocket epithelium, despite conventional periodontal treatment. Nd:YAG and diode lasers are able to eradicate intra- and extracellular bacteria from these sites, suggesting that they can be considered suitable devices to improve the clinical outcome of periodontal disease.

Introduction

Chronic periodontitis is the most common inflammatory disease of the soft and hard tooth supporting tissues. Nowadays, it is accepted that chronic periodontitis is a multifactorial disease, resulting from complex interactions among periodontopathogenic bacteria, present in the plaque biofilm and into the cells of the sulcular and buccal gingival epithelia, host defense mechanisms, and local and systemic factors, modulating the establishment and progression of the disease.1,2 Delayed or inappropriate treatments can lead to periodontal tissue injury, progressive alveolar bone destruction, and, eventually, tooth loss. In industrialized countries, chronic periodontitis affects up to 20% of the adult population and is the main cause of edentulism and a major public health concern.1,2 Periodontopathogens include many bacterial species, among which the most aggressive are Porphyromonas gingivalis, Fusobacterium nucleatum, Eikenella corrodens, Treponema denticola and Aggregatibacter actinomycetemcomitans.3–5 These bacteria, through the release of multiple virulence and pathogenic factors, such as lipopolysaccharide (LPS), activate and sustain the host inflammatory response eventually causing alveolar bone resorption.6 Moreover, most periodontopathogenic bacteria are capable of binding to and entering gingival/buccal epithelial cells, where they can escape immune reaction and traditional antiseptic therapies.8–12 Recent evidence suggests that bacteria can internalize within epithelial cells not only of the periodontal pocket but also of the outer gingiva,13 thus creating a microbial reservoir that can favor post-treatment relapses and chronicization of periodontitis.14–19
In the last decade, the application of laser medicine for the treatment of oral diseases has attracted considerable attention. Laser therapy offers several advantages over conventional surgical treatments, such as a noninvasive approach; selective targeting; repeatability; marked bactericidal effect; reduced pain, inflammation, and edema; improved and accelerated wound healing; and reduced need for antibiotics and local anesthetics. Growing evidence indicates that Nd:YAG, Er:YAG, CO₂, and diode lasers in phototherapeutic mode can be used for the treatment of inflammatory periodontal diseases. In particular, Er:YAG is also effective for removing calculus and debris adherent on the root surface. Principle, laser therapy should be capable of effectively targeting periodontal tissues infected by bacteria, including the epithelial sanctuaries. On the basis of these beliefs, in the present study we analyzed and compared the bactericidal and morphological effects of different laser devices widely used in medicine and dentistry (diode GaAs, erbium- or neodymium-doped yttrium aluminum garnet [Er:YAG, Nd:YAG], and carbon dioxide [CO₂] lasers), in the gingiva outside the periodontal pocket of patients treated with conventional therapy for chronic periodontitis.

Methods

Subjects

Sixteen patients (10 males and 6 females; 40-65 age range; mean range 55.6 years) with moderate-to-severe chronic periodontitis were included in this study. The protocol was designed in compliance with the ethical guidelines of the Declaration of Helsinki, as amended in Edinburgh in 2008, and was approved by the Ethical Committee of the Faculty of Medicine, University of Florence, Italy. The patients underwent a screening visit during which they gave written informed consent to their enrollment in the study. Exclusion criteria were: systemic diseases (diabetes mellitus, cancer, HIV, metabolic and endocrine diseases), pregnancy and lactation, chronic high-dose steroid therapy, radiation or immunosuppressive therapy, heavy smoking (more than 10 cigarettes/day), orthodontic treatments, extensive carious lesions, and antibiotics taken within the 6 months preceding the study. The enrolled patients were visually examined and the depth of periodontal pockets was measured with a conventional manual periodontal probe (Hu-Friedy, Chicago, IL) at six sites per tooth. Clinical measurements included: clinical attachment level (CAL), pocket probing depth (PD), and gingival index and bleeding on probing (GI/BOP).

Conventional periodontal treatment

The patients were subjected to supragingival and subgingival scaling and root planning (SRP) with a combined use of hand (Hu-Friedy, Chicago, IL) and ultrasonic instruments, under local anesthesia. During SRP, a subgingival irrigation with chlorhexidine (CHX; 0.3% in water, 10 mL per quadrant for an approximate period of 5 min for each selected site) was performed, followed by gentle brushing with a sterile gauze rinsed in 0.3% CHX for supragingival antiseptis. The patients received oral hygiene instructions corresponding to their individual needs.

Cytodiagnostic assay

Before laser irradiation, samples of exfoliated cells were taken from each patient in proximity to the free gingival margin with a sterile microcurette and processed for cytodiagnostic fluorescence analysis, as detailed previously. Briefly, the LIVE/DEAD BacLight bacterial viability kit (Invitrogen Molecular Probes, Milan, Italy) was used by this method, dead bacteria stained red, whereas the viable ones stained green. This method allowed us to detect additional diagnostic parameters, such as the occurrence of inflammatory leukocytes, erythrocytes, and altered gingival epithelial cells. The collected material was smeared on a histological slide, fixed in 90% ethanol, air dried and stained with 1 mL of the fluorescent dye solution for 2 min at 37°C. After thorough rinsing in distilled water, the samples were immediately observed under a Leica 4000B fluorescent microscope (Leica Microsystems, Milan, Italy). The results were combined with the standard clinical parameters to obtain an objective, semiquantitative scoring of disease severity, as reported in Table 1. Some samples were also examined by a Leica TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) to detect the exact cellular location of bacteria. To this aim, a series of optical sections, 0.4 μm thick, were taken through the depth of the cells and projected onto a single image.

Photoablative laser treatments

To remove the contaminated periodontal tissues, the patients were laser irradiated on the inner and outer free gingiva using the following devices:

1. Er:YAG (OpusDuo ECTM, Lumenis, Milan, Italy)
2. CO₂ (OpusDuo ECTM, Lumenis)
3. Nd:YAG (Pulse Master 600 IQ, American Dental Technologies Inc., Corpus Christi, TX)
4. Diode laser (4x4 Dental Laser, General Project, Montespertoli, Italy)

The irradiation parameters, chosen on the basis of those widely accepted for the treatment of periodontitis, are specified in Table 2. Energy output of the lasers was measured with a power meter before each procedure. Epi protection of the operator, assistant, and patients was assured by wearing safety glasses. Treatments were performed with the patient under local anesthesia (articain HCl, Ultracain, Frankfurt, Germany) in all circumstances for Er:YAG and CO₂ lasers, and on patient’s request for diode Nd:YAG lasers. Air flow cooling was used to minimize the photothermal effects of laser irradiation. Of note, laser therapy was performed by expert operators (M.G., L.L.) who were not involved in the subsequent analysis of data.

Biopsy collection and morphological analysis

Upon admission and after laser treatments, small biopsies of gingival soft tissues, ~2×2 mm, including surface epithelium and lamina propria, were taken with the patient under local anesthesia (articain HCl) using a biopsy punch, 2-mm diameter, from the buccal aspect of the gingiva, taking care not to expose the marginal alveolar bone and
periostium. Usually, biopsies were taken at the oral sites in which there was a therapeutic indication to reduce the periodontal pockets: hence, their number and tooth site varied from patient to patient. The biopsies (n=32, with 16 taken before and 16 \[4 per laser type\] after laser treatments) were fixed by immersion in 4% (w/v) formaldehyde in 0.2M phosphate-buffered saline, pH 7.4, dehydrated in graded ethanol, and embedded in paraffin. Five \[\mu m\]-thick sections were stained with hematoxylin and eosin, viewed, and photographed under a light microscope (Nikon, Tokyo, Japan). Semiserial sections of each sample were made and submitted to immunohistochemistry to reveal intercellular cell adhesion molecule (ICAM)-1 expression by the vascular endothelium, using a mouse monoclonal anti-human ICAM-1 antiserum (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200) and goat anti-mouse Alexa-488-conjugated IgG (Molecular Probes Inc., Eugene, OR; 71:200). The immunostained samples were counterstained with propidium iodide (Molecular Probes) and observed under the Leica TCS SP5 confocal microscope. Some small gingival samples were also fixed in 4% cacodylate-buffered glutaraldehyde, post-fixed in 1% phosphate-buffered osmium tetroxide, dehydrated in graded ethanol, and embedded in EPON\[812\] epoxy resin. Ultrathin sections, 0.7 \[\mu m\]-thick, and stained with uranyl acetate and lead citrate, were examined under a JEM 1010 electron microscope (Jeol, Tokyo, Japan) at 80 kV.

Quantification of bacteria by real-time polymerase chain reaction (PCR) analysis

Genomic DNA was extracted from formalin-fixed paraffin-embedded histological sections according to the RecoverAll Total Nucleic Acid Isolation Protocol (Ambion-Applied Biosystems, Foster City, CA). Slides were kept in xylene for 10 min at 50°C and air dried for 10 min. Tissue sections were detached from slides by scraping, collected in Eppendorf tubes, incubated in xylene at 50°C for 3 min to remove paraffin from the tissue, and centrifuged at 13,000 rpm for 5 min at room temperature. The supernatant was discarded and the pellet washed twice in 96% ethanol. Pellets were treated with the digestion buffer and protease following manufacturer's instructions for 48 h. As indicated in the protocol, increasing the incubation time up to 48 h at 50°C usually resulted in recovery of DNA with increased functionality. RNAse was added to tubes transferred from 50°C to room temperature for 1 h. On these samples, genomic DNA was extracted with phenol/chloroform method and spectrophotometrically quantified with NanoVue Plus Spectrophotometer (GE Healthcare, Milan, Italy). The numbers of 16S rDNA-gene copies of \(P.\ gingivalis, A.\ actinomycetemcomitans, F.\ nucleatum, T.\ denticola, Prevotella intermedia,\) and \(E.\ corrodens, 16S \)rDNA-gene copies of total bacterial cells and human DNA copies were quantified using SsoFast EvaGreen Supermix (Bio-Rad, Milan, Italy) following manufacturer's instructions. All reactions were performed in a final volume of 15 \[\mu L\] containing 10 ng of total genomic DNA (5 \[\mu L\] of sample at 2 ng/\[\mu L\]) and primers at 300 nM final concentration, using CFX96 Cycler (Bio-Rad) programmed as follows: 2 min 98°C for initial heat activation, 40 cycles of 5 sec at 98°C for denaturation, and 20 sec at 60°C for annealing/extension steps. Melting curves were also collected in the temperature range 65-95°C.
Negative controls without DNA and standard samples were included in each real-time plate. Data were analyzed to provide an estimate of the total number of bacterial cells. When a gene was used as target for quantification of a specific bacterium, we assumed that the 16s rDNA gene copy numbers and overall genome size were basically similar among the different bacteria analyzed.29,30 The standard curves for each bacterial strain were built on serial 5·dilutions, starting from 10 ng of species-specific genomic DNA (DSM, Milan, Italy), corresponding to nearly 10·106 cells. The standard curves were performed in triplicate and quality parameters were analyzed systematically. The DNA starting quantity determined by comparing cycle threshold (Ct) values with standard curves were converted to theoretical bacterial numbers, normalized to human DNA.

**Results**

**Pretreatment**

Histological and cytodiagnostic analysis of the gingival samples taken outside the periodontal pocket (Fig. 1) in periodontitis patients performed before the treatment showed marked abnormalities (Table 1). The histological changes mainly consisted of: focal epithelial ulceration, diffuse infiltration of inflammatory cells in the lamina propria, and dilated blood vessels (Fig. 2A). By confocal microscopy, the endothelial lining of capillaries and post-capillary venules showed a marked immunostaining for ICAM-1, a typical endothelial adhesion molecule regulating leukocyte trafficking during inflammation (Fig. 3A). The morphological damage was associated with severe bacterial contamination of the gingival tissues, as judged by real-time PCR analysis (Fig. 4A,B). In particular, high DNA levels of periodontopathogenic species, namely *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum*, *T. denticola*, *P. intermedia*, and *E. corrodens*, were detected in all the samples. In particular, the cytofluorescent analysis performed on exfoliated gingival

![FIG. 1. Representative image of gingival biopsy sites in a diode laser-treated (arrow) and untreated area (arrowhead). Inset: detail of the biopsy specimen.](image-url)
epithelial cells showed numerous bacteria (cocc, bacilli, spirochetes), located both at the cell surface and within the cytoplasms, as well as several erythrocytes and polymorphonuclear leukocytes (Fig. 4A,B). Of note, the presence of intracellular bacteria endowed into gingival 9 epithelial cells was further confirmed by confocal and transmission electron microscopy (Fig. 5C,D).

Laser treatment
Histological and cytodiagnostic analysis of the gingival samples from the patients, which were performed after photoablative laser irradiation, showed an almost complete eradication of bacterial contamination, in terms of total bacterial and specific periodontopathogenic DNA (Fig. 4A,B). However, substantial histological differences were observed among the different laser devices used. In particular:

1. Er:YAG laser caused an incomplete ablation of the gingival squamous epithelium associated with dilatation and rupture of the underlying blood microvessels (Fig. 2B). This finding was consistent with the sustained bleeding observed during the treatment.
2. CO₂ laser yielded a complete removal of the squamous epithelium accompanied by a diffuse heat-induced coagulation of the papillary connective tissue and microvessels (Fig. 2C).
3. Nd:YAG laser also caused a complete removal of the squamous epithelium, in the absence of appreciable changes of the stromal and microvessel components of the lamina propria (Fig. 2D). Of note, microvessels appeared collapsed, consistently with reduced bleeding at treatment, and showed a significant reduction of ICAM-1 expression (Fig. 3B).
4. The diode laser (λ 810 nm) yielded similar effects as those of the Nd:YAG laser, inducing a complete ablation of the surface epithelium, prominent microvessel constriction associated with ICAM-1 downregulation, and negligible stromal coagulation (Figs. 2E and 3C). Even in this case, intraoperative bleeding was minimal.

Discussion
There is a general consensus that periodontopathogenic bacteria can invade host gingival epithelial cells and establish an intracellular niche, which is critical for their survival mechanisms. This relationship with the host cells is initially innocuous, but can potentially became dangerous, being the prerequisite for the spreading to neighboring cells. This is a crucial issue in periodontology, because the silent persistence of periodontopathogens within oral epithelial cells surrounding the gingival pocket, ostensibly uninvolved...
in the disease at clinical observation, can explain the frequent post-treatment relapses and the need for extended oral decontamination.\(^8,11,14\) Although laser treatment has generated considerable interest among dentists and the public, there is still substantial controversy on the actual clinical efficacy of laser treatment over SRP and conventional surgical curettage for the therapy of chronic periodontitis.\(^10,20,31–33\) This controversy likely exists for two main reasons: 1) the use of non-univocal laser irradiation protocols, such as different wavelengths and setting parameters, which do not allow a reliable comparison of results;\(^20,32,33\) and 2) the fact that most of the previous studies limit the laser treatment to periodontal pocket curettage,\(^31,32\) which has been recently stated to be substantially ineffective in a consensus report of the American Academy of Periodontology.\(^34\) In such a view, the data of the present study provide evidence that periodontopathogens, such as *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum*, *T. denticola*, *P. intermedia*, and *E. corrodens*, can persist into the epithelial cells outside the periodontal pocket of patients treated with conventional therapy for chronic periodontitis, and that laser treatment is suited to achieve de-epithelization of the infected gingiva. Moreover, combining the microbiological data and morphological analysis, we have compared the effects of the different laser treatments to define the most appropriate irradiation protocols for successful bacterial removal with minimal tissue damage. We found that Er:YAG, although being adequate to control microbial contamination, caused prominent blood vessel dilatation associated with marked bleeding at treatment. This vascular modification could favor the systemic spread of periodontopathogenic bacteria and their harmful by-products (such as LPS), which represent a major concern in the treatment of periodontitis, as they may predispose the patients to cardiovascular disease. Indeed, recent clinical studies have reported the presence of *P. gingivalis* DNA and rRNA in carotic and coronary atherosclerotic plaques,\(^35\) and there is in *vitro* evidence that *P. gingivalis*-derived LPS promotes leukocyte adhesion to vascular endothelial cells.\(^36\) Moreover, Er:YAG laser failed to completely and selectively ablate the gingival epithelium, accounting for the persistence of intracellular bacteria in the remnant gingival tissue. On the other hand, CO\(_2\) laser, although being capable of achieving a complete de-epithelization, caused heat-induced coagulation of the

![FIG. 3. Confocal immunofluorescence analysis of ICAM-1 expression by gingival microvessels (arrows) before (A) and after Nd:YAG (B) and diode λ 810 nm (C) laser irradiation. Note the ICAM-1 (green) is downregulated in the microvascular endothelium of the treated samples. Nuclei (red) are counterstained with propidium iodide. Red blood cells show cyan autofluorescence. Original magnification ×630. Bars = 5 μm.](image)

![FIG. 4. Real-time polymerase chain reaction (PCR) of (A) total bacterial DNA and (B) specific DNAs of periodontopathogens before and after treatment with the noted lasers.](image)
papillary connective tissue and microvessels, thus delaying gingival healing and representing a favorable substrate for bacterial re-growth.

Among the tested devices, the Nd:YAG and diode (λ 810 nm) lasers gave the best results. At the reported irradiation parameters, both instruments yielded a complete removal of the gingival surface epithelium, without causing major signs of stromal damage and microvesel dilation. These features indicate that Nd:YAG and diode lasers are capable of removing the epithelial sanctuaries, which represent a barrier against traditional antisepsis methods and host immunity defenses. Of note, epithelial removal by Nd:YAG and diode laser irradiation was accompanied by microvesel constriction, possibly related to direct vaso-motor effects and/or deactivation of local pro-inflammatory cytokines by laser light.\textsuperscript{37,38} This feature provides obvious clinical advantages, as it can prevent bleeding, reduce the chances for systemic bacterial spreading during the treatment, and favor the formation of a dry surgical field for direct visual control in deep pockets and better access for SRP procedures. Moreover, compared with the pretreatment findings, Nd:YAG and diode lasers were capable of reducing ICAM-1 immunolabelling by the vascular endothelium. This finding, together with our previous observation that Nd:YAG and diode lasers are capable of inactivating bacterial LPS,\textsuperscript{28,39} a potent pro-inflammatory mediator, further underscore that Nd:YAG and diode lasers, by reducing endothelial activation and leukocyte extravasation into the gingival stroma, can exert prominent anti-inflammatory effects on the gingival/periodontal tissue.

Conclusions and Summary

The present findings show that Nd:YAG and diode lasers are effective for the immediate removal of the contaminated gingival epithelium outside the periodontal pocket in patients with chronic periodontitis, and suggest that, when used as a complement to conventional therapy, they may be adequate to attain periodontal health and reduce the incidence of post-treatment relapses. Future long-term, clinical trials are needed to support the efficacy of Nd:YAG and diode laser as an adjunctive therapeutic approach to periodontal disease.

Author Disclosure Statement

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