Original

Lethal photosensitization in microbiological treatment of ligature-induced peri-implantitis: a preliminary study in dogs

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Abstract: This pilot study evaluated, by culture testing, the effectiveness of lethal photosensitization for the microbiological treatment of peri-implantitis in dogs. Experimental peri-implantitis was induced by ligature placement for 2 months. Following ligature removal, plaque control was instituted by scrubbing with 0.12% chlorhexidine daily for 12 months. Subsequently, mucoperiosteal flaps were elevated for scaling the implant surface. Microbial samples were obtained with paper points before and after treatment of implant surfaces by means of 100 μg/ml toluidine blue O (TBO,) and were exposed, for 80 s, to light with a wavelength of 685 nm from a 50 mW GaAlAs diode laser. The mean initial and final bacterial counts were 7.22 ± 0.20 and 6.84 ± 0.44 CFU/ml, respectively for TVC ($P < 0.0001$); 6.19 ± 0.45 and 3.14 ± 3.29 CFU/ml for *P. intermedia/nigrescens* ($P = 0.001$); 5.98 ± 0.38 and 1.69 ± 2.90 CFU/ml for *Fusobacterium* spp. ($P = 0.001$); and 6.07 ± 0.22 to 1.69 ± 2.94 CFU/ml for beta-hemolytic *Streptococcus* ($P = 0.0039$). It may be concluded that lethal photosensitization resulted in a reduction of the bacterial count. Complete elimination of bacteria was achieved in some samples. (J. Oral Sci. 45, 17-23, 2003)

Key words: Dental implants; laser therapy; osseointegration; periodontal pathogens; peri-implantitis; photodynamic antimicrobial chemotherapy.

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Introduction

Early studies have documented the excellent long-term prognosis of osseointegration (1). However, several different etiologic factors are associated with dental implant failures, such as poor surgical management, failure to achieve osseointegration, premature loading, biomechanical overload (2), and main peri-implant infection, due to colonization by bacteria such as *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum,* and *Bacteroides forsythus* (3). Dental implant failure, or peri-implantitis, is defined as an inflammatory process affecting the tissues around a in function dental implant, resulting in loss of supporting bone (4).

Several therapeutic strategies can be applied for the treatment of peri-implantitis (5-7). In some of these *in vivo* studies, clinical, radiographical and histological evaluations were performed without considering the microbiological

features before and after contamination of the implant surface.

Discontamination by mechanical (7), chemical (5) and physical (8,9) methods have been used.

The physical method, utilizing a low-power laser following the application of a photosensitizing substance, such as toluidine blue O (TBO) (10), has been used to treat periodontal diseases (11-14) and peri-implantitis (8,9). The mechanism by which TBO plus laser irradiation kills microorganisms such as *Porphyromonas gingivalis, Prevotella intermedia, Actinobacillus actinomycetemcomitans,* and *Fusobacterium nucleatum* has not yet been established. However, it is believed that lethal photosensitization of these microorganisms may involve changes in their membranes and/or plasma membrane proteins, as well as DNA damage mediated by singlet oxygen (13).

The purpose of this study, therefore, was to evaluate, by culture testing, the viability of microorganisms, following lethal photosensitization, during the microbiological treatment of ligature-induced peri-implantitis in dogs.

Materials and Methods

Animals and implants

The outline of the experiment is presented in Fig. 1. A total of six consecutively treated mongrel dogs (24-months old with an average weight of 18 kg) with ligature-induced peri-implantitis around 19 dental implants were treated in this study. Animal selection, management, and surgical protocol followed routines approved by the Dental School of Araraquara Institutional Animal Care and Use Committee.

All mandibular premolars were extracted to create space for dental implants. After three months of healing, 36 dental implants with four different surfaces and three different implant systems: commercially pure titanium implants-CP, 3*i*® Implants Innovations, Palm Beach Gardens, FL, USA; titanium plasma sprayed-TPS, *Esthetic plus* ITI®, Straumann AG, Waldenburg, Switzerland; hydroxyapatite-HA, Calcitek®, Sulzer Medica, Carlsbad, CA, USA; hybrid surface-machined titanium in the three screws and acid-etched-acid, *Osseotite*®-3*i*® Implants Innovations, Palm Beach Gardens, FL, USA were installed in each quadrant of the mandible.

Three months after fixture installation, healing abutment connections were installed, according to the instructions of each dental implant system. After a plaque control program for two months and healing of the soft tissue, cotton floss ligatures were placed around the dental implants and sutured in the peri-implant mucosa, not only to facilitate plaque accumulation, but also to hold the ligatures in position. Tying further ligatures at 20 day-intervals for a period of 60 days accelerated peri-implant bone loss. At 60 days, when approximately 40% of the initial bone support was lost, the ligatures were finally removed.

A 12-month plaque control program was initiated by means of scrubbing daily with 0.12% chlorhexidine and scaling the abutment surface once a month. At the end of this period, natural peri-implantitis progression was observed, and only 19 dental implants (5 CP, 6 TPS, 3 HA, and 5 acid) remained osseointegrated.

Treatment

A crestal incision was made through the mucosa and buccal and lingual full-thickness flaps were elevated. The abutments were removed and the granulation tissue present in the bone craters around the dental implants was curetted with a plastic curette (Fig. 2). The implant surface was then rinsed with physiological saline solution and the first peri-implant microbial sample was taken. TBO (100 (µ/ml) was then injected into the peri-implant defect as far as the bony border with a thin needle; the TBO was left in place for 1 minute and then carefully drawn off again. The stained area was subsequently irradiated with a GaAlAs
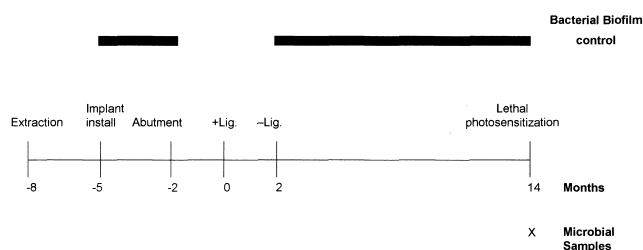

Fig. 1 Outline of the experiment. Ligatures were placed around the dental implants at 0 months (+Lig.) and removed at 2 months (-Lig.). Treatment of peri-implantitis was performed at 14 months by photodynamic therapy.

Fig. 2 Clinical view of peri-implant defects after debridment.

diode laser (IR 500-Laser Beam, Brazil) with a measured power output of 50 mW (Fig. 3). This laser emits radiation in collimated beams (2 mm^2) with a wavelength of 685nm, for 80 s and a total energy of 4 J (energy density, 200 J/cm^2). The diode laser was focalized in contact with the mesial, distal, buccal and lingual surfaces by a scanning method for 20 s on each face, making a total of 80 s. The second microbial sample was then obtained.

Microbial Samples

The paper points were removed and placed into 3-ml vials containing VMGAIII anaerobic transport medium (15). All samples were collected by the same operator and coded by an assistant to provide blind identification. The microbiological procedures were initiated within 24 hours.

The samples were centrifuged for 60 s and were serially diluted 10-fold in peptonated water to between 10^{-1} and 10^{-6} for quantitative evaluation of CFU/ml and to obtain isolated colonies for qualitative identification. Aliquots of 0.1 ml of the dilutions were plated onto Enriched Tryptic Soy Agar (ETSA) (16) and Tryptic Soy-Serum-Bacitracin-Vancomycin agar (TSBV) (17) in a standard manner. ETSA plates were incubated in anaerobic jars containing a mixed gas atmosphere (85% N_2, 10% H_2, 5% CO_2) at 37°C for 7 to 10 days. TSBV agar plates were incubated in a 5% CO_2 atmosphere for 5 days at 37°C. The bacterial species were identified from anaerobic cultures based on gram-stain, aerotolerance, colony morphology, esculin hydrolysis (18), nitrate reduction, indole production, [alpha]-glucosidase and N-benzoyl-DL-arginine-2-naphthylamide (BANA) hydrolysis (19), oxidase and catalase activities. Total viable count (TVC) and cultivable microbiota, including *Porphyromonas gingivalis, Prevotella intermedia/nigrescens, Fusobacterium* spp., beta-hemolytic *Streptococcus. Actinobacillus actinomycetemcomitans*
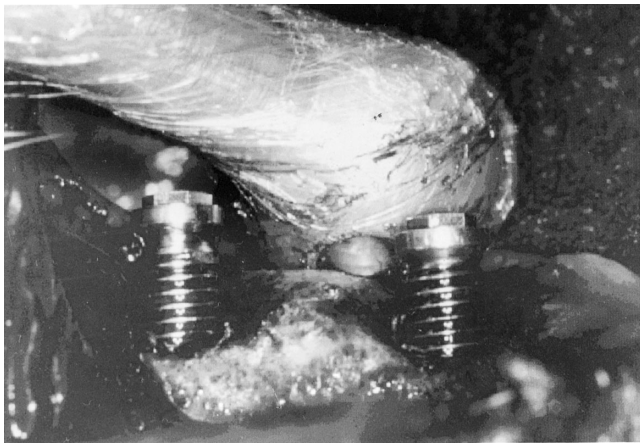
detection was performed based on colony morphology and positive catalase tests (20).

Data Analysis

The TVC were transformed into colony-forming units per ml (CFU/ml) using predetermined conversion factors to account for dilution and the size of the evaluated surface on the plate.

Data were then analyzed for each dental implant. Differences between groups and bacterial species were assessed by Wilcoxon's signed rank-test ($P < 0.05$). Microorganism analysis was performed after logarithmic transformation of TVC for each periodontal pathogen.

Results

P. gingivalis and *A. actinomycetemcomitans* were not detected in any peri-implant microbial sample.

The mean initial bacterial count ranged around 7.22 ± 0.20 CFU/ml for TVC, 6.19 ± 0.45 CFU/ml for *P. intermedia/nigrescens*, 5.98 ± 0.38 CFU/ml for *Fusobacterium* spp., and 6.07 ± 0.22 CFU/ml for beta-hemolytic *Streptococcus*.

The means measured following lethal photosensitization were even lower; 6.84 ± 0.44 CFU/ml for TVC (Fig. 4), 3.14 ± 3.29 CFU/ml for *P. intermedia/nigrescens* (Fig. 5), 1.69 ± 2.90 CFU/ml for *Fusobacterium* spp. (Fig. 6), and 5.01 ± 1.90 CFU/ml for beta-hemolytic *Streptococcus* (Fig. 7). Significant decreases in counts following lethal photosensitization were observed for TVC ($P < 0.0001$), *P. intermedia/nigrescens* ($P = 0.001$), *Fusobacterium* spp. ($P = 0.001$) and to beta-hemolytic *Streptococcus* ($P =$

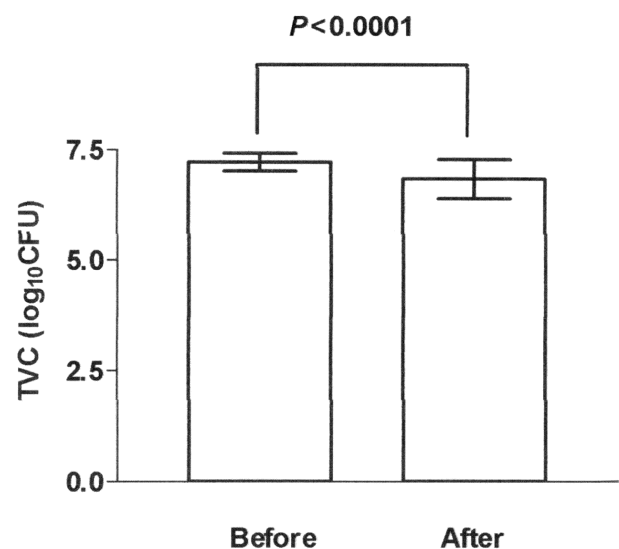Fig. 3   Clinical view of peri-implant defect and dental implant surface after stained by TBO.

Fig. 4   Mean and standard deviation of the effect of lethal photosensitization on the viability of bacteria (TVC) in peri-implant microbial samples.
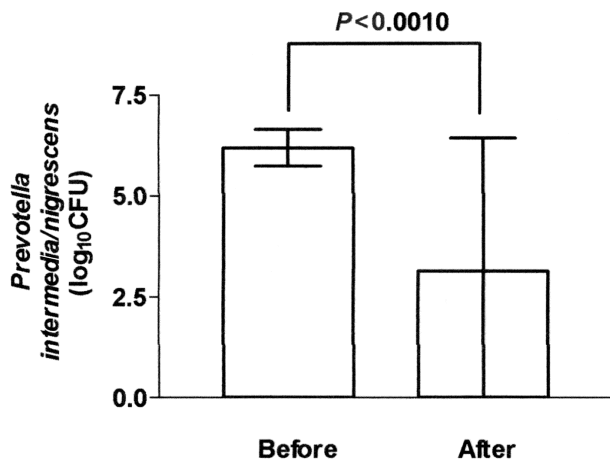
Fig. 5 Mean and standard deviation of the effect of lethal photosensitization on viability in all samples positive for Prevotella intermedia/nigrescens.
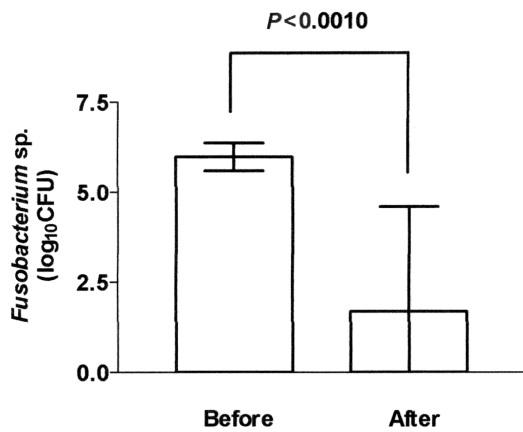

Fig. 6 Mean and standard deviation of the effect of lethal photosensitization on viability in all samples positive for Fusobacterium spp.
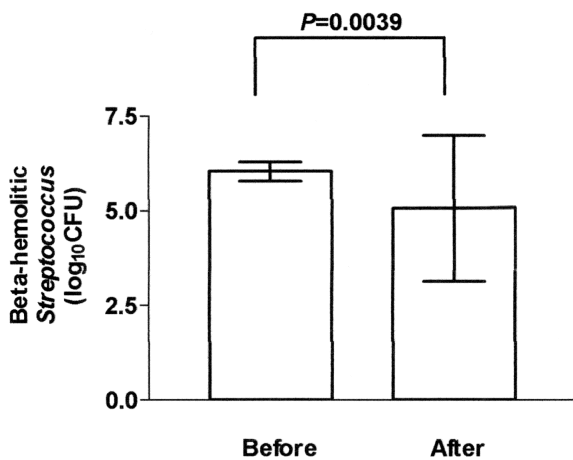

Fig. 7 Mean and standard deviation of the effect of lethal photosensitization on viability in all samples positive for beta-hemolytic Streptococcus.

0.0039).

Complete bacterial elimination was achieved in *P. intermedia/nigrescens* (6 out of 12 samples), *Fusobacterium* spp. (6 in 11 samples), and beta-hemolytic *Streptococcus* (1 in 9 samples) (Table 1).

Discussion

Several studies have demonstrated the bactericidal effect of high-power laser on contaminated dental implant surfaces (21-23). This energy is specifically absorbed by water molecules, which cause the water-rich tissue to be preferentially vaporized, in bacterial cytoplasm this effect causes cell lyses.

In addition, studies (8,9,24) have shown the effectiveness of lethal photosensitization in decreasing the viable count of periodontal pathogens in peri-implantitis lesions without damage to the dental implant surface.

This preliminary study attempted to examine whether lethal photosensitization, which has been shown to be effective in eliminating periodontal pathogens (*in vitro*) on contaminated titanium implants surfaces (8), is also effective *in vivo* (9,24). The results of this preliminary study indicate that photosensitization with TBO plus irradiation

Implant Surface	TVC Before	TVC After	P.intermedia/nigrescens Before	P.intermedia/nigrescens After	Fusobacterium sp. Before	Fusobacterium sp. After	Streptococci Before	Streptococci After
CP	7.18	7.00	6.40	6.40	6.18	0.00	5.70	5.40
	7.40	7.35	6.18	0.00	5.87	0.00	6.30	6.00
	7.30	6.40	6.40	6.30	6.30	0.00	6.08	5.70
	7.30	6.09	-	-	-	-	-	-
	7.40	7.35	-	-	-	-	-	-
TPS	7.18	6.40	6.07	0.00	6.30	0.00	6.18	5.40
	7.47	7.30	6.40	6.09	6.40	6.30	6.40	6.18
	7.40	7.10	6.70	6.57	6.00	0.00	6.18	5.69
	6.87	6.57	6.18	6.00	-	-	5.87	5.40
	7.47	7.40	-	-	-	-	-	-
	7.18	7.18	-	-	-	-	-	-
HA	7.00	6.70	6.10	0.00	5.70	0.00	5.87	0.00
	7.18	7.10	5.40	0.00	-	-	-	-
	7.00	6.70	-	-	-	-	-	-
Acid	7.10	6.40	6.30	0.00	5.30	0.00	6.10	5.40
	7.30	6.70	6.87	6.40	5.70	0.00	-	-
	6.87	6.70	5.30	0.00	6.40	6.17	-	-
	7.57	7.44	-	-	5.55	0.00	-	-
	7.04	6.10	-	-	-	-	-	-

Table 1 CFU/ml after logarithmic transformation, before and after lethal photosensitization (-: Species were not identified in this site.)

with a GaAlAs diode laser (200 J/cm^2) result in a statistical reduction of periodontal pathogens of up to a maximum of 1 log step.

These results confirm those obtained by other studies that use various photosensitizers in combination with a low-power laser to induce bacterial reduction (25,26,27). Periodontal pathogens, such as *Fusobacterium nucleatum, Prevotella intermedia and Actinobacillus actinomycetemcomitans* were killed after photodynamic therapy (28). Furthermore, the dye/laser combination was demonstrated for the first time, to be able to reduce the beta-hemolytic *Streptococcus* mean count. However these data should be considered with caution due the sample size utilized in this pilot study and also due to absence of control group. In addition, other possibility that reduction of bacterial density in a fluid that filled the ligature-induced peri-implant defect can also caused by physical removal of the microorganisms prior to the second sample collection by stream of the TBO solution while staining and suctioning since it is well know that bacteria attach to the infragingival surfaces quite loosely.

Many factors may interfere in the laser irradiation, these include the capacity for light absorption by the microorganism, the wavelength of the laser, the physiological state of the bacteria, the emission form of the laser, the time of exposition to the laser, the pH of the medium, the staining of the area to be irradiated, water content, thermal conductivity and the organic matrix (29). In our study we could conclude that biofilm present on dental implants surfaces was susceptible to photodynamic treatment and their variables.

Various drugs have been used over the last few years in association with low-power lasers to promote their bactericidal effects, since the low-intensity lasers by themselves do not have the capacity to cause significant reductions in the microorganisms as they induce a photochemical reaction rather than a thermal reaction. However, the association of these lasers with photosensitizers causes an alteration in the viabilities of different microorganisms (26,29,30), confirmed by our data. Several authors conclude that neither low intensity laser irradiation nor photosensitizers alone can kill these bacteria. Haas et al. (8) evaluated, by microbiologic examinations, the effectiveness of lethal photosensitization in different implant surfaces. They conclude that the group that utilized TBO plus laser was effective while the others groups (TBO alone, Laser alone, saline solution) were not effective. These results agree with the studies conducted by Bhatti et al. (31) and Bhatti et al. (8). In these works the authors evaluated not only the distribution of TBO but also the dosimetric effect and physiologic factors on lethal photosensitization of *P. gingivalis*. We can also cite Sarkar and Wilson (12), and Dortbudak et al. (9), who agree with the statement that lethal photosensitization is effective when the photosensitizer was used plus laser irradiation.

Although the mechanism by which the low-power lasers cause bacterial reduction when associated with photosensitizers is not totally clarified, some authors believe that when the laser is strongly absorbed locally, due to the sensitization of the bacterial photoreceptors by the stains, an oxygen molecule with a high degree of atomic excitation (oxygen triplet = O_3) is produced, but only has a bactericidal effect upon the production of free radicals or singlet oxygen (29). The reason why an oxygen molecule affects only the bacteria is because the light dose required to kill bacteria treated with TBO is far lower than that causing toxicity in cultured human keratinocytes and fibroblasts (32). In addition to this important effect is the presence of the selectivity of the laser by the use of specific stains for each wavelength, due to the different degrees of absorption by the stains (33).

As well as being non-toxic to man, the ideal photosensitizer must be able to absorb strongly at the wavelength of light used, have high excitation efficiency (a high probability of triplet state formation per photon absorbed) and a relatively long (several microseconds) triplet state (29). Wilson et al. (33) screened a number of compounds for their ability to sensitize oral bacteria to killer by low power laser. These compounds include TBO, methylene blue, aluminum disulphonated phthalocyanine, thionin, crystal violet and dihaematoporphyrin ester (29).

This study utilized sensitization to TBO, since other studies (25,31,34) have demonstrated the effectiveness of this stain in association with red spectrum-wavelength lasers on bacterial viability, probably due to the fact that TBO strongly absorbs visible spectrum wavelengths (red). This finding agrees with the theory that the bactericidal effect of lasers depends upon their wavelength (29). The wavelength used to kill periodontal pathogens in ligature-induced peri-implantitis in this study was shown to be effective; however additional investigations must be conducted.

In addition, this approach to killing periodontal pathogens offers some advantages over the use of conventional antimicrobials: avoids development of resistance among target organisms to the photochemically-generated free radicals thought to be responsible for bacterial killing and, unlike antiseptics and antibiotics, there would be no need to maintain high concentrations of the TBO in the peri-implant defects for long periods.

Within the limits of this pilot study, it may be concluded that microbiological peri-implantitis treatment by lethal

photosensitization reduced and eliminated, in some samples, the periodontal pathogens involved in this pathological condition. Further studies will be necessary to evaluate the amount of re-osseointegration after lethal photosensitization.

Acknowledgments

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