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Original Research

Antimicrobial Effects of Two Different Photodynamic Therapies on Nitriding-treated and Non-treated Titanium Surfaces

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Abstract:

Background: This study evaluated bacterial decontamination on nitriding-treated (etched with nitric acid) (TiT) and non-treated titanium (TiNT) machined discs using photodynamic therapy (PDT). An implant's surface roughness has a significant impact on bacterial plaque accumulation, as rough surfaces encourage biofilm formation. The surfaces of treated titanium implants tend to be a more asperous. Initial bacterial adhesion begins in areas of surface free of energy and wettability and inside the grooves of the roughened surfaces, from where it is difficult to eliminate plaque. The aim of this in vitro study was to evaluate two PDT protocols on both treated and TiNT surfaces, contaminated with Staphylococcus aureus or Escherichia coli.

Materials and Methods: Low-level laser (LLL) and methylene blue or light emitting diode (LED) and curcumin were used to decontaminate 60 machined titanium discs. ANOVA and Tukey's multiple comparison tests were applied.

Results: Our results showed that PDT reduced the number of bacteria on titanium discs, regardless of surface treatment (TiT and TiNT) ($P < 0.05$). There was no statistically significant difference between the tested groups (LLL and LED).

Conclusion: Based on these results, PDT could be considered an additional treatment option. Furthermore, in vitro and prospective clinical trials are necessary for confirmation of these results.

Key Words: Light emitting diode, low-level laser, photodynamic therapy, titanium discs, treated titanium surfaces

Introduction

With the growing popularity of titanium implant treatments and the increasing number of performed surgeries in recent years, short-term and long-term complications with implants have also increased.[1] The prevalence of peri-implant diseases was evaluated on The Consensus Report of the Sixth European Workshop in Periodontology and revealed an incidence of mucositis of up to 80% and of peri-implantitis between 28% and 56%.[2,3]

The literature outlines evidence that the microbial biofilm associated with peri-implantitis is similar to that of periodontitis, with high levels of non-motile anaerobic Gram-negative bacteria and spirochetes.[1,3]

Implant decontamination by mechanical, chemical and physical methods is used in the treatment of peri-implantitis. However, conventional mechanical treatment (air-powder abrasive treatment, mechanical cleaning with curettes or scalers or the application of plastic-coated ultrasonic scalers) may fail to eliminate bacteria in hard-to-reach areas, such as implant threads.[4,5] Furthermore, the roughness of implant surfaces poses an obstacle in decontamination. The implant's surface roughness has a significant impact on the quantity of the bacterial plaque formed. The surfaces of treated titanium implants tend to be a more asperous. In addition, initial bacterial adhesion starts in areas of surface free of energy and wettability (characteristics of titanium), as the presence of free metal ions contributes to the roughness of the surface and hence, bacterial adhesion. Furthermore, it is difficult to remove plaque from inside the grooves of the roughened surfaces.[1,6]

A recent study demonstrated that hydrothermally synthesized TiO2-anatase coatings, which are advantageous in terms of corrosion resistance, inflammatory potential, and osteogenesis, significantly reduced the *Escherichia coli* adhesion on the surface of implants (approximately 50% less for TiB, hydrothermally treated titanium, versus non-treated titanium [TiNT]).[6]

The antimicrobial effects of laser light (neodymium-doped yttrium aluminum garnet, erbium-doped: Yttrium-aluminum-garnet, CO_2, low-level laser [LLL]) on implant surfaces has been demonstrated.[4,7] Light emitting diode (LED) can potentially provide a novel decontamination method for both Gram-positive and gram-negative species.[8]

Therefore, an alternative to the treatment of peri-implantitis is photodynamic therapy (PDT). PDT is a modality based on the activation of exogenous photosensitizing agents by a light source in combination with photosensitizers (e.g., toluidine blue, methylene blue [MB], curcumin).[9] Curcumin is a natural yellow pigment extracted from the rhizomes of *Curcuma longa* and has a wide range of pharmacological effects, including anti-inflammatory, anticarcinogenic, and anti-infection properties.[10]

PDT generates bactericidal effects against aerobic and anaerobic bacteria (*Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Streptococcus mutans, Staphylococcus aureus, or E. coli*).[4] Besides the inactivation of pathogenic bacteria, it is essential to remove cytotoxic cell components such as endo- and exotoxins from implant surfaces.[7] Bacterial adhesion to the implant surface can be augmented by PDT, thus providing an acceptable alternative for the treatment of peri-implantitis.

The aim of this *in vitro* study was to evaluate two PDT protocols on both nitriding-treated and TiNT surfaces, contaminated with *S. aureus* or *E. coli*.

Materials and Methods

Titanium discs

A total of 60 titanium discs were used in this study. The machined titanium discs (width of 2 mm and 6 mm diameter) were manufactured for this study (Implants Kopp, Curitiba, Brazil). The surface roughness of each disc was measured 3 times by a rugosimeter (Mitutoyo SJ-201). The surface within the valleys was, additionally, measured in both the parallel and perpendicular directions to analyze the local asperity features and determine the roughness. Average roughness (Ra) values are reported in the Results section.

Bacterial culture and inoculation disc

The standard strain of *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) were stored in Positivo University Microbiology Laboratory (Curitiba, Brazil). The bacteria were grown overnight in test tubes containing 5 ml of a medium of Brain-Heart Infusion broth (Difco, São Paulo, SP, Brazil) and incubated aerobically at 37°C (Fanem, São Paulo, SP, Brazil) for 24 h. Microbial growth was adjusted to a 0.5 McFarland scale prepared for standard bacterial inoculum containing approximately 1.5×10^8 bacteria to infect the titanium discs. Before inoculation, the cells were dispersed in the solution with a test tube shaker (Vortex, Araraquara, SP, Brazil).

The sterile titanium discs were separated into two groups - nitriding-treated (etched with nitric acid (TiT) and TiNT titanium surfaces. Each group was contaminated on one side of the titanium discs with 10 ml of *S. aureus* and *E. coli* solution. After contamination, the discs were incubated for 1 h at 35°C for adhesion of bacteria on the titanium surface.

Treatment protocol

After contamination with *S. aureus or E. coli,* the discs were randomly divided into subgroups, according to the therapy, totaling 12 groups. Table 1 shows the distribution of each group, and the treatment they received.

Two PDT therapy protocols were evaluated. One therapy involved LLL at a wavelength of 685 nm (DSP, Brazil), capable of delivering 6.0 J/cm^2 of power, radiating 10 µl of MB at a concentration of 1% on the contaminated surface of the titanium disc. Similarly, the LED, with a wavelength of 420 to 480 nm (Shuster dental materials, Santa Maria, RS, Brazil), was capable of delivering 1250 mW/cm^2 of power, radiating 10 µl of a curcumin solution on the contaminated surface of the titanium discs. The curcumin was weighed to obtain a stock solution of 8 mM in sterile saline, and 0.5 ml of the supernatant was added to 0.5 ml of bacterial suspension, making a final concentration of 4 mM. After the decontamination protocol, each disc was inoculated aseptically into individual test tubes containing 5 ml of sterile saline, sonicated for 1 min for dispersion of bacterial cells that survived. An aliquot of 100 µl of each solution was inoculated into test tubes containing sterile brain heart infusion (BHI) broth. All tubes were incubated for 24 h to allow microbial growth, prior to recording of the turbidity by a Shimadzu spectrophotometer (model UV-1601PC).[11]

The control groups infected with *S. aureus* or *E. coli* did not undergo decontamination processes; however, they underwent sonication procedures and were inoculated in BHI and incubated as described above.

After the incubation period, all tubes were stored in a refrigerator at a temperature of 1-5°C until the measurement of absorbance by spectrophotometer. A quartz cuvette was employed in spectrophotometric scanning. The device was calibrated with distilled water and standardized with standard solutions containing bacteria (maximum concentration 1.050 bacteria/mL), and the absorbance was read at a wavelength (λ) of 540 nm (Figure 1). The data recorded in a spreadsheet

Table 1: Distribution of titanium discs according to PDT after bacterial contamination.

Contamination	***Escherichia coli***		***Staphylococcus aureus***	
Ti discs	**TiT**	**TiNT**	**TiT**	**TiNT**
Therapy	LLL+MB (*n*=5)	LLL+MB (*n*=5)	LLL+MB (*n*=5)	LLL+BM (*n*=5)
	LED+curcumin (*n*=4)	LED+curcumin (*n*=5)	LED+curcumin (*n*=5)	LED+curcumin (*n*=5)
	No therapy (*n*=5)	No therapy (*n*=5)	No therapy (*n*=5)	No therapy (*n*=5)

Ti: Titanium, TiT: Nitriding-treated (etched with nitric acid), TiNT: Non-treated titanium, LLL: Low-level laser, LED: Light emitting diode, MB: Methylene blue, PDT: Photodynamic therapy specifically designed for the purpose of tabulating the results for statistical analysis.

The results of the study were derived from parametric tests since the data were a good fit to a normal distribution (Shapiro-Wilk test). ANOVA and Tukey's multiple comparison tests were applied; $P < 0.05$ was established for rejection of the null hypothesis.

Results

The average roughness (Ra) values demonstrated no statistical difference between machined discs from the same groups (TiNT or TiT). When we compared values between TiT (0.832 μm) and TiNT (0.295 μm), the difference in average roughness was statistically significant ($P < 0.05$).

Analysis of the results showed significant differences in spectrophotometer measurements ($P < 0.05$) between the TiT and TiNT groups without light therapy with LLL and LED; and no statistical difference between LLL and LED (Table 2). All untreated titanium discs that were contaminated with bacteria (*S. aureus* or *E. coli*) showed higher values on spectrophotometer analysis (Graph 1a and b).

Discussion

Conventional treatment of titanium implants by mechanical instrumentation (dental curettes, ultrasonic scalers, air-powder abrasive) and/or chemical procedures (chlorhexidine digluconate 0.12%) to produce a healthy surface for osseointegration remains challenging. The elimination of bacterial toxins from the implant surfaces becomes increasingly difficult once the roughness of the implant's surface facilitates bacterial adhesion and colonization.[1,12] In the present study, we have selected *S. aureus* and *E. coli* because they are considered the gold standard to compare microbial decontamination, once they represent the entire group of gram positive and gram negative.

Table 2: Spectrophotometer analysis on titanium nitriding-treated and TiNT discs according to the light therapy.

Bacteria	Titanium disc	Light therapy	Spectrophotometer analysis (nm)
E. coli	TiT	No therapy	1.48^{b}
E. coli	TiT	LLL	1.19^{c}
E. coli	TiT	LED	1.31^{c}
E. coli	TiNT	No therapy	1.51^{b}
E. coli	TiNT	LLL	1.19^{c}
E. coli	TiNT	LED	1.17^{c}
S. aureus	TiT	No therapy	1.97^{b}
S. aureus	TiT	LLL	1.08^{a}
S. aureus	TiT	LED	0.56^{a}
S. aureus	TiNT	No therapy	0.64^{a}
S. aureus	TiNT	LLL	0.55^{a}
S. aureus	TiNT	LED	0.92^{a}

Different letters indicate statistical significance ($P<0.05$). *E. coli: Escherichia coli, S. aureus: Staphylococcus aureus*, LLL: Low-level laser, LED: Light emitting diode, TiNT: Non-treated titanium, TiT: Nitriding-treated (etched with nitric acid)

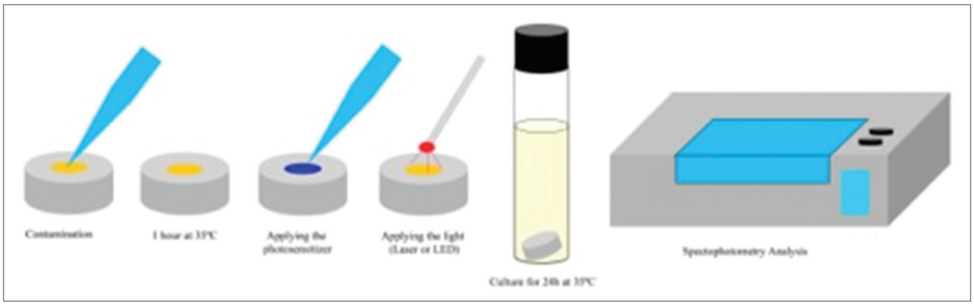
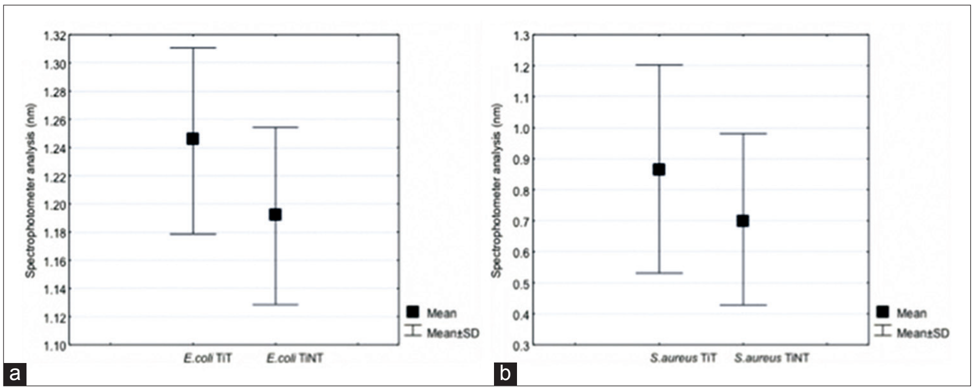


Figure 1: Methodological sequence: bacterial infection (10 ml of *Staphylococcus aureus* and *Escherichia coli* solution) on one side of the titanium discs. Photosensitizing application (low level laser+curcumin or light emitting diode+methylene blue) to decontaminate the surface of titanium discs. Incubation period and measurement of absorbance by spectrophotometer analysis.



Graph 1: (a) *Staphylococcus aureus* group (P=0.0986), (b) *Escherichia coli* group (P=0.2801). There was no statistically significant difference between microbial growth for groups non-treated titanium and Nitriding-treated (etched with nitric acid).

In this study, there was a statistically significant difference in average roughness between TiT (0.832 μm) and TiNT (0.295 μm). However, there was no difference between bacterial (*S. aureus* or *E. coli*) growth or adhesion between treated and non-treated discs. Indeed, all 60 discs in this study had a similarly infected surface. It is well-known that bacterial adhesion to implant surfaces is not only a critical first step in biofilm formation but also a trigger in the development of mucositis and peri-implantitis.[6] It is suggested that treated titanium implant discs offer protection against bacterial adhesion, producing less contact area between the bacterium and the coating, but it was not possible to confirm this in the present study. Results in this study showed statistically significant differences between TiT and TiNT with no light therapy. Indeed, when *S. aureus* and *E. coli* groups were compared, a difference in bacterial adhesion was noted. This observation may be explained by the similar degree of wetting between *E. coli* and the titanium coating. *E. coli* may have a greater adhesion to the treated surface of the implant etched with nitric acid. Nitriding-treated coatings on titanium may favor bacterial adhesion due to the peculiar topography and poor wettability of these coatings. Hydrophobic surfaces increase the number of attached bacteria, compared with hydrophilic surfaces. The influence of surface modification on bacterial adhesion to titanium has been studied and corroborates these findings.[6]

Evidence suggests that the composition of the subgingival biofilm is similar in both teeth and implants.[16,13] In particular, *S. aureus* appears to play an important role in the development of peri-implantitis.[12,3] This bacteria shows a high affinity for titanium.[3]

PDT acts mainly by the generation of reactive oxygen species and/or singlet oxygen, which can damage the cellular cytoplasmatic membrane, producing cellular death.[14] PDT reduced the number of bacteria on TiNT discs, compared with the control ($P < 0.05$). In the present study, no statistically significant difference between LLL and LED was noted from both TiT and TiNT groups. Due to numerous variable irradiation parameters, it is very difficult to compare these results to other investigations.[7] The PDT used in the present study reduced, but did not completely eliminate, bacterial contamination from machined titanium discs. It demonstrated better results than no therapy at all, but only reduction and not elimination. No difference was noted in the TiNT group treated with LLL, LED or no therapy ($P > 0.05$). This suggests that PDT had no effect on Gram-positive bacteria. Moreover, in previous studies, *S. aureus* showed a high affinity for titanium implants, which may be the reason for the difficulty of decontamination.[3]

These results may be explained by the fact that curcumin dye may exert antibacterial and anti-inflammatory effects without exposure to additional light. Moreover, curcumin-mediated antimicrobial PDT can be used at low doses with considerable antibacterial effect, due to its broad absorption peak (range 300-500 nm).[4,10] In this study, LED absorption ranged between 420 and 480 nm. The efficacy of the use of MB in eliminating various microorganisms has been demonstrated in previous studies.[15]

Antimicrobial PDT may have better access to regions that are unable to be reached by conventional treatment, such as implant threads, and the bacterial selectivity of the photosensitizer prevents damage to host tissues at the site of infection.[16]

PDT should be considered as an additional treatment option. Due to the fact that it is a relatively new approach, it is difficult to compare data from literature addressing variable irradiation parameters and different photosensitizers. Investigations vary in type and concentration of the photosensitizer, type of laser used, wavelength, power, and irradiation time. This study evaluated two PDT (LLL and LED) protocols on both nitriding-treated and TiNT surfaces, contaminated with *S. aureus* or *E. coli*. It was observed that LLL and MB or LED and curcumin could be an acceptable alternative for peri-implantitis treatment. Additional *in vitro* evaluations prior to prospective clinical trials may be necessary.

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