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Antimicrobial efficacy of photodynamic therapy and light-activated disinfection on contaminated zirconia implants: an *in vitro* study

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Research highlights

► The antimicrobial effect of photodynamic therapy and light assisted disinfection is evaluated.

► Photodynamic therapy is successful in decontaminating zirconia implant surfaces .

► Light activated disinfection is successful in decontaminating zirconia implant surfaces.

Photodynamic therapy and light activated disinfection do not damage the implant surfaces

Abstract

Background: We aimed to evaluate the antimicrobial efficacy of photodynamic therapy (PDT) and light-activated disinfection (LAD) on zirconia dental implants contaminated with three bacterial species and investigate if the PDT and LAD cause implant surface alterations. *Methods:* Seventy-two zirconia dental implants were contaminated with a bacterial suspension of *Prevotella intermedia, Actinomyces actinomycetemcomitans,* and *Porphyromonas gingivalis.* The implants were subsequently randomly divided into four groups (n=12 dental implants/each) according to the decontamination protocol: Group 1 (PDT1) - PDT (660 nm, 100 mW) with toluidine blue; Group 2 (PDT2) - PDT (660 nm, 100 mW) with phenothiazine chloride dye; Group 3 (LAD) - light emitting diode (LED) with toluidine blue; and Group 4 (TB) - toluidine blue without the application of light. Implants in the positive control (PC) group were treated with a 0.2% chlorhexidine-based solution, and implants assigned to the negative control (NC) group did not undergo any treatment. Each implant was then placed in tubes containing

phosphate buffered saline (PBS) and vortexed for 60 s to remove the remaining bacteria from the implant surface. After 10-fold serial dilutions, 30 μ l of the suspension was plated on Brucella agar plates. After 72 hours, the colony forming units (CFU) were counted. Distinctive colonies were confirmed with MALDI Biotyper. The implants were analyzed using scanning electron microscope (SEM) to evaluate the possible surface alterations due to PDT or LAD.

Results: All study groups had significant reductions in the number of CFUs compared with the NC (p<0.05). PDT1, the PDT2, and the LAD groups had the largest bacterial reduction with respect to each bacterial species separately and the total bacterial count, and they were more efficient compared with the TB group (p<0.05). SEM analysis did not reveal any alterations of the implant surface after the treatment procedures.

Conclusion: Both PDT protocols and LAD showed high and equal effectiveness in decontamination of zirconia dental implants.

Keywords: Photodynamic therapy, light-activated disinfection, dental implants, decontamination, laser

Introduction

Titanium dental implants are currently the gold standard in dental implantology because of their biocompatibility and well-documented scientific results [1–3]. However, there are some concerns regarding their dark gray color, which can be visible through the peri-implant soft tissue, especially when a thin gingiva biotype is present or when there occurs desorption of the buccal plate [3,4]. Furthermore, titanium dental implants might cause galvanic adverse effects after contact with saliva and, even though rare, allergic reactions [5,6]. Due to these disadvantages, the focus has shifted toward new implant technologies. Recently, high-strength zirconia ceramics have been developed as an alternative material for dental implants. In addition to their tooth-like color and high strength, they also have higher fracture toughness compared to other ceramics [4,7,8]. According to some studies, zirconia implants induce lesser inflammatory response and bone resorption compared with the titanium particles, which suggests good biocompatibility comparable to that of titanium implants [2,3].

Similar to titanium dental implants, zirconia dental implants are affected by periimplantitis affects as well. The initial cause of peri-implantitis is microorganisms that are similar to the microbiota causing periodontitis; most of them being spirochetes and non-motile Gramnegative bacteria, such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, and *Treponema denticola* [9]. When treating periimplantitis, implant surface decontamination is a precondition for successful regenerative processes to take place [10]. Techniques of implant surface decontamination can be physical or chemical. Chemical methods include the localized use of anti-microbial solutions (chlorhexidine, tetracycline, citric acid, hydrogen peroxide, etc.) [10]. Physical methods include mechanical decontamination and decontamination using laser or other alternative light sources.

Photodynamic therapy (PDT) is a photochemical decontamination method based on a chemical interaction between light, a photosensitizer, and oxygen. Its decontamination potential is based on the activation of a photosensitive dye by the laser light [11]. As a result, the photosensitizer transcends from a low-energy ground state to a high-energy state, leading to the generation of cytotoxic reactive oxygen species and singlet oxygen that are toxic to the bacteria [11–13]. Many *in vitro*, animal, and clinical studies have already shown the non-invasive PDT to be successful and safe as an adjunct therapeutic protocol for titanium peri-implantitis [12,14–16]. Although diode lasers are the main light sources used for photodynamic therapy, the use of light emitting diodes (LED) devices as an alternative light source for light-activated disinfection (LAD) has recently been promoted [15,17].

The aims of the study were to evaluate the efficacy of PDT and LAD against multibacterial species colonization on zirconia dental implants and to analyze the possible alterations of the implant surfaces as a result of PDT and LAD.

Materials and Methods

Study sample

The study sample consisted of 72 sterile zirconia dental implants (whiteSKY,Bredent[®], Senden, Germany) with a diameter of 4.0 mm and length of 12 mm. The approval for the study was obtained from the Ethics Committee of the School of Dental Medicine, University of Zagreb.

Bacterial contamination of dental implants

All microbiological procedures were performed at the laboratory of the Department of Clinical and Molecular Microbiology, University Hospital Centre Zagreb.

A bacterial suspension was prepared from three bacteria species: *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*. The strain of *P. intermedia* was isolated from a clinical sample at the University Hospital Centre. *A. actinomycetemcomitans* (ATCC[®] 33384) and *P. gingivalis* (ATCC[®] 33277) were purchased from The Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Germany as dry frozen cultures. The bacteria were grown separately in Columbia agar for 72 hours. A bacterial suspension was prepared for each of the bacterial species and mixed together in a joint suspension using thioglycolate broth. A density of 600 nm (equivalent of 1×10^8 CFU/ml) was set by optical densitometer (Densimat, Biomerieux, Marcy l'Etoile, France).

Every implant was placed in a sterile 1.5-ml Eppendorf tube (Eppendorf, Hamburg, Germany) containing 300 µl of the prepared bacterial suspension and incubated in anaerobic conditions for 72 hours using GasPak[®] Anaerobic system (Becton, Dickinson and Co, Maryland, USA). Implants were immersed in their full length in the bacterial suspension.

Antimicrobial protocols

After the incubation period, the implants were taken out of the anaerobic chamber conditions and randomly divided into four groups (n=12 implants per group) and two control groups (n=12 implants each).

Group 1. Photodynamic therapy (PDT1)

The implants were treated with a diode laser (Laser $HF^{\mathbb{R}}$, Hager Werken, Duisburg, Germany) with a 320-µm optical flat fiber tip and a toluidine blue-based dye (155 µg/ml, Laser $HF^{\mathbb{R}}$ Paro-PDT solution).

Group 2. Photodynamic therapy (PDT2)

The implants were treated with a diode laser (Helbo[®] Therapielaser, Helbo Photodynamic Systems GmbH & Co KG, Grieskirchen, Austria) and a 3D fiber optic tip (HELBO 3D Pocket Probe, Helbo Photodynamic Systems GmbH & Co KG, Austria) with phenothiazine chloride dye (10 mg/ml, Helbo[®] Blue photosensitizer).

Group 3. Light-activated disinfection (LAD)

The implants were treated with a modified LED curing light (Optilight LD[®], Gnatus, Brazil). The pre-existing blue LED light was replaced with a red LED light (LZ1-00R205, LED Engin Inc.[®], San Jose, USA). A toluidine blue solution (Biognost[®], Zagreb, Croatia) was used as a photosensitive dye.

The parameters for PDT1, PDT2, and LAD are presented in Table 1.

The implants in thePDT1, PDT2, LAD groups were first coated with the photosensitizers and left for 60 s; then, they were rinsed with sterile saline solution. In order to standardize the irradiation treatment protocols for all implants, the implants were placed in a rotational electric motor (Shenzhen Powerful Electronics, Shajing, China) with a power of 12V, a current of 120mA, and a rotating speed of 10 rounds per minute. A sterile implant holder included in the implant packaging was fixed to the electric motor, and then the implants were placed on that holder. The light source was fixed on a static holder and placed approximately 5 mm away from the surface of the rotating implant; the light treatment time for every implant was 60 s (Figure 1).

Group 4. Toluidine blue treatment

The implants were immersed in a photosensitive dye - toluidine blue (Biognost[®], Zagreb, Croatia) solution (1mg/ml) - for 60 s; subsequently, they were rinsed with sterile saline solution to remove the excess dye.

In the positive control group (PC), the implants were immersed in 0.2% chlorhexidine gluconate solution (Curasept ADS[®] Curaden International AG, Kriens, Switzerland) for 60 s. After the removal from the chlorhexidine solution, the implants were rinsed with sterile saline to remove the remaining solution.

The implants in the negative control group (NC) did not undergo any treatment. In order to standardize the time in aerobic conditions for all groups, the implants were, after removal from the bacterial suspension, kept in room conditions for 60 s before microbiological analysis.

Microbiological analysis

Immediately after the treatment procedures, each implant was placed in a 1.5-ml Eppendorf test tube containing 500 μ l of phosphate buffered saline (PBS) and vortexed for 60 s to remove the remaining bacteria from the implant surface. From each tube, 100 μ l were transferred to 100 μ l of Mueller Hinton broth, and a volume of 20 μ l of PBS was transferred to a microplate well containing 180 μ l of broth creating a 10-fold dilution. Tenfold serial dilutions were performed by using 96-well microtiter plates; 30 μ l of the suspension from each well was then inoculated to Brucella agar plates. The plates were incubated in anaerobic conditions for 72 hours, and colony forming units (CFU) were counted. Macroscopically, distinctive colonies were confirmed with MALDI Biotyper (Bruker Daltonics, Germany).

Scanning electron microscopy analysis

After microbiological analysis, one random implant from each group and one sterile non-treated implant of the same type were chosen for scanning electron microscopy (SEM). The implants for the SEM analysis were stored in 2% paraformaldehyde for 2 hours. Then, the implants were dehydrated in increasing concentrations of ethanol (60%, 75%, and 95%) for 30 minutes in each one, and then they were left to dry all night. Prior to capturing the images, the implants were coated with gold and palladium sputter (SC7620 MiniSputter Coater, Quorum Technologies Ltd, UK). The surface of the implants was observed using SEM (Vega TS5136MM, Tescan, Brno, Czech Republic). The SEM images were taken at 1:250 magnifications, and all the images were taken between the fourth and fifth thread. A visual comparative analysis was done to compare the treated implants against a sterile, non-treated, same type of zirconia implant for possible surface alterations or damage.

Statistical Analysis

To determine the difference between the groups with respect to each bacterium separately and the total count of bacteria; the obtained data were compared by analysis of variance test (ANOVA). Multiple comparisons between the applied methods were done by the Tukey test. The level of significance was set at 5%. Because of the large differences in the standard deviations between the groups, the data were transformed using the following formula:

$$L = log_{10}(N+1).$$

To calculate the bacterial reduction and the reduction in percentage compared to the NC group, the following formula was used:

$$1-\frac{T}{c}=100\cdot\left(1-\frac{T}{c}\right)\%$$

where T stands for the mean value of each group and C stands for the NC group.

All calculations were done using the statistical package SAS system for Windows (Release 8.02, SAS Institute Inc., Cary, NC, USA).

Results

The results showed statistically significant differences between the groups with respect to each bacterial species separately and the total number of bacteria (p<0.05).

The bacterial reduction results are presented in Table 1. In terms of the total number of CFUs, all groups showed statistically significant difference when compared with NC (p<0.05), with the reduction of more than 99% in each group. Among all the groups, PDT1, PDT2, and LAD had greater bacterial reduction with respect to each bacterium separately and for the total count of bacteria. In addition to the difference to NC group, these three groups were significantly superior over the TB group (p<0.05). The TB group did not have statistically significant difference for *A*. *actinomycetemcomitans* compared to NC.

Intergroup analysis did not show significant differences between PDT1, PDT2, and LAD with respect to the total number of bacteria and number of each bacterial species separately (p>0.05). The SEM images obtained from PDT1, PDT2, and LAD groups did not show any surface alterations when compared with the image taken from the sterile implant of the same type; on visual examination, they appeared to be the same as the surface of the sterile implant (Figure 2).

Discussion

Photodynamic therapy is a promising alternative for the treatment of periodontal and periimplant diseases. With the increasing number of titanium and zirconia implants placed every year, there is a rising need to investigate new treatment options for the treatment of periimplantitis.

The lack of a clear protocol for treating peri-implantitis has directed the attention of the scientific community toward the use of photodynamic therapy as a treatment option or an adjuvant treatment for peri-implantitis in the recent years [14,18,19].

Since one of the main purposes of treating peri-implantitis is to decontaminate the implant surface, the effect of photodynamic therapy and light activated disinfection was evaluated on *in vitro* contaminated zirconia dental implants in this study.

The main goal of the study was to evaluate if PDT and LAD are effective when compared with the conventional disinfection with chlorhexidine solution (PC). According to our best knowledge, there are no studies published yet that evaluated the antimicrobial effect or surface alterations after the use of PDT or LAD on zirconia implants. Hence, our results are compared with previous studies done on titanium surfaces or *in vitro* grown bacterial biofilms.

Our results showed that, even though there are differences among them, all study groups had significantly lower bacterial counts compared to NC. In many studies done on titanium surfaces, using PDT, LAD, or only photosensitizer (TB) was shown to be effective in the bacterial reduction; however, the reductions were lower than the results obtained in our study [10,17,20].

This difference in results might be due to the fact that our study was done on zirconia implant surfaces, and according to other studies, the affinity of bacteria to attach to zirconia is significantly lower than affinity to attach to titanium surfaces due their difference in surface

properties, such as surface roughness and surface free energy [21,22]. We assume that the bacteria were not attached strongly to the implant surface after 72h of incubation. In addition to PDT and LAD, the rinsing of the photosensitizer might have caused additional detachment of the bacteria from the implant surface.

When comparing the study groups among themselves, the most effective were PDT1, PDT2, and LAD. In addition to NC, they differed significantly from the TB group. When compared to PC, even though they had lower bacterial counts, there were no differences among them.

Marotti et al. [18] in their *in vitro* study showed that photodynamic therapy is effective in reducing the bacteria from the titanium implant surfaces. Similarly, Chan et al.[23] reported a 95–99% kill rate of various bacteria grown in *in vitro* conditions after the use of diode laser in combination with methylene blue as a photosensitizer. Our results are in accordance with these studies.

It is worth noting that the results obtained from the LAD group are comparable to PDT1 and PDT2, which can suggest that even with alternative light sources, such as light emitting diodes, an effective antibacterial effect can be achieved. The antimicrobial effect of LAD has been shown also on previous studies [24,25].

With respect to the use of different photosensitizers among PDT1, PDT2, and LAD, we could not find any differences in our study. This, however, is very difficult to conclude from an *in vitro* study due to the fact that different bacteria have different absorption affinity towards different photosensitizers. In addition, there are also bacteria that can produce endogenous photosensitizers (eg, *Porphyromonas gingivalis*) [24].

Surface alterations and damage of implant surface can interfere with the re-osseointegration of the implant, hence making the treatment less likely to succeed [26]. According to our results, PDT and LAD do not cause any structural changes on the implant surfaces. There are no studies that have evaluated the effect of PDT or LAD on the zirconia implant surface. However, our findings are in accordance with a previous study done on titanium implants [15].

Conclusion

Within the limited scope (*in vitro* contamination and decontamination of the implant surfaces) of this study, PDT and the LAD are effective treatment options for decontamination of zirconia dental implants surfaces.

Declarations of interest: none

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FIGURE LEGENDS:

Figure 1. The implant placed on the rotational electric motor and then treated with the light source (PDT1) 5 mm away from the implant.

Figure 2. Comparison among the a) sterile implant and the implants treated with b) PDT1, c) PDT2, and d) LAD; SEM images at 1:250 magnification rate.



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TABLES:

Table.1 Parameters of PDT1, PDT2, and LAD

	PDT1	PDT2	LAD		
Light Source:	Diode Laser (LaserHf)	Diode Laser (Helbo)	Light emitting diode		
Wavelength:	660 nm	660 nm	660 nm		
Photosensitizer:	Toluidine blue based	Phenothiazine chloride	Toluidine blue dye		
	dye	dye			
Power Output:	100 mW	100 mW	200 mW		
Power Density:	124.3 W/cm ²	35.37W/cm ²	0.71 W/cm ²		
Working Mode:	Continuous	Continuous	Continuous		
Irradiation time:	60 s	60 s	60 s		

Table 2. Mean and standard deviation for each of the bacteria separately and the total bacterial count presented in logarithmic form.

	A. actinomycetem.			P. gingivalis		P. intermedia			Total			
Group:	Mean	SD	p*	Mean	SD	p*	Mea	SD	p*	Mean	SD	p*
PDT1	0.4ª	0.8	< 0.001	0.4 ^b	0.8	< 0.001	0.8 ^b	0.9	< 0.001	0.9 ^b	1.0	< 0.001
PDT2	0.4ª	0.6		0.3 ^b	0.5		0.5 ^b	0.7		0.7 ^b	0.8	
LAD	0.8 ^a	1.1	_	0.6 ^b	0.7	_	0.8 ^b	0.9	_	1.1 ^b	1.2	_
TB	2.4	1.3	_	1.9 ^a	1.1	_	2.3ª	1.2		2.9ª	1.2	_
РС	1.2ª	1.0	_	0.9 ^{ab}	1.2	_	1.5 ^{ab}	1.5		1.8 ^{ab}	1.5	_
NC	5.9	0.7	_	5.7	1.0	_	5.9	1.3		6.7	0.9	_

* - p-value for ANOVA test

abc - result of post-hoc comparison (Tukey test). Means (groups) with the same letter in superscript were not significantly different. The presence of the same letter or letters in two or more groups means that there was no significant difference between them.