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Antimicrobial efficacy of photodynamic therapy using two different light sources on the titanium-adherent biofilms of *Aggregatibacter actinomycetemcomitans*: An *in vitro* study

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A R T I C L E I N F O

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A B S T R A C T

Background: Antimicrobial photodynamic therapy (aPDT), is a promising approach proposed as an adjunct for the decontamination of dental implant surfaces. This study aimed to investigate the effect of aPDT with laser or light emitting diode (LED) compared with conventional chlorhexidine treatment on the titanium-attached biofilms of *Aggregatibacter actinomycetemcomitans*.
Methods and materials: Thirty-six acid-etched and sandblasted (SLA) titanium discs were allocated to six groups and incubated with the titanium-adherent biofilms of *A. actinomycetemcomitans* as follows. Negative control (no treatment applied), positive control (0.2% chlorhexidine solution), 0.1 mg/mL Toluidine Blue [TBO] group, aPDT-treated groups subjected either to diode laser with a wavelength of 635 nm wavelength or LED with the peak wavelength of 630 nm with TBO as photosensitizer and sterile control (not contaminated). Following sonication and transferring the specimens to the microplate, the number of colony-forming units (CFUs) per disc was calculated. Data were analyzed using one-way analysis of variance (ANOVA) and additional post hoc tests with the level of significance set at $P < 0.05$.
Results: aPDT using TBO + LED was significantly more effective ($0.93 \pm 0.24 \times 10^4$) in the suppression of *A. actinomycetemcomitans* compared with TBO + Laser ($2.65 \pm 0.7 \times 10^4$). However, the lowest mean of CFU count was found in sterile, and chlorhexidine groups, respectively ($P < 0.0001$) and the highest bacterial count was observed in the negative control group ($P < 0.0001$).
Conclusions: LEDs and diode lasers have a lower ability to suppress *A. actinomycetemcomitans* biofilms compared to 0.2% chlorhexidine *in vitro*. However, the aPDT with the use of LED as a light source and TBO as a photosensitive agent could be an appropriate alternative to conventional chlorhexidine treatment.

1. Introduction

Reconstruction of the edentulous sites with implant-supported prostheses exhibits a survival rate of 97% after 14 years [1,2]. Nonetheless, this triumph has been influenced by the growing debate on peri-implantitis [3]. Peri-implantitis is defined as the polymicrobial inflammatory condition of the peri-implant soft tissues combined with progressing loss of the supporting bone [4]. Recent microbiological methods have revealed the microbial nature of peri-implantitis which mainly comprises of gram-negative anaerobes such as *Porphyromonas gingivalis*, *Prevotella intermedia/nigrescens*, *Actinomyces* species and *Aggregatibacter actinomycetemcomitans* [5]. Distinctively, *A. actinomycetemcomitans* is an opportunistic periodontal pathogen with several virulence factors and is capable of resisting the mechanical debridement owing to its protective extracellular matrix [6].

Numerous surgical and non-surgical approaches have been proposed to treat peri-implantitis with the primary intention of eliminating bacterial contamination and impeding bone resorption [7]. However, current roughened implant body surfaces have made the removal of the biofilm from the surface extremely challenging via mechanical decontamination alone [7]. Thus, antibiotics, antiseptics, and laser treatments have been advocated as therapeutic supplement alternatives in

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the non-surgical and surgical treatment of peri-implantitis [8]. Antimicrobial photodynamic therapy, also known as aPDT, is a state-of-the-art approach that employs photosensitizer and low-level energy source to destroy pathogenic bacteria [9]. The activation of the photosensitizer with a specific wavelength results in lethal alterations to the target bacteria [10]. Various studies have confirmed that lethal photosensitization of bacteria could be accomplished *in vitro* without any harm to the implant titanium surfaces [11,12]. Besides, it seems improbable that resistance to aPDT will arise since its bactericidal activity is because of singlet oxygen and other reactive species such as hydroxyl radicals, which involve a variety of cellular targets [13]. By the way, numerous photosensitive agents, accompanied by different light sources of energy, have been utilized to evaluate their effect on pathogenic bacteria [14,15] and predictable outcomes are not always observed since there are a considerable quantity of parameters such as irradiation doses, photosensitizer concentration, and inoculum concentration [16]. This study intended to investigate the effect of aPDT with laser or light emitting diode (LED) on the biofilm formation ability of *A. actinomycetemcomitans*.

2. Methods and materials

2.1. Study samples

Thirty-six sandblasted and acid-etched (SLA) titanium discs (Servo-Dental GmbH & Co. KG, Germany) with the surface roughness of 1–2 μm and the diameter of 8 mm and thickness of 2 mm were used. The surface of the disks was equivalent to the exposed area in a 4.1-mm diameter implant following 2-mm of vertical bone loss [17].

2.2. Bacterial strain and cultivation

A. actinomycetemcomitans ATCC® 33384 was cultivated in microaerophilic conditions in supplemented Tryptic Soy Broth (sTSB) (Difco Laboratories, Detroit, MI, USA), with 5% defibrinated sheep blood, 5 mg/L of hemin, 1 mg/L of Menadione (all purchased from Sigma-Aldrich Co., Ltd., Dorset, United Kingdom), and 5 g/L of yeast extract (Merck KGaA, Darmstadt, Germany) to a final concentration of 10^8 CFU/mL. Afterward, SLA titanium discs were submerged in 1 mL of the cultured suspension and then incubated at 37 °C in microaerophilic conditions for two days [18].

2.3. Photosensitizer and light sources

A stock solution of Toluidine blue O (TBO) (Sigma-Aldrich, Steinheim, Germany) with the concentration of 0.1 mg/mL was prepared in sterile 0.9% (wt/vol) NaCl and kept under dark conditions before implementation. AlGalnP diode laser (Konftec, Taiwan) at a wavelength of 635 nm and a light-emitting diode (LED) (FotoSan, CMS Dental APS, Copenhagen, Denmark) at a wavelength of 630 ± 10 nm and power density of 2000–4000 mW/cm^2 were used as light sources. The detailed characteristics of light sources are as follows: For the LED device, the tip diameter was 8 mm with the energy density of 60–120 J/cm2 (90 J/cm^2 in average), and spot area of 0.502 cm^2. For the 635 nm diode laser, the tip diameter was 7 mm with the spot area of 0.384-cm^2 and output power of 220 mW. Power density was 0.44 w/cm^2 considering the 1 mm distance between the light source and the specimen. The energy density and total energy of the diode laser was 13.2 J/cm^2 and 5.1 J, respectively. The output powers of all wavelengths were measured by a power meter (Laser Point s.r.l, Milan, Italy).

2.4. aPDT procedure

After washing the discs with phosphate-buffered saline (PBS) to remove planktonic bacteria, they were randomly allocated to six groups; each consisted of six SLA titanium disc as followed:

Group 1 (Neg-C); Assigned as a negative control in which no treatment protocol was performed.

Group 2 (Pos-C); Assigned as a positive control in which SLA discs immersed in chlorhexidine 0.2% solution for 5 min at 37 °C under dark conditions.

Group 3 (TBO-DL); TBO + Laser irradiation group in which TBO solution with the concentration of 0.1 mg/mL used for 5 min at 37 °C under dark conditions and then irradiated with a diode laser (DX62, Konftec, Taiwan).

Group 4 (TBO-LED); TBO + LED irradiation group in which TBO solution with the concentration of 0.1 mg/mL used for 5 min at 37 °C under dark conditions and then irradiated with LED device (FotoSan®, CMS Dental APS, Copenhagen, Denmark).

Group 5 (TBO only); Consisted of SLA discs immersed in TBO dye with the concentration of 0.1 mg/mL was used for 5 min at 37 °C under dark conditions.

Group 6 (Ster-C); In order to investigate the contamination of discs with other bacteria throughout the experiment, six discs were assigned as sterile control under similar incubation conditions.

In the aPDT-treated groups, (TBO-DL and TBO-LED) 200 μl of appropriate photosensitizer were added to the suspension. The discs were then kept in the dark for five minutes before irradiation, allowing photosensitizers to absorb into the bacterial cells. Irradiation was performed in a laminar flow hood (Besat, Tehran, Iran) in the dark under aseptic condition. Laser and LED devices were placed in a constant distance of 1 mm, perpendicular to the surfaces of the discs for 30 s.

2.5. Determination of colony forming units per milliliters (CFU/mL)

After treatment, all discs were washed with sterile PBS solution and then transferred to microtubes containing one mL of sTSB medium. In order to remove the superficial biofilms from the discs, the microtubes were sonicated (SinapTec, Stuttgart, Germany) for 30 s at the 50 Hz frequency with the power of 150 W. 10 μl of the generated suspension was inoculated into 96-well containing microplate (TPP, Trasadingen, Switzerland), each containing 100 μl of sTSB medium. Finally, each bacterial growth was quantified after 48 h of incubation at 37 °C by plating 10-fold dilutions on supplemented Trypticase Soy Agar (Difco Laboratories, Detroit, MI, USA).

2.6. Statistical analysis

Statistical tests were performed using SPSS ver. 22.0 software. Kruskal-Wallis tests and one-way ANOVA were used to determine the difference between the groups and the Post-hoc additional analysis were used for pairwise comparisons. P-value of < 0.05 was considered as statistically significant.

3. Results

This study was conducted on 36 SLA titanium discs contaminated with *A. actinomycetemcomitans*. The effect of different treatment protocols on the biofilm formation ability of *A. actinomycetemcomitans* is presented in Table 1.

In the sterile control (Ster-C) and positive control (0.2% chlorhexidine) groups, the CFU/mL level was zero and close to zero, respectively, indicating roughly complete removal of the bacteria and approving the sterility and decontamination of the discs. It was also observed that the most contaminated group was negative control (Neg-C) group in which no treatment protocol was applied.

The TBO-LED protocol significantly reduced microbial load compared to the TBO + laser and TBO only groups, demonstrating that the LED was superior in reducing the number of *A. actinomycetemcomitans* colonies compared to diode laser ($P < 0.0001$ according to Kruskal-Wallis test). It should be noted that the results of sterile and chlorhexidine control groups were excluded from statistical analysis due to

Table 1
CFU/mL levels in experimental groups. TBO-DL: Toluidine blue O plus diode laser; TBO-LED: Toluidine blue O plus light emitting diode; TBO: Toluidine blue O; Neg-C: Negative control group; CHX: Chlorhexidine 0.2% solution; Ster-C: Sterile control group.

Groups	Minimum	Maximum	Mean ± SD
TBO-DL	1.6×10^4	3.8×10^4	$2.65 \pm 0.7 \times 10^4$
TBO-LED	6.0×10^3	13.0×10^3	$0.93 \pm 0.24 \times 10^4$
TBO	6.0×10^4	11.1×10^4	$77.1 \pm 18 \times 10^4$
Neg-C	21.6×10^5	37.1×10^5	$284 \pm 59 \times 10^4$
CHX	< 100	< 1000	92 ± 115
Ster-C	0	0	0
P-value	0.0001*		

* shows a statistically significant result.

Table 2
Results of the post hoc test between experimental groups according to P-value. The level of significance was set to 0.05. TBO-DL: Toluidine blue O plus diode laser; TBO-LED: Toluidine blue O plus light emitting diode; TBO: Toluidine blue O; Neg-C: Negative control group.

Groups	Neg-C	TBO	TBO-DL	TBO-LED
Neg-C	–	0.001	0.001	0.0001
TBO	0.001	–	0.004	0.002
TBO-DL	0.001	0.005	–	0.013
TBO-LED	0.0001	0.002	0.013	–

*shows a statistically significant result.

the possible corruption of the test. By the way, One-way ANOVA showed that the difference between negative control (Neg-C) group, TBO, TBO-DL, and TBO-LED was also significant ($P < 0.0001$) (Fig. 1). Post-hoc test similarly showed that pairwise comparison between all experimental groups was significant (Table 2).

4. Discussion

The present study was conducted to investigate the antimicrobial effect of PDT using two distinctive light sources against *A. actinomycetemcomitans*. The results showed significant reductions in colony counts of this bacterium on the titanium surfaces in all studied groups. The highest reduction was observed with the use of chlorhexidine solution, and the lowest rate was seen in TBO only group. It was also found that the LED showed significantly higher efficacy in eliminating the bacteria compared to the diode laser.

Several studies have been carried out to find an alternative method for conventional treatment of peri-implantitis, which consists of mechanical debridement of the involved implant surfaces [8]. Of all the bacteria engaged in the periodontal and peri-implant diseases, *A. actinomycetemcomitans* plays a very significant part, especially in aggressive forms of the disease [6]. The disadvantages of mechanical therapy with systemic antibiotics are the difficulty in obtaining direct access and visualization of the area [19] and the unfeasibility of complete elimination of bacteria, as well as the struggle in maintaining the appropriate concentration of local antibiotics, which in turn can escalate the incidence of opportunistic infection [20].

Numerous advantages in the use of aPDT as an antimicrobial treatment approach have been specified in the literature. The photosensitive agent used in aPDT is administered exclusively to the contaminated site and therefore reduces adverse effects to a minimum amount [14]. It has also been shown that aPDT has no destructive impact on the peripheral soft and hard tissues [21]. Komerik et al. [13]

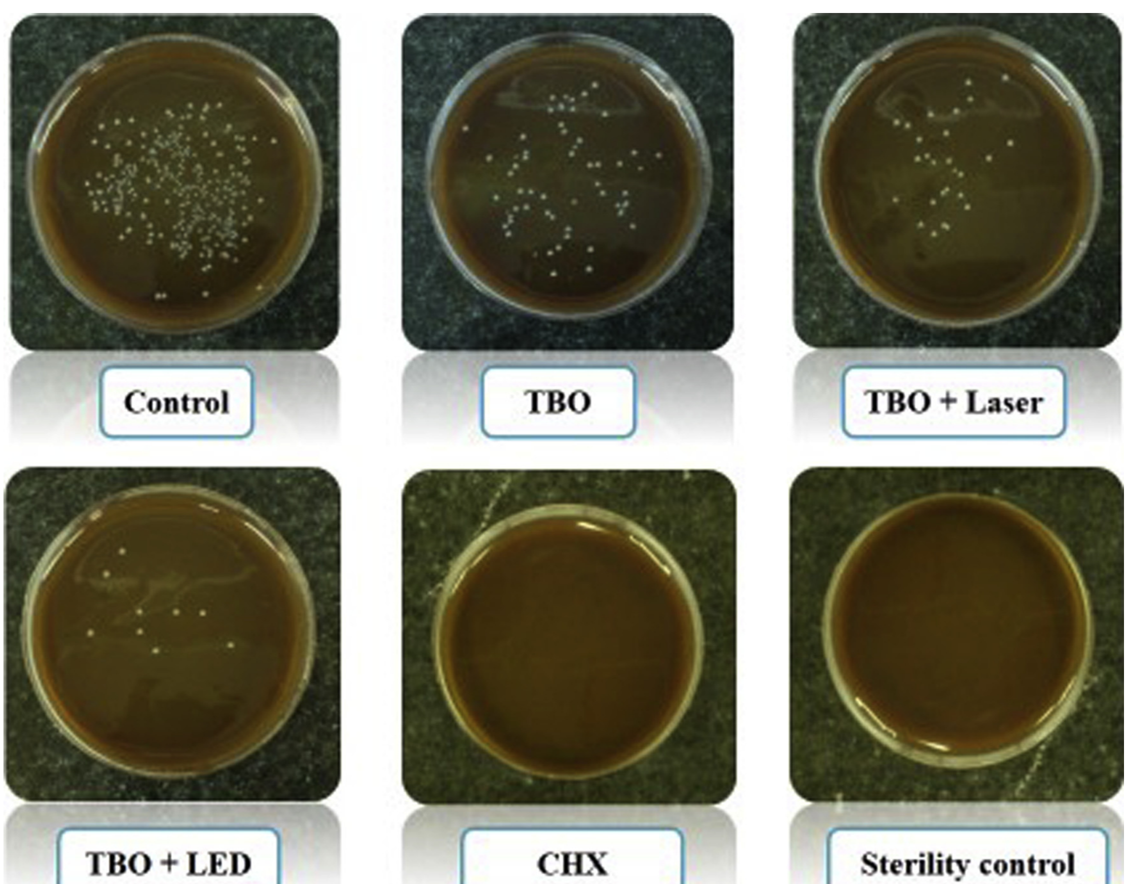

Fig. 1. Efficacy of different treatment modalities in the reduction of colony counts (CFU/ml) of *A. actinomycetemcomitans*.

in an animal study demonstrated that following the application of aPDT in combination with TBO on periodontal tissues, no alteration in the structure of periodontium, such as connective tissue ulceration or inflammation, even with high concentrations of TBO occurred. Authors also stated that aPDT plus TBO significantly reduced the *P. gingivalis* without inducing any tissue damage.

Selection of TBO as a photosensitizing agent in this study was based on evidence of biological stability, photochemical efficacy, environmental sustainability and minimal side effects [22]. Chan and Lai showed that this substance, after activated by a laser, only increases the temperature from 0.5 °C to 3.9 °C in the peripheral tissues and thus is safe to use in the clinical setting [19].

Mattiello et al. [23] stated that aPDT comprised of TBO and diode laser with the wavelength of 660 nm was effective in reducing the CFU/mL of *Streptococcus sanguinis* and *A. actinomycetemcomitans*, but in the present study diode laser showed less efficacy in reducing *A. actinomycetemcomitans* colonies compared to LED.

Hamblin and Hansan argued that the physiology of Gram-negative bacteria justify their susceptibility to the laser [21], as the cell wall contains an internal cytoplasmic membrane and an outer membrane that creates a physical and functional barrier between the bacteria and the outer environment and is responsible for maintaining the configuration of the cell. Previously, Rovaldi et al. [24] had found that the outer wall of Gram-negative bacteria is more resistant to aPDT than Gram-positive bacteria and that this defensive barrier interferes with the absorption of photosensitive substances. However, due to the positive charge of the TBO, it merely adheres to the outer wall of the Gram-negative bacteria and interacts with its lipopolysaccharide [25], and thus the TBO is a suitable photosensitive agent to destroy Gram-negative bacteria [26].

An early study by Chan and Lai [19] showed that the ability of the laser to eliminate periodontal pathogens depends on their strain. *Fusobacterium nucleatum* and *A. actinomycetemcomitans* appeared to be more resistant to aPDT than *P. gingivalis, P. intermedia* and *S. sanguinis*. Authors found that the number of periodontal pathogens, which exposed 30 s to diode laser with an output power of 665 nm in combination with 0.01 mg/mL TBO, significantly reduced (71–88%). In the current study, although TBO was applied at a concentration of 0.1 mg/mL, almost similar reduction was observed for *A. actinomycetemcomitans*. The researchers also pointed out that increasing the exposure time to 60 s combined with the energy density of 21.2 J/cm^2 could reduce the number of bacteria by 99–100 percent. In the case of *A. actinomycetemcomitans* and *F. nucleatum*, this increase in time leads to a decrease of 95–96%. The similar observation in the present study can be clarified by the same rationalization, since the 30-second time application of the light sources, provided better results for 630 nm LED.

Chui et al. [27] supposed that in aPDT, the homogeneity of the light source and the photosensitive agent is of great importance. In this study, the concentration of 0.1 mg/mL of TBO was used as it is confirmed to be the most effective on particular oral strains when applied with LED [26].

Several studies have been conducted with different photosensitive agents and light sources [27–29]. However, Monteiro et al. [30] did not observe any significant differences in the groups treated with a diode laser (660 nm) or LED (632 nm), though the examined strain was *Staphylococcus aureus*, a Gram-positive bacterium.

Marotti et al. [31] showed that the diode laser with a wavelength of 660 nm in combination with methylene blue dye with exposure times of either 3 or 5 min significantly reduced the bacteria residing on the implant surface. Contamination of implant surfaces in this study was performed by the saliva collected from a patient with periodontitis. Therefore, this reduction in microbial load in the anaerobic environment does not indicate the type and strain of the bacteria, though it shows the positive effects of aPDT. Moslemi et al. [32] also showed the effectiveness of a diode laser with the wavelength of 630 nm plus 0.1 mg/mL TBO against *A. actinomycetemcomitans* in planktonic conditions, which is in line with the findings of the current study. However, the bacteria assessed in that study were in the planktonic form that does not represent the oral environment precisely.

One of the noteworthy findings of the present study was the statistically significant reduction in the number of the bacteria in TBO-treated group compared to the negative control group. In a study by Damante et al., it was demonstrated that TBO for aPDT might have an acidic pH (pH ≈ 4), which stimulates enamel and dentin demineralization [33]. In a similar study, Piccirillo et al. showed that TBO was covalently bound to an activated silicone polymer through an amide condensation reaction, and the new polymers with covalently attached dye displayed significant bactericidal activity against *Escherichia coli* and *S. epidermidis* with a 99.99% reduction in viable bacteria after four minutes exposure to a low power laser [34]. Altogether, the observed antibacterial activity of TBO dye might be due to its acidic pH and chemical properties.

In the present study, 0.2% chlorhexidine showed the highest reduction in bacteria among the studied groups. The results were in support of the antibacterial properties of this substance, as Ccahuana-Vásquez [35] showed that administration of 0.12% chlorhexidine twice a day can eliminate a significant percentage of vital bacteria in biofilms and hinder its expansion. Similarly, in a study by Saffarpour et al. [36] the most significant reduction in the bacterial colonies was found in the chlorhexidine group. However, in their study, LEDs showed comparable results with the laser that may be because of the application of two different photosensitive agents for each of the experimental groups, but in the current study, the same substance was used for both light sources. Also, in a study by Nunez et al., authors concluded that the ionic strength is an essential variable in the effectiveness of aPDT [37]. Authors demonstrated that the solution used to dilute the dye is very vital and may affect the dimerization status of the dye. According to this study, TBO dissolved in water generated 2% more singlet oxygen than saline solution. Thus, the lower bactericidal capacity of aPDT compared with chlorhexidine solution in the present study might be related to the solution used to dilute the dye. Also, the better results of LED may be because of a higher chance to attain the absorption band of TBO diluted in saline solution.

Gianelli et al. [38] and Marotti et al. [31] reported that the efficacy of chlorhexidine is equal to or less than aPDT, which may be due to variations in the concentration of applied chlorhexidine. Nonetheless, there are some concerns regarding the use of chlorhexidine in conjunction with mechanical debridement. Some studies have demonstrated the cytotoxic effects of chlorhexidine on host cells. Gianelli et al. [38] showed that although chlorhexidine was washed away from the surface of the titanium discs before the culturing, the damage to host cells was still observed on the adjacent macrophages. However, discs exposed to aPDT exhibited macrophages with normal morphology. Also, chlorhexidine is capable of adhering to the implant surfaces and is gradually released over time [39]. Subsequently, the anti-bacterial properties and side effects persist for up to 24 h after washing.

One of the limitations of this study was the investigation of the efficacy of the different modalities on the only single bacterium. Besides, the application of different concentrations of photosensitive agents were not the objectives of this research. Nevertheless, the findings of this research can be resumed in clinical settings, as both diode laser and LED demonstrated a significant reduction in the colony counts of *A. actinomycetemcomitans*.

5. Conclusion

Within the limitations of this study, it was found that LEDs and diode lasers have a lower capacity to eliminate *A. actinomycetemcomitans* biofilms compared to 0.2% chlorhexidine *in vitro*. However, because of the concerns regarding the cytotoxicity of the chlorhexidine, the aPDT with the use of LED as a light source and TBO as a photosensitive agent could be an appropriate alternative.

Conflict of interests

The authors declare no conflict of interests and confirm that this article is an original work and is not under publication nor reviewing elsewhere.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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