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Efficacy of antimicrobial photodynamic therapy for elimination of *Aggregatibacter actinomycetemcomitans* biofilm on Laser-Lok titanium discs

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ABSTRACT

Background: Antimicrobial Photodynamic therapy (aPDT) is a novel modality suggested for treatment of peri-implantitis. This study aimed to assess the effect of aPDT with toluidine blue (TBO) and indocyanine green (ICG) and 635 nm and 808 nm diode laser on *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) biofilm formed on Laser-Lok titanium discs.

Materials and Methods: Eighty sterile Laser-Lok titanium discs were inoculated with A. actinomycetemcomitans to form biofilm and were randomly divided into 8 groups (n = 10) of control, chlorhexidine (CHX), TBO, ICG, 635 nm diode laser with 220 mW power, 808 nm diode laser with 250 mW power, 100 μg/mL TBO + 635 nm diode laser and ICG + 808 nm diode laser. Number of colony forming units (CFUs) on the surface of each disc was counted after the intervention. Data were analyzed using the Kruskal-Wallis test.

Results: Significant differences were noted in colony count among the eight groups after the intervention (P = 0.001). Pairwise comparisons with adjusted P value test showed that aPDT with TBO + 635 nm laser and ICG + 808 nm laser caused significant reduction of bacterial biofilm compared to the control group (P = 0.0001). TBO alone caused significant reduction of biofilm compared to the control group (P = 0.004). No other significant differences were noted (P > 0.05).

Conclusion: Within the limitations of this study, the results showed that aPDT is a potential modality for decontamination of implant surface and reduction of *A. actinomycetemcomitans* biofilm in vitro. In this study, aPDT with TBO + 635 nm diode laser and ICG + 808 nm diode laser decreased the bacterial load on titanium discs.

1. Introduction

With the increased demand for implant treatment, dental clinicians are now dealing with challenges related to management of peri-implant disease. The prevalence of peri-implant diseases is increasing [1] and a retrospective study reported a prevalence of 80% for peri-implant mucositis and 28% for peri-implantitis [2]. Bacteria involved in development of peri-implant diseases are the same pathogenic microorganisms causing periodontitis, which include *Staphylococcus aureus*, *capnocytophaga*, *spirochete*, anaerobic Gram-negative rods, *Fusobacterium nucleatum, Porphyromonas gingivalis (P. gingivalis)*, *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) [3]. Decontamination of implant surfaces is a difficult task. Several modalities are employed for this purpose depending on the severity of bone loss. The suggested modalities include mechanical methods such as the use of curettes, ultrasonic scalers and air powder abrasion and chemical methods such as the use of citric acid, hydrogen peroxide, ethylenediaminetetraacetic acid, chlorhexidine (CHX), local and systemic antibiotics and irradiation of Er:YAG and CO2 lasers [4]. If efficient treatment is not performed, progression of bone loss often leads to implant loosening and its subsequent explantation, which has significant financial, biological and psychological impacts on patients [5].

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Some of the suggested modalities for implant surface decontamination may damage the implant surface or generate resistant bacterial species [6,7].

Due to multifactorial nature of peri-implantitis, different methods of therapy, different study design and population, there is no consensus on a certain therapy as gold standard so far [4]. In this respect, different modalities are being to introduce with a same goal that is control and/or stop the process of peri-implantitis.

Controversial reports also exist regarding implant surface modifications due to laser therapy [8–10]. High-level lasers elevate the temperature and may alter implant surface. Also, laser equipment is expensive [11].

Antimicrobial photodynamic therapy (aPDT) is a novel, non-invasive photochemical modality suggested for elimination of periodontal pathogens, which has gained increasing popularity in the recent years [12,13]. It was first used for treatment of basal cell carcinoma in 1905 [14]. In aPDT, non-toxic chemical agents, known as photosensitizers, are used along with low level light energy as in diode laser or light-emitting diode to eliminate microorganisms. This modality has three main advantages: (I) it does not cause bacterial resistance, (II) it provides adequate antibacterial efficacy at the required site and (III) it does not damage the adjacent sound tissue due to its local effect [15].

At present, toluidine blue (TBO) and indocyanine green (ICG) are two photosensitizers used in aPDT [16]. The results of previous studies regarding the effects of TBO and ICG on *A. actinomycetemcomitans*, which is an important periopathogenic microorganism playing a role in peri-implantitis, have been controversial [17–21]. Considering existing controversy in this respect and assuming aPDT as a relatively new, noninvasive treatment approach, this study aimed to assess the efficacy of aPDT with TBO and ICG in combination with two wavelengths of diode laser for decontamination of Laser-Lok titanium discs inoculated with *A. actinomycetemcomitans* biofilm in vitro.

2. Materials and methods

In this *in* vitro, experimental study, 80 sterile Laser-Lok titanium discs with 10 mm diameter, 1 mm thickness and 8 μm grooves (BioHorizons, Birmingham, USA) were randomly divided into 8 groups (n = 10; six experimental groups, one negative control and one positive control) by block randomization.

Formation of *A. actinomycetemcomitans* biofilm on the discs:

A. actinomycetemcomitans (ATCC33384) was obtained in lyophilized form from the Microbiology Department of Tehran University, cultured in modified trypticase soy broth (TSB; Merck, Darmstadt, Germany) containing 3% yeast extract. It was then incubated at 37 °C and 5% CO2 in a jar/AnaeroPack-CO_2 system (Mitsubishi Gas Chemical Group, Tokyo, Japan) for 48 h [22].

The discs were inoculated with 1 mL of *A. actinomycetemcomitans* bacterial suspension with a final concentration of 10^7 colony forming units (CFUs)/mL in TSB and incubated at 37 °C and 5% CO2 in a jar/AnaeroPack-CO_2 system for 48 h [22]. In order to remove planktonic bacteria from the surface of the discs, the discs were rinsed with 5 mL of sterile phosphate buffered saline (PBS, pH:7) under aseptic conditions.

The discs were then subjected to different treatments in the study groups as follows:

Group 1. Control group: Discs with *A. actinomycetemcomitans* biofilm did not undergo any treatment) and were only rinsed with sterile PBS for 30 s.

Group 2. Chlorhexidine (CHX): Discs with *A. actinomycetemcomitans* biofilm were exposed to 0.2% CHX (Iran Najo, Tehran, Iran) for 5 min and were then rinsed with sterile PBS for 30 s [23].

Group 3. TBO: Discs with *A. actinomycetemcomitans* biofilm were exposed to 100 μg/mL TBO (Sigma, Taufkrichen, Germany) at 37 °C in the dark for 5 min and were then rinsed with sterile PBS for 30 s [24].

Group 4. ICG: Discs with *A. actinomycetemcomitans* biofilm were exposed to 1 mg/mL ICG (Sigma, Taufkrichen, Germany) at 37 °C in the dark for 5 min and were then rinsed with sterile PBS for 30 s [25].

Group 5. Discs with *A. actinomycetemcomitans* biofilm were rinsed with sterile PBS for 30 s and were then subjected to diode laser irradiation (DX62; Konftec, New Taipei, Taiwan) with 635 nm wavelength, 220 mW power, 13.14 J/cm^2 energy density at 1 mm distance with 8 mm tip for 30 s in a circular motion [24].

Group 6. Discs with *A. actinomycetemcomitans* biofilm were rinsed with sterile PBS for 30 s and were then subjected to diode laser irradiation (DX82; Konftec, New Taipei, Taiwan) at 808 nm wavelength, 250 mW power, 14.94 J/cm^2 energy density at 1 mm distance with 8 mm tip for 30 s in a circular motion [25].

Group 7. Discs with *A. actinomycetemcomitans* biofilm were exposed to 100 μg/mL TBO (Sigma, Taufkrichen, Germany) at 37 °C in the dark for 5 min and were then rinsed with sterile PBS for 30 s. Next, they were irradiated with 635 nm diode laser as in group 5 [24].

Group 8. Discs with *A. actinomycetemcomitans* biofilm were exposed to 1 mg/mL ICG (Sigma, Taufkrichen, Germany) at 37 °C in the dark for 5 min and were then rinsed with sterile PBS for 30 s. They were then subjected to diode laser irradiation as in group 6 [25].

Next, the discs were transferred into microtubes containing 1 mL of modified TSB. In order to remove biofilm from the disc surface, the microtubes containing the discs were sonicated at 50 Hz frequency and 150 W power for 30 s (Sinaptec, Germany). Next, 10 μL of the suspension was transferred into a round-bottom 96-well microtiter plate (TPP, Trasadingen, Switzerland) containing 90 μL of modified TSB. After two-fold serial dilution, 10 μL of each dilution was spread-cultured on sheep blood agar plates. The plates were incubated at 37 °C under microaerophilic conditions in a jar/AnaeroPack − CO2 system for 48 h. The colony count (CFUs/mL) of each group was calculated according to the protocol suggested by Miles and Misra [26].

Antimicrobial efficacy was evaluated by reduction in colony count (CFUs/mL) following culture. The percentage of reduction in colony count (CFUs/mL) was calculated using the following equation:

$$R = (A–B)/A \times 100$$

Where A is the number of colonies at baseline (before treatment), B is the number of colonies after the treatment and R is the percentage of reduction in colony count (CFUs/mL).

The mean and standard deviation of *A. actinomycetemcomitans* colony count were calculated and analyzed using SPSS version 22 (SPSS Inc., IL, USA). The groups were compared using the Kruskal-Wallis test. Pairwise comparisons were carried out with adjusted P value test.

3. Results

The groups were significantly different in terms of colony count and efficacy of decontamination of Laser-Lok surfaces (P = 0.001) such that the highest decontamination (the greatest reduction in colony count) was noted in CHX (100%) and the lowest in 635 nm diode laser group (50.61% ± 11.26%, Table 1). According to the Kruskal-Wallis test, decontamination in CHX group was significantly greater than that in 635 nm diode laser, 808 nm diode laser, control and ICG groups (P = 0.002, P = 0.0001, P = 0.0001 and P = 0.0001, respectively). Also, TBO + 635 nm laser group caused significantly higher decontamination compared with 635 nm laser, control and 808 nm laser groups (P = 0.006, P = 0.0001, P = 0.0001, respectively). The difference between TBO and control groups was also significant (P = 0.004). The difference between ICG + 808 nm laser and 635 nm laser and control groups was significant as well (P = 0.004 and P = 0.0001, respectively). No other significant differences were noted (Table 2).

4. Discussion

This study aimed to assess the effect of aPDT with TBO and 635 nm

Table 1
Percentage of decontamination of Laser-Lok discs (reduction in colony count) in experimental groups compared to the control group.

Group	Percentage of decontamination compared to the control group Mean ± SD	P-value
TBO	96.19 ± 1.41	0.0001*
ICG	84.01 ± 4.33	
635 nm laser	50.61 ± 11.26	
808 nm laser	74.49 ± 10.11	
TBO + 635 nm laser	99.73 ± 0.12	
ICG + 808 nm laser	99.32 ± 0.15	
CHX	100.00	

Table 2
Pairwise comparison of the groups Each node shows the sample average rank of new group (material + laser).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.
CHX-TBO + laser 635	10.050	10.381	.968	.333	1.000
CHX-ICG + Laser 808	19.950	10.381	1.922	.055	1.000
CHX-TBO	30.400	10.381	2.929	.003	.095
CHX-ICG	41.300	10.381	3.979	.000	**.002**
CHX-Laser 808	48.800	10.381	4.701	.000	**.000**
CHX-Laser 635	59.500	10.381	5.732	.000	**.000**
CHX-Control	70.000	10.381	6.743	.000	**.000**
TBO + Laser 635-ICG + Laser 808	−9.900	10.381	−.954	.340	1.000
TBO + Laser 635- TBO	20.350	10.381	1.960	.050	1.000
TBO + Laser 635-ICG	31.250	10.381	3.010	.003	.073
TBO + Laser 635-Laser 808	38.750	10.381	3.733	.000	**.005**
TBO + Laser 635-Laser 635	49.450	10.381	4.764	.000	**.000**
TBO + Laser 635-Control	59.950	10.381	5.775	.000	**.000**
ICG + Laser 808- TBO	10.450	10.381	1.007	.314	1.000
ICG + Laser 808- ICG	21.350	10.381	2.057	.040	1.000
ICG + Laser 808- Laser 808	28.850	10.381	2.779	.005	.153
ICG + Laser808- Laser 635	39.550	10.381	3.810	.000	**.004**
ICG + Laser 808-Control	50.050	10.381	4.822	.000	**.000**
TBO- ICG	−10.900	10.381	−1.050	.294	1.000
TBO- Laser 808	−18.400	10.381	−1.773	.076	1.000
TBO- Laser 635	−29.100	10.381	−2.803	.005	.142
TBO- Control	39.600	10.381	3.815	.000	**.004**
ICG + Laser 808	−7.500	10.381	−.723	.470	1.000
ICG- Laser 635	−18.200	10.381	−1.753	.080	1.000
ICG- Control	28.700	10.381	2.765	.006	.159
Laser 808-Laser 635	10.700	10.381	1.031	.303	1.000
Laser 808- Control	21.200	10.381	2.042	.041	1.000
Laser 635- Control	10.500	10.381	1.012	.312	1.000

Each row tests the null hypothesis that the Sample1 and Sample 2 distributions are the same.
Asymptotic significances (2- sided tests) are displayed. The significance level is 0.05.

diode laser, ICG and 808 nm diode laser on *A*. actinomycetemcomitans biofilm formed on Laser-Lok titanium discs. The results confirmed the antibacterial effects of aPDT with TBO and ICG for reduction of *A. actinomycetemcomitans* on the surface of Laser-Lok titanium discs.

Laser-Lok implants enable better adhesion of fibroblasts to their surface for a superior tissue seal [27]. Also, minimum crestal bone loss around Laser-Lok implants preserves the soft tissue and improves esthetics [28]. In the current study, a significant reduction was noted in A. actinomycetemcomitans colony count in TBO, ICG + 808 nm laser and TBO + 635 nm laser groups compared with the control group. The greatest reduction in colony count was noted in CHX group while minimum reduction was noted in 635 nm laser group. Our findings confirmed the efficacy of aPDT with TBO and ICG and diode laser for reduction of *A. actinomycetemcomitans* colony count. *A. actinomycetemcomitans* is a major pathogen in development of peri-implantitis. Evidence shows that aPDT decreases the level of tumor necrosis factor-alpha and interleukin-1B [29]. Clinical studies have shown reduction in level of cytokines in gingival crevicular fluid following PDT [30,31]. In aPDT, light not absorbed by the bacteria results in scattering of chromatophores present in implant adjacent tissue and causes analgesic effects. It also resolves inflammation and enhances peri-implant soft and hard tissue healing [32]. Evidence shows that cationic, anionic and neutral photosensitizer molecules can play a significant role in killing of Gram-positive bacteria. Cationic photosensitizers can eliminate tens and thousands of Gram-negative bacteria [33]. Gram-negative bacteria have an internal cytoplasmic membrane along with an external membrane, which decrease the penetration of photosensitizer [33]. Phenothiazine dyes, such as TBO, are positively charged due to the presence of quaternary nitrogen atoms [34]. Due to the positive charge, TBO bonds to the external membrane of Gram-negative bacteria [35] and is therefore, a suitable photosensitizer for elimination of Gram-negative bacteria particularly the black-pigment bacteria [36]. Pourhajibagher et al. [24] showed that 25 μg/mL and 100 μg/mL TBO for 5 min caused a significant reduction in *Porphyromonas gingivalis* count.

ICG is an anionic photosensitizer, which is water soluble and relatively non-toxic [37]. Its photothermal effect is greater than its photochemical effect [38]. It can efficiently eliminate bacteria from deep periodontal pockets due to its photothermal effect [32]. Its absorbance peak is at 800 nm [39]. Thus, ICG plus 810 nm laser is suitable for PDT [40].

Mattielo et al. [41] evaluated the effect of aPDT with 0.01% TBO combined with 660 nm diode laser (30 mW, 3-minute time) on *A. actinomycetemcomitans*. aPDT caused a significant reduction in *A. actinomycetemcomitans* count but TBO alone was not effective for this purpose. The latter finding was in contrast to our results since TBO in our study significantly decreased the *A. actinomycetemcomitans* count; this difference may be attributed to the use of different concentrations of TBO. Nastri et al. [42] evaluated the effect of PDT with TBO photosensitizer on *A. actinomycetemcomitans* culture and biofilm. They showed that 10 μm concentration of TBO along with 830 nm diode laser (10.6 J/cm^2 energy density, 0.1 W power, for 60 s) caused reduction of *A. actinomycetemcomitans* culture by 99.98% and *A. actinomycetemcomitans* biofilm by 96.2%. They showed greater reduction in bacterial count compared with our study probably due to the use of bacterial suspension and creation of biofilm on a surface different from titanium discs. Bohem et al. [43] demonstrated that *A. actinomycetemcomitans* had the highest absorbance peak in presence of 10 μm concentration of ICG for 5 min. aPDT with 810 nm diode laser with 0.1 W and 0.5 W power and 80 and 400 W/cm^2 energy density for 5 s caused a reduction in *A. actinomycetemcomitans* in culture medium. Laser and ICG alone did not decrease the bacterial count. Reduction in bacterial count by the use of 0.1 W laser (90%) was lower than the value in our study, which may be due to the shorter duration of using diode laser (5 s) and its higher power (150 mW) in our study, which could have been associated with its photothermal effect [44]. Pourhajibagher et al. [25] demonstrated that ICG at 62.5 and 1000 μg/mL concentrations for 5 min caused 23.2% and 93.7% reduction in *P. gingivalis* count, respectively. Diode laser irradiation with 810 nm wavelength and 62.5 J/cm^2 energy density for 2 min caused a reduction in *P. gingivalis* count by 37%. Topaloglu et al. [20] showed positive efficacy of aPDT with ICG for reduction of *Staphylococcus aureus* (95%) and *Pseudomonas aeruginosa* (99%). Their findings were generally in line with ours; however, they did not mention the laser parameters and evaluated wild and resistant

microbial suspension of different bacterial species. Saffarpour et al. [45] reported that aPDT with ICG and 810 nm diode laser with 300 mW power and 2.38 W/cm^2 power density caused a significant reduction in *A. actinomycetemcomitans* count on implant surfaces. Also, CHX showed greater antibacterial efficacy compared to other groups. Their findings were in line with ours although they evaluated SLA implant surfaces. Prates et al, [46] in an in vitro study demonstrated up to 99% reduction in *A. actinomycetemcomitans* colony count when malachite green was used as photosensitizer in aPDT. Their study was conducted on bacterial suspension. Hass et al, [47] in an in vitro study on titanium discs showed that PDT with TBO and diode laser at 905 nm wavelength caused complete elimination of P. gingivalis and *A. actinomycetemcomitans*. Their study is the only one reporting complete elimination of bacteria following aPDT. None of the tested protocols in our study could completely eliminate *A. actinomycetemcomitans*; thus, aPDT can be used as an adjunct to periodontal therapy for elimination of *A. actinomycetemcomitans*. In vitro studies have reported variable percentage of reduction in periopathogenic microorganisms by PDT; however, the results of clinical studies have been controversial [48,49]. For instance, Esposito et al. [48] showed that similar efficacy of periodontal treatment protocols in presence and absence of aPDT. Deppe et al. [49] reported that aPDT in patients with severe peri-implantitis (5–8 mm pocket depth) did not prevent bone resorption compared with patients with moderate peri-implantitis (< 5 mm pocket depth). One explanation for controversial results regarding the efficacy of PDT can be the difference in severity of peri-implantitis and its definition. Repetition of aPDT (4–8 times) and treatment time also vary in clinical studies. Azarpazhooh et al. [50] demonstrated that aPDT had no effect on treatment of periodontitis. However, they called for clinical trials in this regard. In general, it seems that aPDT has advantages in peri-implantitis. Resistance to aPDT has not been reported due to its mechanism of action based on singlet oxygen and other reactive species. Also, the application of aPDT for detoxification of implant surfaces does not cause any damage [12,13,15,18,20].

Further studies with different photosensitizers and different laser parameters are required to determine the most efficient combination for aPDT. Moreover, the efficacy of aPDT for elimination of orange-complex bacteria such as *Prevotella* and *Fusobacterium* and red-complex bacteria such as *P. gingivalis*, *Tannerella forsythia* and *Treponema denticola* should be evaluated in future studies.

5. Conclusion

This study showed that aPDT is an efficient modality for decontamination of Laser-Lok implant surfaces and reduction of *A. actinomycetemcomitans* biofilm *in vitro*. In this study, aPDT with TBO + 635 nm diode laser and ICG + 808 nm diode laser decreased the bacterial load on Laser-Lok titanium discs.

Declaration of Competing Interest

None to declare.

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