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Research Paper

Comparative study of the effect of different exposure parameters of 635nm diode laser and toluidine blue O in eliminating *Aggregatibacter actinomycetemcomitans* biofilm from titanium implant surfaces

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ABSTRACT

Background: The aim of this study was to investigate the effects of antimicrobial photodynamic therapy (PDT) using 635 nm diode laser irradiation with an energy density of 6 to 30 J/cm^2 and toluidine blue O (TBO) as a photosensitizer on the viability of *Aggregatibacter actinomycetemcomitans* attached to the surface of titanium implants.

Materials and methods: Titanium implants contaminated with *A. actinomycetemcomitans* were treated with TBO alone or in combination with different exposure parameters (light doses of 6 - 30 J/cm^2 at 635 nm) and 0.2 % chlorhexidine (CHX). After treatment, colony forming units (CFUs)/ml were determined to assess PDT efficacy. The structure of the biofilm of *A. actinomycetemcomitans* was analyzed by field emission scanning electron microscopy (FESEM).

Results: Under optimal conditions, the colony count was reduced by ~90 %. Treatment with CHX was somewhat more effective (colony formation was reduced by ~95 %), but this agent has adverse effects that can be avoided with PDT.

Conclusion: This study confirms the efficacy of PDT against *A. actinomycetemcomitans* depending on the light dose. Treatment with TBO + 635 nm diode laser has an effect that may be equivalent to that of CHX, but perhaps with fewer adverse effects.

1. Introduction

While there is an increasing use of dental implants, there can be complications associated with infections. Currently, dentists are faced with the challenge of controlling peri-implant diseases, which often occur in the form of peri-implantitis and peri-implant mucositis with the presence of bacteria [1]. The prevalence of peri-implant mucositis and peri-implantitis was 46.83 % and 19.83 %, respectively [2].

Peri-implantitis can thus be defined as a poly-microbial or mixed anaerobic infection [3]. *Aggregatibacter actinomycetemcomitans* is one of the bacteria associated with peri-implantitis infections. It expresses several virulence factors such as lipopolysaccharide, fimbriae, and toxins that trigger a host response that may be related to the pathogenesis of the disease [4].

Treatment of peri-implantitis includes infection control, cleaning of the implant surface, regeneration of lost tissue, and control of microbial plaque [5]. Many surgical and conservative methods have been proposed for the treatment of peri-implantitis, which aim to remove bacterial contamination and prevent bone resorption [6]. However, complete removal of bacterial biofilm from rough implant surfaces is simply not possible. Furthermore, this tool can damage the implant surfaces and weaken the osteoblasts in the reconstruction of the surrounding bone [7].

Antimicrobial photodynamic therapy (PDT) is a non-invasive method that can be used alongside mechanical methods to treat peri-implantitis. In PDT, a harmless light of the appropriate wavelength is used to stimulate photosensitizers in the presence of molecular oxygen to generate an excited state and reactive singlet oxygen. Various

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parameters are involved in the treatment, such as irradiation dose, type of implant surface material, light source, concentration, and type of photosensitizer, etc. [8]. Developing a phototherapeutic strategy needs the right combination of photosensitizer, light source, appropriate light parameters, and drug to light interval [9–11]. Optimization of the antimicrobial effect of PDT might be achieved by standardizing of the PDT protocol [12].

The laser is a monochromatic and coherent light source. The monochromaticity of lasers permits for irradiation with the exact wavelength at which the photosensitizer reaches its maximum absorption capacity. Coherence makes it possible to guide the light through optical fibers into the contaminated areas. Diode lasers are favored in the application of PDT for the inactivation of bacteria due to their compactness, simplicity, low cost, and effectiveness [13]. In addition, it has a biostimulant activity which supports regeneration process [14]. Diode lasers with a wavelength of 635 nm have so far been used for PDT of bacteria with encouraging results [15,16].

Toluidine blue O (TBO) is a small molecule with a high water-solubility. Its cationic form, hydrophilic nature, and tendency to form dimers make it easier for bacteria to bind to it [17]. TBO is also tasteless and odorless and has low toxicity to human cells [11,17]. The concentration of TBO in aqueous solutions determines its maximum absorption. Therefore, at a suitable concentration, TBO can be excited with light sources emitting electromagnetic waves in the range of 600–660 nm [17]. The possibility of decontaminating implants with TBO-PDT has been confirmed in several studies [15,16,18,19].

Determining the optimal parameters for biofilm control ensures that the target areas are treated with an appropriate dose to kill pathogens. There is no doubt that dosimetry is an important issue for high efficacy of PDT. The lack of optimal parameters may hinder the control of biofilm and may be associated with failure to achieve clinically acceptable parameters [11]. Accordingly, this study aims to determine the most effective light energy dose to reduce the *A. actinomycetemcomitans* biofilm formed on the implant surface using TBO and a 635 nm diode laser.

2. Methods and materials

2.1. Bacterial strain and growth conditions

A. actinomycetemcomitans ATCC 700,685 was grown at 37 °C with 5 % CO_2 and under oxygen-limited conditions in brain heart infusion (BHI) broth (Ibresco, Iran) supplemented with 0.5 % yeast extract (Ibresco, Iran), 5 mg/ml hemin (Sigma-Aldrich, Dorset, UK), and 1 mg/ml vitamin K.

2.2. Biofilm formation on titanium implants

The titanium implants were placed in the 24-well microplates with 0.9 ml BHI broth, and inoculated with 0.1 ml of bacterial suspension (1.5×10^7 CFU/ml). The titanium implants were incubated at 37 °C for 72 h under oxygen-limited conditions for biofilm formation. After that, the titanium implants were washed with phosphate-buffered saline (PBS) for removing planktonic cells.

2.3. Description of the study groups

The test groups consisted of 48 titanium implants (4.1 × 10 mm, Dentium, Seoul, Korea) which were divided into 8 groups, with Group A (3 implants) considered as a negative control group, as the implants contaminated with *A. actinomycetemcomitans* were not subjected to any treatment. Treatment with TBO (Merck KGaA, Darmstadt, Germany) alone (Group B; 7 implants) was as follows: the implants contaminated with *A. actinomycetemcomitans* were subjected to TBO at a concentration of 0.1 mg/ml for 5 min at ambient temperature and under dark conditions. Groups C-G (7 implants) were used to compare the efficacy of the different energy densities of the 635 nm diode laser; therefore, the implants contaminated with *A. actinomycetemcomitans* were treated with TBO as described in group B. Subsequently, all PDT groups were irradiated with a diode laser (Wiser 3, Doctor Smile, Brendola, Italy) with a wavelength of 635 nm and an output power of 100, 200, 300, 400, and 500 mW at an irradiation time of 30 s. The energy densities (fluence) were, respectively: 6, 12, 18, 24, and 30 J/cm^2. Group H (3 implants) was considered as a positive control group and the implants contaminated with *A. actinomycetemcomitans* were immersed in 0.2 % chlorhexidine (CHX) for 1 min at room temperature.

2.4. Determination of CFU/ml

To determine the *A. actinomycetemcomitans* populations colonizing the titanium implant surface before and after treatment, contaminated implants were placed in 1.5 ml Eppendorf tubes containing 1 ml PBS and then vortexed for 60 s. Next, 10 µl of the pool was serially diluted (10^{-1} to 10^{-5}) and 10 µl of each was spread on BHI agar (Ibresco, Iran) plate. The plates were incubated at 37 °C with 5 % CO_2 and under oxygen-limited conditions. After incubation for 48 h, each plate was counted.

2.5. Field emission scanning electron microscopy (FESEM) observations of the biofilm on titanium implants

Each titanium implant with the *A. actinomycetemcomitans* biofilm before and after treatment was fixed with 2.5 % glutaraldehyde and 1 % osmium tetroxide for 1 h at 4 °C. Samples were washed with PBS and dehydrated with an ascending ethanol series (25, 50, 70, 100 %). A gold-palladium layer was then applied to the samples and observed with FESEM (TESCAN MIRA 3, Czech).

2.6. Statistical analysis

The mean and standard deviation of the microbial variables were calculated using SPSS version 26. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tamhane post hoc test. A statistically significant difference was determined if a p value was < 0.05.

3. Results

The mean bacterial load of the titanium implants before and after treatment is shown in Fig. 1. The bactericidal effect of TBO plus 635 nm diode laser with an energy density of 6 and 12 J/cm^2 on titanium implants contaminated with *A. actinomycetemcomitans* was almost the same ($p = 0.77$ and 0.58, respectively) and there was no significant difference between them ($p = 0.98$). However, their effect was significantly lower than that of a 635 nm diode laser with an energy density of 18, 24, 30 J/cm^2, and CHX ($p < 0.001$). The bactericidal effect of 635 nm diode laser with an energy density of 18 or 24 J/cm^2 was similar and no significant difference was found between them ($p = 1.00$). In the 635 nm diode laser group with an energy density of 30 J/cm^2, a maximum bactericidal rate was achieved, similar to CHX ($p < 0.001$). There was a significant effect between 635 nm diode laser with an energy density of 30 J/cm^2 and 635 nm diode laser with an energy density of 6 or 12 J/cm^2 ($p < 0.001$). However, this difference was not significant for a 635 nm diode laser with an energy density of 18 and 24 J/cm^2 ($p = 0.10$ and 0.28, respectively).

The implants treated with TBO and the most effective dose of energy density and the implants treated with CHX were subjected to FESEM for further investigation (Fig. 2). The titanium implant without any contamination is shown in Fig. 2a. As can be seen in Fig. 2b, large clusters of *A. actinomycetemcomitans* cells and thick accumulations of biofilm can be observed on the untreated titanium implant. In the PDT group in which the implant was treated with TBO and a higher energy density (30 J/cm^2), the biofilm appeared thinner and smaller than the untreated biofilm (Fig. 2c). Only a few scattered bacterial cells were

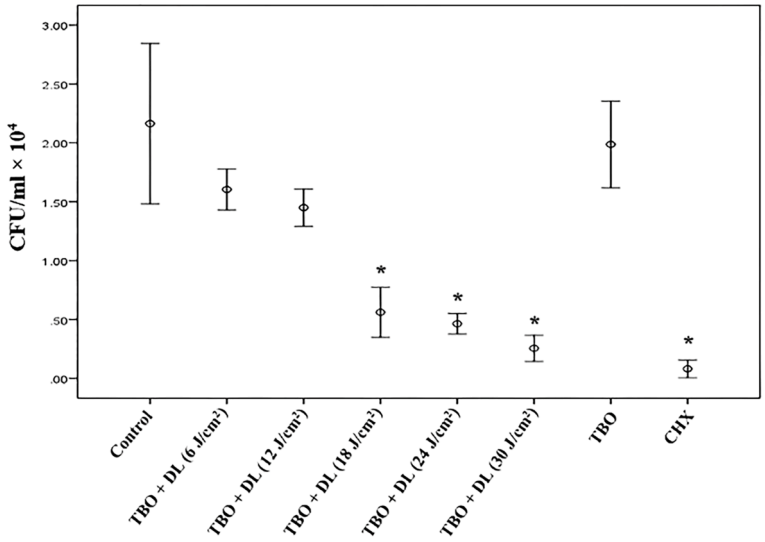


Fig. 1. Plot of mean bacterial load (CFU/ml) in each experimental group. Implant-adherent biofilms of *Aggregatibacter actinomycetemcomitans* before and after treatment were used for CFU/ml measurements. CFU/ml: colony forming unit per milliliter, nm: nanometers, DL: diode laser, J/cm^2: joules per square centimeters, TBO: toluidine blue O, CXH: 0.2 % chlorhexidine. *: significantly different from control.

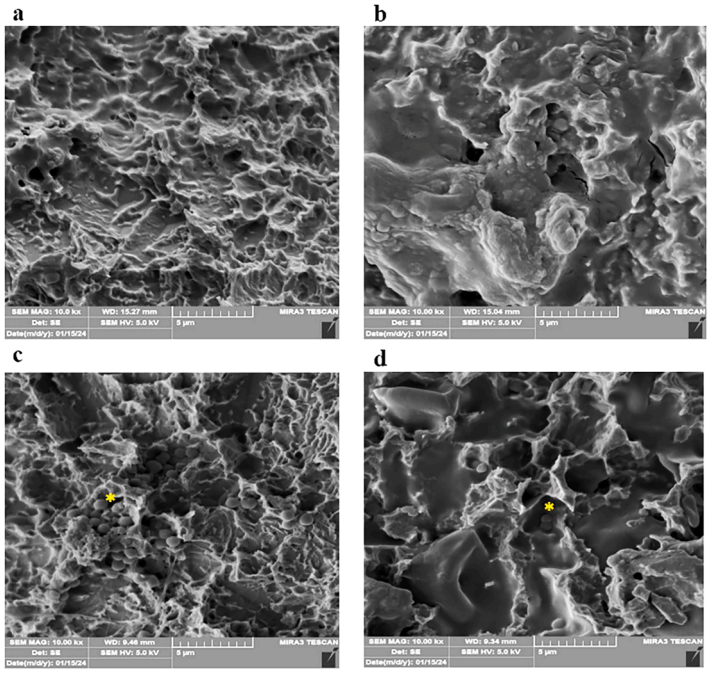


Fig. 2. Field emission scanning electron microscope images of *Aggregatibacter actinomycetemcomitans* biofilm developed over dental implants. (**a**) Titanium implant surface without any contamination (**b**) Biofilm without treatment is shown as a control, (**c**) Biofilm after PDT with TBO + 635 nm diode laser irradiation at 30 J/cm^2 (500 mW), (**d**) Biofilm after treatment with 0.2 % chlorhexidine. All figures are demonstrated at a magnification of 10,000 × . The length of the bar represents 5 μm. The asterisks indicate the presence of *A. actinomycetemcomitans* on the implant surface, the results of which differ significantly from the control.

observed in the titanium implant treated with CHX (Fig. 2d).

4. Discussion

The clinical success of PDT in the treatment of peri-implantitis has already been reported [20,21]. In PDT on the exposed implant surface, the photosensitizer is applied with a syringe from the bottom of the pocket around the implant and laser irradiation is performed with a flexible tip [20]. Measurement of total light dose delivered to the target is important when performing PDT. In conventional clinical settings, PDT is prescribed by the light power density (W/cm^2) and total light dose (J/cm^2) [22]. Despite numerous laboratory studies, the optimal settings for the parameters of light sources have not yet been clarified [17]. In this report, the effect of the different energy density when using 635 nm diode laser and TBO on *A. actinomycetemcomitans* was compared.

Overall, the energy density of 30 J/cm^2 was by far the most effective energy density, with much higher fluences of the 635 nm diode laser required to achieve a high level of killing. Such an approach provides the possibility to increase the effectiveness of the destruction of *A. actinomycetemcomitans* many times over. The determination of the most suitable fluence has been the subject of several studies [11,23–27].

The experiments with TBO showed a strong effect of PDT on cell killing of *A. actinomycetemcomitans*, and this was particularly evident when the light dose was increased. The cell viability of *A. actinomycetemcomitans* at an energy density of 6 J/cm^2 was 74.07 %, while at an energy density of 30 J/cm^2 a cell viability of 11.57 % was observed, and a high killing efficiency was found after CHX treatment (cell viability = 3.70 %). In fact, a significant killing of *A. actinomycetemcomitans* cells was only observed at an energy density of 18 J/cm^2 and above. In agreement with the data previously reported, increasing the light energy dose was more effective in killing the microorganisms [11,23–27]. Balhaddad et al. treated *Streptococcus mutans* biofilm with different doses of light energy (36, 108, and 180 J/cm^2) and different incubation times before irradiation. The results showed that increasing the energy dose, but not the incubation time, was associated with biofilm reduction. In addition, this study demonstrated a low cytotoxic effect of TBO up to 100 μg/ml on the macrophage cell line (viability > 70 %) [11]. Li et al. stated that fluence-dependent killing of *Staphylococcus epidermidis* when irradiated at the higher red-light doses of 20 or 30 J/cm^2. In addition, SEM images showed that the number and size of colony masses decreased with increasing light dose in the TBO-PDT group [27]. Souza et al. demonstrated that the highest reduction in log CFU/ml of *Candida albicans* observed with increasing energy density of the laser from 15.8 to 39.5 J/cm^2 using methylene blue, malachite green, and TBO as photosensitizers [25]. The explanation for these results could be that the generation of reactive oxygen species by a high light dose can diffuse into the cell and cause cell death [28,29].

Although CHX has been used extensively to treat peri-implantitis, no study has been able to show that it is superior to other decontamination agents in terms of clinical treatment outcomes [30]. Its use has a number of drawbacks, including discoloration, taste disruption, and a slight increase in calculus formation [31,32]. Kotsakis et al. concluded that the use of CHX is not recommended for the decontamination of titanium surfaces [33]. In PDT, defects such as risk post-surgery, surface topography alterations, and the presence of remnants on the implant surfaces are generally not present [34]. Several methods for the decontamination of implants include mechanical forces (such as conventional ultrasonic scaler and air-powder abrasive system), chemical methods (such as the use of hydrogen peroxide, citric acid, and CHX), antibiotics (systemic and local), disinfectants and lasers [6]. The current literature does not indicate which protocol is more effective for the decontamination of implants and the removal of biofilms [35]. PDT does indeed reduce the microbial load associated with peri-implant disease and may be considered for the decontamination of implants after implantation [21, 36]. However, due to the microbiological characteristics of peri-implantitis, it is necessary to evaluate the disinfection protocols used in the treatment of this disease [35].

One limitation is that although the strong effect of PDT has been confirmed *in vitro*, some environmental factors present *in vivo*, such as inflammatory mediators or limited O_2 availability, may reduce this effect *in vivo*. Multispecies biofilms may behave differently. In addition, light transmission to the site of infection may be affected by local factors. The results are not surprising but are important for the design of experiments on PDT in implant infections. It should not be forgotten that TBO has been used in PDT for a long time. Given its significant activity in mediating PDI, it may be possible to increase its efficacy by modifying the irradiation parameters. The data do not reflect all aspects that can be directly transferred to clinical trials, but nevertheless predict the parameters for successful treatment in trials.

5. Conclusions

PDT based on TBO is almost as effective as CHX *in vitro* at a light dose of 30 J/cm^2. It remains to be seen whether *in vivo* studies will confirm the efficacy, which could depend on the way the light enters the implants after implantation.

CRediT authorship contribution statement

Mahnaz Arshad: Project administration, Funding acquisition, Conceptualization. **Faraz Joshan:** Methodology, Conceptualization. **Nasim Chiniforush:** Resources, Methodology, Conceptualization. **Shima Afrasiabi:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Data curation.

Declaration of competing interest

The authors declare no conflict of interest.

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