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Effect of Photodynamic Therapy Using Toluidine Blue on Eikenella corrodens and Aggregatibacter Actinomycetemcomitans Biofilms Adhered to Titanium Discs: An In Vitro Study

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ABSTRACT

Background and aim: Antimicrobial photodynamic therapy (aPDT) has been suggested as a novel technique for decontamination of exposed implant surfaces. We aimed to evaluate the effect of aPDT on sandblasted, large-grit, acid-etched (SLA) titanium discs contaminated with *Eikenella* corrodens (Ec) and *Aggregatibacter* actinomycetemcomitans (Aa).

Materials and methods: In this in-vitro study, twenty-four sterile SLA titanium discs were contaminated with Ec (PTCC® 1391) and Aa (ATCC® 33384) and were randomly divided into the following groups: aPDT-treated group consisted of 12 discs submerged in 1 ml of toluidine blue and exposed to a low-level laser; negative control group comprised of 6 discs rinsed with physiological saline, and positive control group included 6 discs submerged in 2 ml of 0.2% chlorhexidine (CHX). After serial dilution, each sample was cultivated in an anaerobic environment (24 hours for Ec and 48 hours for Aa). Microbial reduction rate was calculated through colony-forming unit (CFU) counting according to Kruskal-Wallis test.

Results: The number of colonies for both bacterial strains in the aPDT-treated group was significantly reduced compared to the negative control group, showing the bactericidal potential of aPDT with toluidine blue ($P<0.0001$). The 0.2% CHX group showed a significantly smaller amount of colonies ($CFU=1.089\times10^7$ for Ec and 3×10^7 for Aa) compared to the aPDT-treated group ($CFU=3.73\pm1.19\times10^9$ for Ec and $52\pm13.6\times10^5$ for Aa; $P<0.00001$).

Conclusion: aPDT with toluidine blue significantly reduces Ec and Aa contamination of SLA titanium discs; however, 0.2% CHX showed the highest bactericidal potential and is still considered the gold standard in antimicrobial treatment of peri-implant diseases.

Original Article

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Introduction:

The prevalence of peri-implant diseases has been reported to be as high as 80% for peri-implant mucositis[1-4] and 28%-56% for peri-implantitis.[5,6] Given the severity of bone resorption in peri-implantitis, nonsurgical treatments such as scaling and root planing, topical and systemic antibiotics, and lasers such as carbon dioxide (CO2) and erbium-doped yttrium aluminium garnet (Er:YAG) are suggested.[7-9] Lack of proper and timely treatments leads to complications such as pain, implant loosening, and bone resorption, which will eventually lead to implant loss. [10,11]

Currently, a novel noninvasive photochemical method for eliminating periodontopathogens, called antimicrobial photodynamic therapy (aPDT), is of great interest.[12-14] aPDT has been primarily used in cancer treatment by Von Tappeiner and Jesionek in 1903.[15] This method combines the application of non-toxic chemical photosensitizer agents with low-level light energy.[16] aPDT is preferred over antibiotic therapy for three reasons including elimination of bacterial resistance, delivering precise concentrations to the area, and being harmless to the surrounding tissues due to the narrowed effect.[17] Thus, aPDT is considered one of the most promising techniques in the treatment of peri-implant tissue infection.[17]

It has been suggested that aPDT can eliminate the bacteria on the implant surface through photochemical and photothermal properties.[18-20] aPDT consists of three components including a light source, a photosensitive agent, and free radicals.[21] Once the photosensitive agent is stimulated by its optimal wavelength, it turns from the low-energy state to a highly energized one. The high triple state half-life causes an interaction between the photosensitive agent and oxygen in tissues and produces free radicals, which lead to bacterial destruction.[9] Several in-vivo and in-vitro studies have revealed that aPDT is useful in the reduction of the microbial number and relieving clinical symptoms; however, studies on periodontal pathogens are limited.[22]

This study aimed to investigate the effect of aPDT on sandblasted, large-grit, acid-etched (SLA) titanium discs contaminated with Eikenella corrodens (Ec) and Aggregatibacter actinomycetemcomitans (Aa) biofilms.

Materials and Methods:

Bacterial strains and cultivation methods:
In this in-vitro experimental study, strains of Ec (PTCC® 1391) and Aa (ATCC® 33384) were grown in blood agar supplemented with vitamin K and 10% horse serum in an incubator (Pasteur Microbiology Laboratory, Tehran, Iran) at 37°C in a microaerophilic environment (24 hours for Ec and 48 hours for Aa). Afterward, the bacteria were forced out of the lyophilized form, and the viability of the strains was ensured. Then, the bacteria were diluted to 10^8-10^9 colony-forming units per milliliter (CFU/ml) with 0.5 McFarland standard of OD600: 0.08-0.1. After the preparation of the microbial suspensions, all discs, including the case and control groups, were exposed to the suspension (Figure 1.A-F). There were a total of three comparison groups. Each control group consisted of six discs; the test group comprised of 12 discs.

PDT procedure:
Twenty-four sterile SLA titanium discs (2.5 mm×10 mm; Dentium Co., Seoul, South Korea) were used in this study. Twelve sterile titanium discs served as an aPDT-treated group of which six discs were contaminated with Ec, and six discs were infected with Aa (Figure 1.A). Then, the discs were submerged in sterile toluidine blue (Sigma-Aldrich, Steinheim, Germany) at a standard concentration (0.01 mg/ml) for 3 minutes [23] and were irradiated with a low-level laser (Foto-San, CMS Dental APS, Copenhagen, Denmark) at a wavelength of 630±10 nm and power density of 440 mW/cm^2.

The diameter of the tip was 8 mm with an energy density of 110 J/cm^2.[24] The device was placed at a fixed distance of 1 mm, perpendicular to the surfaces of the discs for 2 minutes. The output power of the device was 220 mW, which was measured by a power meter (LaserPoint s.r.l., Mi-

measured by a power meter (LaserPoint s.r.l., Milan, Italy).[22,24] Irradiation was performed aseptically under a laminar flow hood (Besat, Tehran, Iran) in the dark (Figure 1.B and C).[14,18,23,24] Six discs were assigned to the positive control group (three contaminated with Aa and three infected with Ec) that were disinfected with 2 ml of 0.2% chlorhexidine (CHX; Nazho, Iran Daru Pharmaceutical Co., Tehran, Iran) for one minute without applying aPDT. Six discs of which three were contaminated with Aa and three with Ec were used as the negative control group and received no treatment.[9,22]

All discs were gently rinsed with 3 ml of physiological saline following treatment to ensure no additional material (including CHX and toluidine blue) is transferred to the culture medium. In order to remove the superficial biofilms from the discs, the microtubes were sonicated (SinapTec, Stuttgart, Germany) for 30 seconds at a 50-Hz frequency with the power of 150 W (Figure 1.D). [24] Moreover, using physiological saline (0.9% sodium chloride (NaCl) solution; Samen Pharmaceutical Co., Mashhad, Iran), serial dilutions (10^{-1}-10^{-6}) were organized in separate test tubes, and 50 µl of each suspension was transferred to the culture medium (Figure 1.E and F).

Then, the plates were cultured in anaerobic condition (24 hours for Ec and 48 hours for Aa). 150 µg of the bacterial suspension was added to each plate, and the number of Aa colonies was multiplied by 20 to calculate the numbers in each ml.[22] However, due to the fineness of Ec colonies,

The plate surfaces were subdivided into 16 equal sections using a protractor, and at least three sections were randomly counted for the number of colonies; the mean value was multiplied by 16 to calculate the number of colonies at the plate surface (Figure 2). The number of colonies at the plate surface was multiplied by 20 to calculate the numbers in each ml. The microbial reduction rate was calculated through CFU counting. Finally, using the below formula, the reduction rate of the microorganisms was calculated: $R=(A-B)/A\times100$

where A is the number of original colonies (negative control), B is the number of secondary colonies (aPDT-treated), and R is the percentage of colony reduction rate.[14,22,23]

Statistical analysis:

After collecting the data, the mean and standard deviation (SD) were calculated. Statistical tests were performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Because the data did not follow a normal distribution, Kruskal-Wallis test was used for comparison of the variables between the groups. Pairwise comparisons were performed by Mann-Whitney-U test in association with Bonferroni correction. $P<0.05$ was considered statistically significant in multiple comparisons. The level of significance in pairwise comparisons was set at $0.05/3=0.0166$.

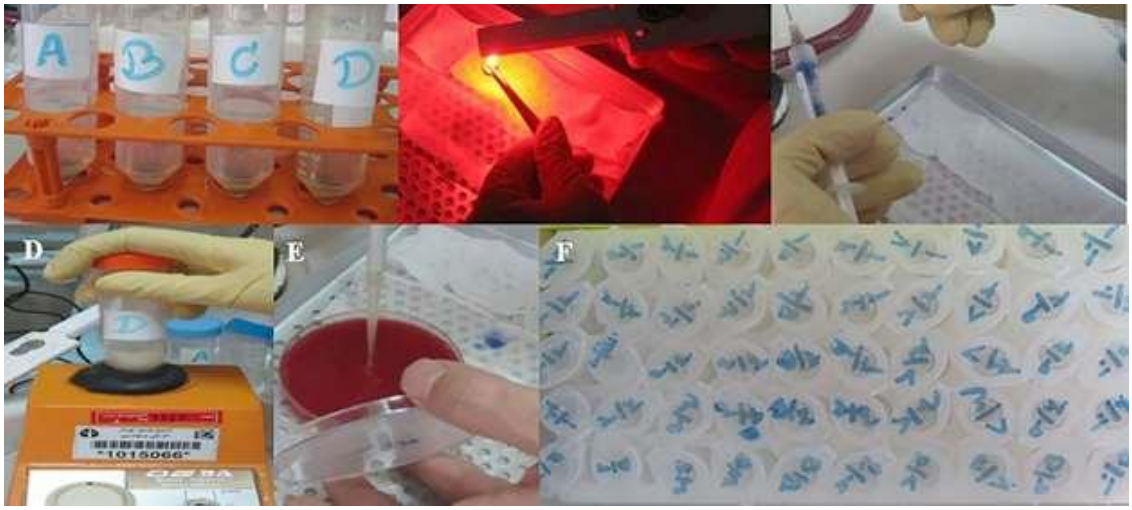

Figure 1. (A) Contamination of the discs with microbial suspensions. (B) Low-level diode laser (FotoSan, CMS Dental APS, Copenhagen, Denmark) at the peak wavelength of 630 nm and maximum output power density of 2000 mW/cm2. (C) The application of toluidine blue. (D) Sonication of the specimens. (E) The culture medium. (F) Serial dilution.

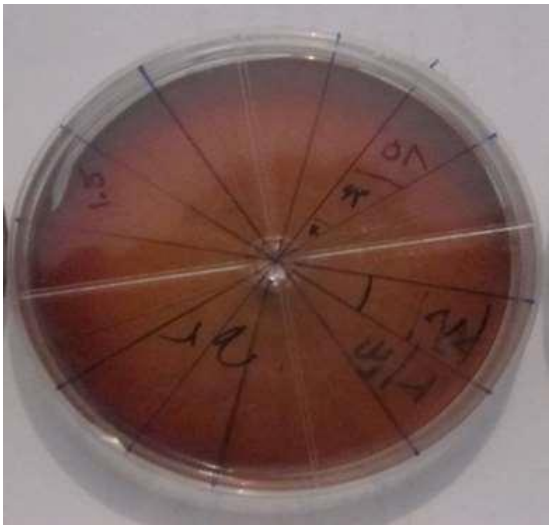

Figure 2. Calculation of the colony-forming units per milliliter (CFU/ml).

Results:

Table 1 shows the number of microbial colonies per ml (CFU/ml) for Ec, and Table 2 shows this number for Aa. The positive control group, which received 0.2% CHX solution, showed the lowest CFU/ml for both bacterial strains (CFU=$1.08^9\times10^7$ for Ec and 3×10^7 for Aa; P<0.0001); the highest CFU/ml was detected in the negative control group (no treatment applied).

Table 1: The number of Eikenella corrodens (Ec) colony-forming units per milliliter (CFU/ml) in the studied groups.

Groups	CFU/ml
Negative control group (physiological saline)	$3.98\pm1.73\times10^9$
aPDT-treated group	$3.73\pm1.19\times10^9$
Positive control group (0.2% CHX)	$1.089\pm0.19\times10^7$
Significance	P<0.0001*

*aPDT=antibacterial Photodynamic Therapy, CHX=Chlorhexidine, *denotes an extremely statistically significant result.*

Also, in the aPDT-treated group, the percentage of Ec colonies per ml was 6.09% lower and the percentage of Aa colonies was 17% lower than the corresponding values in the negative control group (CFU=$3.73\pm1.19\times10^9$ for Ec and $52\pm13.6\times10^5$ for Aa). Kruskal-Wallis test showed that the difference between the negative control group and the aPDT-treated group was significant (P<0.0001).

Table 2: The number of Aggregatibacter actinomycetemcomitans (Aa) colony-forming units per milliliter (CFU/ml) in the studied groups.

Groups	CFU/ml
Negative control group (physiological saline)	$62\pm19.8\times10^5$
aPDT-treated group	$52\pm13.6\times10^5$
Positive control group (0.2% CHX)	$3\pm0.73\times10^7$
Significance	P<0.0001*

*aPDT=antibacterial Photodynamic Therapy, CHX=Chlorhexidine, *denotes an extremely statistically significant result.*

Discussion:

This study showed that aPDT has a positive effect on the reduction of Ec and Aa colonies. This reduction was about 6.9% for Ec and 17% for Aa colonies, which was significant compared to the negative control group.

Due to the structure of implant surfaces, the need to use a complementary non-mechanical treatment to reduce the number of bacterial strains without manipulation of the implant surface is indispensable.[25] Several in-vivo and in-vitro studies have revealed that aPDT is useful in the reduction of the microbial number and alleviating clinical symptoms. It is apparent that the resolution of the clinical signs is a result of eliminating the responsible pathogens.[26]

Mattielo et al showed that using 0.01% toluidine blue in combination with aluminium gallium indium phosphide (AlGaInP) laser could competently eliminate both Aa and Ec but there was no significant reduction in the dye-only group.[22]

The findings of the present study are consistent with the mentioned study but the reduction percentages in the above research were higher. The reasons for the variation between the results might be the wavelength of the applied laser, the duration of irradiation, the type of laser, and the test condition (disc surfaces were not used for

microbial adhesion).

Miyabe et al studied the effect of aPDT on 20 types of Staphylococcus spp.[27] The higher microbial load reduction compared to the present study can be attributed to the employment of microbial suspensions rather than contaminated titanium discs, because the discs have porosities in the range of 2-4 µm. Hence, removal of the bacteria colonized on the discs is more challenging compared to the bacteria in a suspension. In a study by Marotti et al,[23] titanium discs were suspended for 5 minutes in saliva samples collected from patients with peri-implantitis. Then, a low-level laser (660 nm, 30 mW) irradiated the disc surfaces for 3 and 5 minutes (7.2 and 12 J/cm^2). Although using laser alone reduces the number of microbes, nonetheless, a significant difference was reported between the laser-treated group and the aPDT-treated group, with PDT being more efficient. There was no statistically significant difference between the groups with various irradiation times.[23] The method of contaminating the discs is one of the weaknesses of the mentioned study as a 5-minute period is not sufficient for ensuring the adhesion of microbes to the disc surfaces. The low microbial load can justify the statistical difference with the results of the present study.

Another study was conducted by Biondi Filho et al in which the discs were contaminated with Streptococcus sanguinis suspension.[28] The results showed that CHX was very beneficial, and interestingly, microbial reduction in the methylene blue group alone was more than that in the aPDT-treated group.[28] In the cited study, the samples were contaminated with 10 µl of microbial suspension for one hour. Also, the discs were much smaller than the discs used in the present study, and accordingly, decontamination with laser showed better results due to the higher concentration of irradiation in a smaller surface area. Hauser-Gerspach et al carried out a study to examine the in-vitro antibacterial efficacy of two different laser systems (CO2 and diode) applied to two types of bacteria adhered to titanium discs.[29] In this study, Hauser-Gerspach et al considered the microbial suspension as the gingival crevicular fluid (GCF) and compared the bacteria to those adhered to implant surfaces. The results of this study revealed that suspensions of both bacteria were more resistant to the laser compared to the microbes adhered to the disc surfaces. Both CO2 and diode lasers reduced the number of pathogens. However, the CO2 laser with a higher energy level led to surface alterations of the titanium discs. They concluded that the low-level diode laser with a similar bactericidal characteristic is superior due to the lack of titanium destruction.[29] Hass et al conducted a similar study on different implant surfaces using photosensitization and a soft laser.[30] According to this study, aPDT can be used as an easy and inexpensive method in the treatment of peri-implantitis to selectively remove the pathogens. The use of toluidine blue was another similarity of this study with the present study. However, this was the only study that reported the complete removal of pathogens followed by treatment with aPDT. Assessment of different implant surfaces was one of the other merits of the cited study.[30]

In studies by Marotti et al and Biondi Filho et al, the duration of contamination of the samples was reported to be 5 minutes with saliva and one hour with microbial suspensions, respectively.[23,28] In the research by Hauser-Gerspach et al, this duration was considered 2 hours.[29] This extended period can have a significant effect on the number and the volume of the microbes. A smaller number of bacteria on the samples and the discs can undoubtedly lead to the observation of better results from treatment with aPDT.[29]

Another critical factor is the dimensions of the disc surfaces. It is apparent that the smaller the area subjected to the laser, the better the decontamination performance. For example, in the study by Hauser-Gerspach et al,[29] the dimensions of the discs used were 0.5 mm×1 mm, which was five times smaller compared to the discs used in the present study (2.5 mm×10 mm). As a result, the rate of irradiation with a particular dose and duration per unit area was higher. Thus, the microbial reduction efficiency at the end of the treatment was higher. Ec is a gram-negative bacterium with a high growth rate, which has cluster-shaped colonies that grow on top of each other; this can be another reason for the small amount of reduction in the colonies of this microbe after treatment with aPDT. It is also possible that gram-negative bacteria need more exposure time and require a new approach for higher penetra-

tion of photosensitizers into their three-walled membrane; this wall consists of a layer of lipoprotein, a layer of phospholipids, and a layer of lipopolysaccharide.[31]

Hamblin and Hasan discussed that the physiology of gram-negative bacteria justifies their susceptibility to lasers as the cell wall contains an internal cytoplasmic membrane and an outer membrane that create a physical and functional barrier between the bacteria and the outer environment and maintain the cell configuration.[32] Previously, Rovaldi et al had found that the outer wall of gram-negative bacteria is more resistant to aPDT compared to gram-positive bacteria and that this defensive barrier interferes with the absorption of photosensitive substances.[33] However, due to the positive charge of Toluidine Blue O (TBO), it merely adheres to the outer wall of gram-negative bacteria and interacts with its lipopolysaccharide;[34] thus, TBO is a suitable photosensitive agent for destroying gram-negative bacteria.[35]

As mentioned earlier, in our study, the maximum microbial reduction was observed in the 0.2% CHX-treated group. Similarly, Saffarpour et al demonstrated that the 0.2% CHX group had the lowest colony count.[24]

PDT applications have simple concepts but several factors complicate them. Further studies are required for determination of proper and exact parameters for each disease; this includes factors related to photosensitizers such as the transmission method and the duration of application and parameters related to the wavelength, irradiation exposure time, and intensity.

Conclusion:

Within the limitations of this study, it was found that aPDT reduced the CFU of Ec and Aa to 6.09% and 17%, respectively. However, 0.2% CHX showed the highest bactericidal potential and is still considered the gold standard in antimicrobial treatment of peri-implant diseases.

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Compliance whit ethical standards:

The authors declare that this study is self-funded, and informed consent and ethical approval are not applicable to this work.

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