Bactericidal Effect of Erbium-Doped Yttrium Aluminum Garnet Laser and Photodynamic Therapy on *Aggregatibacter Actinomycetemcomitans* Biofilm on Implant Surface

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Purpose: Peri-implantitis is a common complication of dental implants. The first step of treatment is elimination of bacterial biofilm and disinfection of the implant surface. This study sought to compare the effects of an erbium-doped yttrium aluminum garnet (Er:YAG) laser, photodynamic therapy using an indocyanin green-based photosensitizer (ICG-based PS) and diode laser, toluidine blue O (TBO) photosensitizer and light-emitting diode (LED) light source, and 2% chlorhexidine (CHX) on biofilm of Aggregatibacter actinomycetemcomitans *to sandblasted, large-grit, acid-etched (SLA) implant surfaces.* **Materials and Methods:** *Fifty SLA implants were divided into five groups and were incubated with* A actinomycetemcomitans *bacteria to form bacterial biofilm. Group 1 underwent Er:YAG laser radiation (with 10-Hz frequency, 100-mJ energy, and 1-W power); group 2 was subjected to LED (with 630-nm wavelength and maximum output intensity of 2.000 to 4.000 mW/cm^2) and TBO as a photosensitizer; group 3 was exposed to diode laser radiation (with 810-nm wavelength and 300-mW power) and ICG-based PS; and group 4 was immersed in 2% CHX. Group 5 was the control group, and the samples were rinsed with normal saline. The number of colony-forming units (CFU) per implant was then calculated. Data were analyzed using one-way analysis of variance (ANOVA), and the five groups were compared.* **Results:** *Significant differences was found between the control group and the other groups (P < .01). The lowest mean of CFU per implant count was in group 4 (P < .01), and the highest mean belonged to the control group. Photodynamic therapy by TBO + LED and ICG-based PS + diode laser was more effective than Er:YAG laser irradiation in suppression of this organism (P < .01). There was no significant difference between groups 2 and 3.* **Conclusion:** *The antibacterial effect of 2% CHX was greater than that of other understudy methods.* INT J ORAL MAXILLOFAC IMPLANTS 2016;31:e71–e78. doi: 10.11607/jomi.4224

Keywords: *diode laser, Er:YAG laser, implant, implant surface decontamination, laser, photodynamic therapy*

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With the increasing popularity of dental implants, the incidence of complications has increased as well. Peri-implantitis is a common complication encountered in clinical practice. Important steps in the treatment of peri-implantitis are the elimination of bacterial biofilm with minimal surface damage, detoxification of bacterial products, control of inflammatory reactions, and achieving reosseointegration. Elimination of biofilm from the implant surface is done by various chemomechanical techniques (dental curettes, ultrasonic scalers, air-powder abrasive, citric acid) as well as systemic antibiotic therapy.[1] The conventional techniques have some drawbacks, such as alteration of the implant surface, development of bacterial resistance, and limitation in complete microbial plaque removal.[2] Laser irradiation and antimicrobial photodynamic therapy (aPDT) have recently been suggested for treatment of peri-implantitis.[3,4]

The advantages of laser for conventional dental treatments are still a matter of debate. However, laser-based treatments have been proposed as effective methods for implant surface disinfection.[4] If low-power and high-power

lasers are used with correct parameters, lasers with hemostatic and bactericidal effects[4] are able to remove calculus,[4] bacterial lipopolysaccharides (LPS)[4] and smear layer,[5] increase proliferation of periodontal ligament fibroblasts,[6] and improve attachment of osteoblasts.[7] They also improve the bone-implant interface strength.[8]

The erbium-doped yttrium aluminum garnet (Er:YAG) laser has suitable properties for the treatment of oral and dental diseases, because of its capability for removing both soft and hard tissues as well as bacterial biofilm and calculus without causing thermal damage to the adjacent tissues.[9] In addition, the Er:YAG laser is able to remove the granulation tissue around the implant and tooth, debride the root surfaces, and enhance the formation of new bone following periodontal surgery.[10,11] Studies have shown that following Er:YAG laser irradiation, a smear layer does not form on the root surface.[5] Moreover, irradiation with a Er:YAG laser promotes the attachment of osteoblast-like cells on previously diseased implant surfaces.[7,12] Drawbacks include its high cost and large size of the equipment.[13–15] Studies have shown that the Er:YAG laser is suitable for disinfection of implant surfaces.[16]

An alternative technique for disinfection of dental implants is aPDT. It is based on a photochemical reaction comprising three components, including a photosensitizer (PS), oxygen, and light.[17,18] The mechanism of this treatment is based on attachment of PS to the target cells. When light at a specific wavelength is irradiated in the presence of oxygen, the oxygen molecules convert from a low-energy basic state to an excited singlet state, leading to production of singlet and other reactive oxygen species that are toxic for the target cells.[18] These reactions occur in a small environment, making it ideal for achieving selective destruction at the target site.[19] This technique is reproducible and noninvasive, with high target specificity and minimal risk of bacterial resistance following its application.[18–20] Since it is an oxygen-dependent reaction, it has limited application in infected areas with low or no oxygenation. In these situations, aPDT can eliminate microorganisms by causing local hyperthermia.[17] The indocyanine green–based PS (ICG-based PS) used in this study is a new PS that was recently introduced to the dental market. The manufacturer claims that it is mainly composed of ICG, which is a member of the tricarbocyanine family. It has low toxicity, rapid elimination, and also high absorption at the near-infrared region (805 nm), the wavelength that most of the available dental diode laser equipment emit.[21] This dye bonds to biologically active molecules, including antibodies, via its carboxylate group[21] and can target bacterial or cellular components specifically.[21] The mechanism of action of ICG is in two forms: the absorbed laser energy is either transferred by the ICG to active oxygen molecules (phototoxicity) or is transformed into heat, causing thermal damage, membrane degradation, or protein denaturation and consequent cell death.[21] Therefore, use of ICG-based

PS is suggested in areas with low oxygen pressure such as deep periodontal pockets.[17]

Toluidine chloride or toluidine blue O (TBO) is a cationic dye used in dentistry as a PS.[18] Due to its cationic charge, TBO is capable of attaching to the outer membrane of gram-negative bacteria; this explains the high affinity of this dye to the bacteria and killing them (compared to host cells).[18] Studies show that when this PS is activated by the appropriate light, it can effectively eliminate periopatogen bacteria such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis,* and *Fusobacterium nucleatum*.[18,22] The maximum absorption wavelength of this dye is 632 ± 8. Low toxicity and low cost are some of the advantages of this dye.[23]

Also, some studies suggest 2% chlorhexidine (CHX) for treatment of peri-implantitis due to its bactericidal effects.[24]

Several studies have separately investigated the antimicrobial effects of aPDT and laser,[25,26] but only a few studies have compared the efficacy of both methods in removal of peri-implantitis pathogens from the implant surface. Considering the existing controversy in the results of previous studies, this study was designed to evaluate and compare the antimicrobial effects of aPDT, laser, and 2% CHX on the elimination of *A actinomycetemcomitans* biofilm from the implant surface.

MATERIALS AND METHODS

This study was done on 50 implants measuring 4.3 mm in diameter and 10 mm in length with a sandblasted, large-grit, acid-etched (SLA) surface (Dentium, SuperLine).

Bacterial Strains, Culture, and Suspension Condition

Standard *A actinomycetemcomitans* bacterial strains (ATCC 33384 obtained from Rayen Biotechnology) were rehydrated in brain heart infusion (BHI) broth (Merck) and incubated in microaerophilic conditions[27] at 37°C for 48 hours to proliferate. A bacterial suspension with a 6.2×10^7 CFU/mL concentration was prepared by spectrophotometer. For this purpose, an outer diameter of 0.4 for *A actinomycetemcomitans* at a 600-nm wavelength indicates 6.2×10^7 CFU/mL. To ensure the exact number of bacteria, serial dilution was done, and the number of CFU/implant was determined using brain heart infusion (Mueller-Hinton) agar plates.

To prepare bacterial biofilm according to the method described by Zhou et al,[28] implants were immersed in a bacterial suspension for 48 hours.[29] Before contamination, the implants were incubated with sterile human saliva at 37°C for 15 minutes and then transferred to the microbial suspension. Sterile human saliva was prepared as described by Hauser-Gerspach et al.[30] Placement of

Table 1   Distribution of Experimental Groups and Treatment Modality

	Groups				
	Er:YAG	**TB630**	**EM810**	**CHX**	**Control**
Treatment modality	Er:YAG laser irradiation	Photodynamic therapy	Photodynamic therapy	Immersed in chlorhexidine 2%	Rinsed with saline solution
Light source	Er:YAG	LED	Diode laser	–	–
Wavelength	2,940 nm	630 nm	810 nm	–	–
Photosensitizer	–	TBO 0.1 mg/mL	ICG-based PS	–	–
Power density	0.3 W/cm^2	2–4 mW/cm^2	2.38 W/cm^2	–	–
No.	10	10	10	10	10

implants in sterile saliva causes attachment of salivary proteins to the implant surface and formation of pellicle, enhancing the bacterial adhesion to the implant surface, whereby the oral environment is simulated.[30] Just before the decontamination process, implants were washed with 3 mL of saline solution for 30 seconds to wash out the planktonic bacteria with weak adhesion.

Groups

A total of 50 implants were divided into five groups (10 implants in each group) using convenience sampling. Table 1 summarizes the grouping and the decontamination methods for each group.

Light Sources and Photosensitizers

Er:YAG Laser. Er:YAG laser irradiation (Smart 2940D plus, DEKA Laser) with 10-Hz frequency, 100-mJ energy per pulse, and 1-W power, in very short pulse length (230 μs) was used; the maximum power density was 0.3 W/cm^2.

Toluidine Blue O and Light-Emitting Diode

Toluidine blue O (0.1 mg/mL; FtoSan agent medium viscosity, FotoSan, CMS Dental) was used as the PS. Light-emitting diode (LED) was used to activate this medium in a red spectrum (wavelength, 625 to 635 nm; peak, 630 nm; FotoSan 630LED, CMS Dental) with maximum output intensity of 2.000 to 4.000 mW/cm^2, using a disposable blunt tip (4-mm diameter).

ICG-Based PS and Diode Laser

Implants were irradiated by diode laser (810 nm; A.R.C. laser) with a special handpiece with parameters recommended by the manufacturer, power of 300 mW, power density of 2.38 W/cm^2, continuous mood, and ICG-based PS (EmunDo) solution as a photosensitizer (A.R.C. laser).

Decontamination Process and Analysis

The process of decontamination was performed by a skilled calibrated operator. For calibration of the operator, the laser and aPDT procedures (Er:YAG, EM810, TB630

groups) were done on six implants in a pilot study (two implants in each group). In group 1, the Er:YAG laser was irradiated to the implant surface for 60 seconds with water and air spray. Irradiation was done with the fiber-optic tip in a circular motion from the coronal toward the apical direction, so that all implant threads were irradiated. The spot size was 1 mm when the handpiece with contact tip was placed 1 mm above the surface. To match the process, all implants were rinsed again with 3 mL of sterile saline solution for 30 seconds. Figure 1 summarizes the outline of the experiments.

In the TB630 group, the TBO was applied on implants, using a specific syringe for 1 minute. Then, the implants were rinsed with 3 mL of saline solution for 30 seconds according to the manufacturer's instructions. The implant surface was then subjected to LED radiation for 60 seconds according to the manufacturer's instructions.

In the Em810 group, the implants were first soaked in ICG-based PS for 5 minutes, and then the implant surface was irradiated with a 810-nm diode laser from coronal to apical in a circular motion for 60 seconds. Implants were then rinsed with 3 mL of saline solution for 30 seconds (according to the manufacturer's instructions).

In group 4, implants were immersed in sterile plastic Eppendorf tubes containing 3 mL of 2% CHX for 30 seconds. Implants were then rinsed with 3 mL of sterile saline solution for 30 seconds to eliminate the residual CHX. The implants in the control group were only rinsed with 3 mL of saline solution for 30 seconds.

After the procedures, all implants were immersed in 1 mL of BHI broth and ultrasonicated (Branson Ultrasonic) for 5 minutes at 50 Hz and 150 W and vortexed at maximum intensity for 1 minute (Scientific Industries), to separate viable bacteria from the implant surface. The obtained solution was serially diluted with the drop plate method[31] using a sterile saline solution. For colony counting, 50 μL of each dilute was incubated on BHI broth agar under microaerophilic conditions for 48 hours. The number of CFU per implant was then counted by a blind and expert examiner.
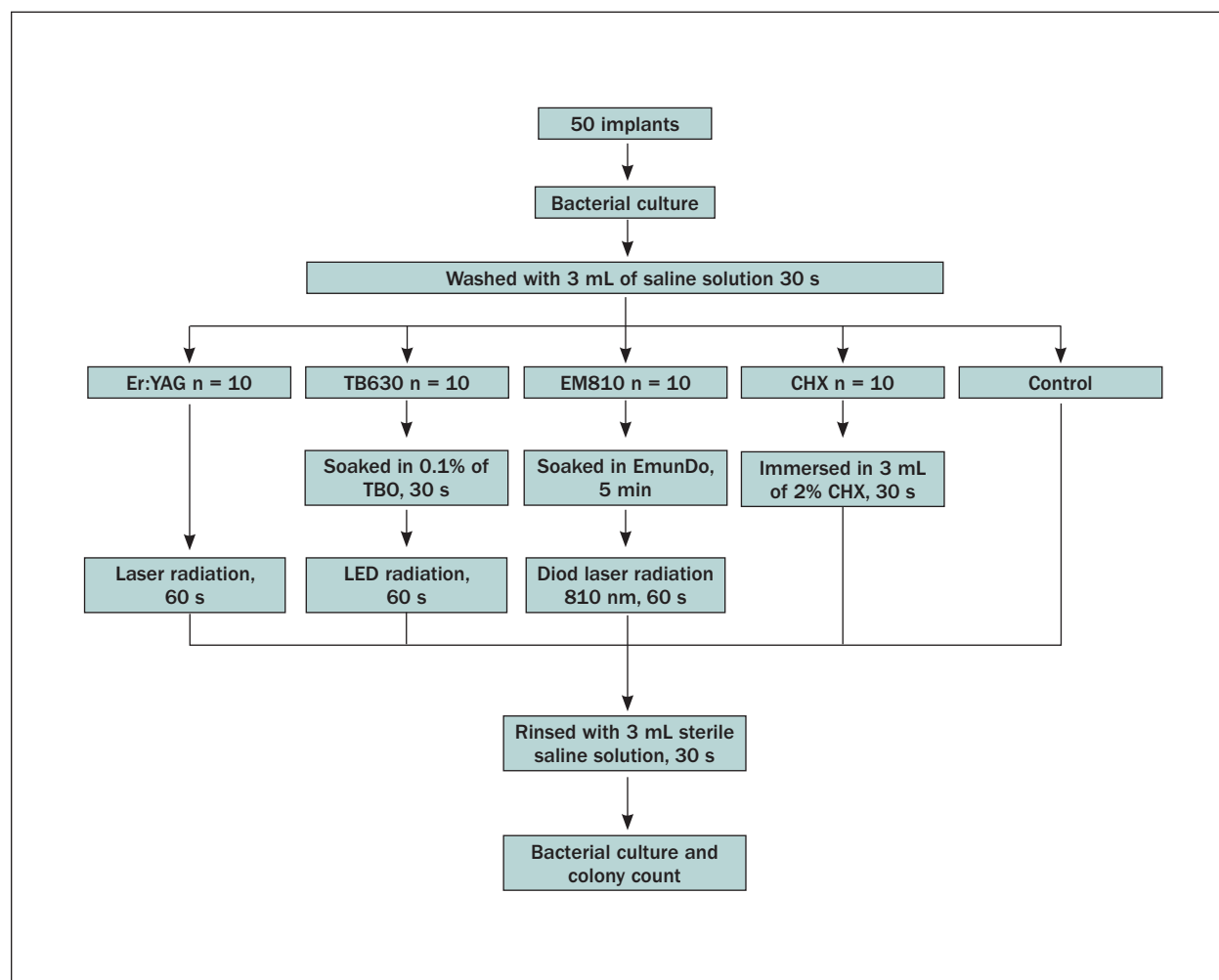
Fig 1   Outline of experiments.

Statistical Analysis

To calculate the residual colony count on implant surface after decontamination, the mean colony count was reported along with the standard deviation. Because the percentages of the colony reduction in the groups were normally distributed (one-sample Kolmogrov-Smirnov test, $P > .05$), a one-way analysis of variance (ANOVA) test was used for comparing them. A Tamhane post hoc test was applied for multiple comparisons due to heterogeneity of variance (Levenes test, $P = .01$). Statistical analyses were applied using SPSS 22 for Windows. P values less than .05 were considered significant.

RESULTS

The mean colony count was significantly different in the understudy groups ($P < .001$). The colony counts after surface decontamination in each group are summarized in Table 2 and Fig 2. The control group had the highest colony count, and the CHX group had the lowest colony count.

Pairwise comparison of the groups showed that the control group had a significant difference from the other groups ($P < .001$), indicating that significant reduction occurred in bacterial count in the other groups. P values are demonstrated in Table 3.

The EM810 and TB630 groups were not significantly different, but the difference between the other groups was statistically significant.

The reduction in the colony count in the Er:YAG group was significantly different from that in the TB630 ($P = .007$), EM810 ($P = .008$), and CHX ($P < .001$) groups and was less than the values in the aforementioned three groups.

DISCUSSION

This study compared the antimicrobial efficacy of aPDT and laser therapy alone in comparison with CHX for elimination of *A actinomycetemcomitans* from the implant surface. The results showed a significant reduction in *A actinomycetemcomitans* colony counts on the implant surface in all treatment groups compared with the control group ($P < .05$). The lowest *A actinomycetemcomitans* count belonged to the CHX, and the highest to Er:YAG. Intergroup comparison revealed no significant difference between the EM810 and TB630 groups ($P = .001$). In other words, TBO plus LED (630 nm) was as effective as ICG-based PS combined with diode laser (810 nm) in elimination of *A actinomycetemcomitans* bacterial biofilm from the implant surface under in vitro conditions. Since no similar study is available for comparison, the results of studies that used different light sources with different physical parameters along with the photosensitizers are discussed, which all are in agreement with the findings of the present study.

Moslemi et al showed that using 0.1 μg/mL TBO in conjunction with LED with a wavelength of 630 nm and energy density of 6 j/cm^2 for 30 seconds could significantly eliminate *A actinomycetemcomitans* in planktonic form.[32] Mattiello et al[33] also reported a significant reduction in *A actinomycetemcomitans* and *Streptococcus sanguinis* colony counts following the use of TBO plus 660-nm diode laser with output power of 30 mW. These results are in accordance with the findings of the present study.

It seems that the gram-negative bacteria are more resistant to aPDT compared with gram-positive bacteria because of the presence of outer membrane in gram-negative bacteria, which hampers the uptake of the PS.[34] However, because of the cationic charge of TBO, it binds to the outer membrane of the gram-negative bacteria and interacts with the LPS.[18] Thus, TBO is a suitable PS for killing the bacteria causing periodontitis and peri-implantitis, particularly the black pigmented bacteria.[35]

The ICG-based PS used in this study is a newly introduced PS for use in the field of dentistry. Boehm Boehm and Ciancio[21] showed that absorption of ICG by *A actinomycetemcomitans* was higher than *P gingivalis*; thus, they believed that ICG was suitable for treatment of periodontitis and peri-implantitis caused by *A actinomycetemcomitans.* Nagahara et al[36] observed that using ICG plus 805-nm diode laser (continuous mode, 0.5 W, 3 to 100 ms) caused a 2-log reduction in *P gingivalis* colony count. In the study by Boehm and Ciancio, using ICG plus 810-nm diode laser with power of 0.1 or 0.5 W and a power density of either 80 W/cm^2 or 400 W/cm^2 for 5 seconds eliminated 20% to 60% of *A actinomycetemcomitans* and *P gingivalis* bacteria from the bacterial culture medium.[21] In general, since the ICG-based PS used in this study is a newly produced material, no similar studies are conducted on it. According to the manufacturer, the bactericidal effect of this PS is mainly due to its photothermal effect; however, the photodynamic effect of this material has gained less attention. Since the oxygen pressure is low at the pocket depth, the photothermal mechanism can be effective for elimination of bacteria from the tooth and implant surface[17];

Table 2   Colony Counts (CFU/Implant) After Decontamination of Implant

Groups	No.	Mean ± SD	Range
Er:YAG	10	6,640.00 ± 1,859.03	3,200.00–8,800.00
TB630	10	3,600.00 ± 1,398.41	1,600.00–5,600.00
EM810	10	3,720.00 ± 1,251.48	2,000.00–5,200.00
CHX	10	1,120.00 ± 795.54	400.00–2,800.00
Control	10	239,600.00 ± 41,158.77	164,000.00–288,000.00
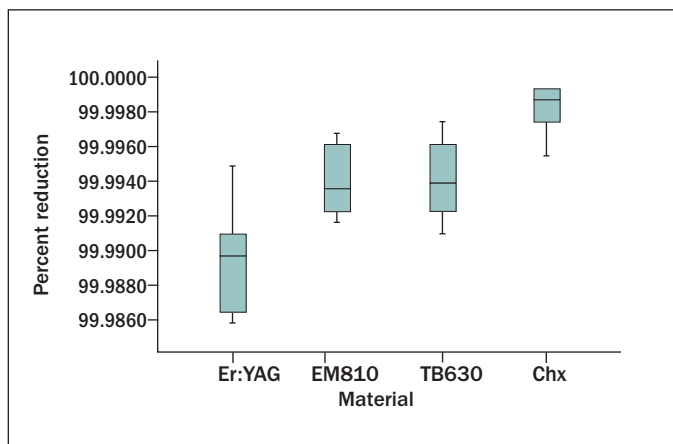
Fig 2 Effects of treatments on percent of reduction in study groups.

Table 3   Statistical Significance of Differences (*P* value) Among 5 Groups

	Er:YAG	EM810	TB630	CHX	Control
Er:YAG	–	.008	.007	< .001	< .001
Em810		–	.999	.001	< .001
TB630			–	.002	< .001
CHX				–	< .001
Control					–

however, superficial thermal damage to the tissues should be investigated in future studies.

As seen in Table 3, the Er:YAG laser had less antimicrobial efficacy than TB630 and EM810 in elimination of *A actinomycetemcomitans* biofilm from the implant surface. It appears that the different effects of the aforementioned three methods are due to the presence of PS. Using PS for treatment of periodontitis or peri-implantitis is advantageous. These materials can penetrate into the implant and root surface porosities that are not accessible by mechanical methods.[18] One drawback of the Er:YAG laser is its small tip, which makes scanning of the entire surface difficult. Successful scanning of the entire surface depends on the expertise and skill of the operator in accurately guiding the handpiece around the implant without missing a point. Although it is difficult to ensure complete scanning of the entire surface, which can compromise the results, using PS decreases this error. The PS marks the bacteria and enables penetration of the laser beam into the porosities and hard-to-reach areas. In the present study, if the clinician was given more time for scanning, more favorable results might be obtained.

The superiority of aPDT over laser alone has been confirmed in the literature. Giannelli et al[3] showed that Er:YAG (with 2,940-nm wavelength, power density of 75.4 W/cm^{2} and pulse energy of 100 mJ for 1 minute), Nd:YAG (with 1,064-nm wavelength in pulsed mode and maximum power density of 264.3 75.4 W/cm^{2} for 1 minute), 810-nm diode laser (in continuous mode with 1 W power and power density of 175.4 W/cm^{2} for 1 minute), 630-nm diode laser (with 0.15-W power and power density of 26.3 W/cm^{2} for 1 minute), and 630-nm LED (in continuous mode with beam power of 0.327 W and 0.344 W/cm^{2} power density for 1 minute) in photoablation mode had much less efficacy for elimination of *P gingivalis* LPS from the surface of titanium discs compared with using diode laser and LED in conjunction with PS in PDT mode. Similarly, the present study emphasized the importance of PS.

Moslemi et al,[32] Marotti et al,[37] and Giannelli et al[3] reported that simultaneous use of laser and PS yielded more favorable results than laser alone. Also, it should be noted that PS, such as methylene blue (MB), TBO, and ICG have no or minimal antimicrobial effect, and the antimicrobial effect reported in the literature is due to the simultaneous use of PS and a light source.[3,21]

In this study, CHX 2% had the highest antimicrobial efficacy. Giannelli et al[3] and Marotti et al[37] reported that the antimicrobial effect of CHX was equal to or lower than that of aPDT. The difference between their results and those of the present study is probably due to the concentration of CHX. Giannelli et al[3] used 0.3%, and Marotti et al[37] used 0.12% CHX. However, several points must be considered when using CHX under in vitro and in vivo conditions. First, CHX is capable of attaching to the surfaces (implant) and gradual release over time.[38] It possesses bactericidal activity even 24 hours after washing.[18,38] Thus, the residual CHX can be transferred to the culture medium and maintain its bactericidal effect, affecting the results. The second point is the cytotoxic effects of CHX on host cells. Giannelli et al[3] showed that when 0.3% CHX was used for disinfection of titanium discs, a severe reduction in longevity of macrophages was observed, although it was washed off the titanium disc surfaces before culture. The microscopic studies by the same author indicated clear signs of cell damage, such as mitochondrial swelling, vacuolation of cytoplasm, and signs of nuclear apoptosis in macrophages adjacent to the discs disinfected with CHX.[39] However, macrophages that were adjacent to titanium discs disinfected with aPDT had normal cytoplasm, organelles, and nuclear chromatin pattern, and the only abnormality observed was slight mitochondrial swelling.[3] Toxic effects of CHX are due to oxidative stress and deranged metabolism of extracellular Ca^{2+} due to mitochondrial dysfunction.[39] The toxic effect of CHX was seen even at the concentration approximately 200-fold below that used in clinic.[40] CHX can induce apoptotic and necrotic/autophagic cell death.[40,41] Studies showed that endothelial cells and fibroblasts are sensitive to CHX, but osteoblasts are the most sensitive cells.[40] Gambarini et al[41] demonstrated that the cytotoxic effect of MB on PDL fibroblasts (whether inactive or activated with a light source) was equal to that of ethylenediaminetetraacetic acid (EDTA) 17% and much lower than the toxicity of 0.3% CHX. Such slight toxicity is not related to light-activated reactive oxygen species.[41]

For evaluation of a therapeutic agent, two aspects must be considered: efficacy and safety, which are both important. Every nontoxic compound is not necessarily safe. Thus, to reach a conclusion, further in vitro and in vivo studies on the effects of CHX on reosseointegration of implants and conditions necessary for reosseointegration are required.

None of the techniques in the present study were capable of complete elimination of *A actinomycetemcomitans* bacteria from the implant surface. Failure in complete elimination of *A actinomycetemcomitans* from the surface means that these methods are inadequate for decontamination of the implant surface and are still an adjunction to conventional treatments to elimination of *A actinomycetemcomitans* biofilm, and more research is needed. Thus, future studies should be conducted on whether longer duration of contact of the implant surface with the photosensitizer influences the effectiveness on surface decontamination. This is particularly true for gram-negative bacteria because they have an outer membrane and greater resistance against dye penetration.[35,37] The present study focused on bactericidal efficacy of laser and aPDT on *A actinomycetemcomitans*

biofilm. Thus, to generalize the result of this study, more studies on the effect of the treatment modalities on the biofilm of other periodontal pathogens are needed. The efficacy of other methods such as flap debridement and air-powder abrasion must be evaluated as well. In this study, the effect of the treatments on the surface biocompatibility, thermal changes, and alterations of the implant surface were not evaluated, so further studies on cell culture and animal models are needed for this purpose.

Finally, the results of in vitro studies may not be generalized to in vivo conditions. The environmental factors, such as variable plaque accumulation, salivation, immune system, limited accessibility, etc, cannot be established in in vitro studies.

CONCLUSIONS

Within the limitations of this study, 2% CHX had superior efficacy for elimination of *A actinomycetemcomitans* biofilm than the other methods under study. Simultaneous use of laser and photosensitizer was found to be a suitable method for disinfection of the implant surface and elimination of *A actinomycetemcomitans* biofilm in comparison with Er:YAG laser alone.

ACKNOWLEDGMENTS

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