Photodiagnosis and Photodynamic Therapy (2015) 12, 131—135

Available online at www.sciencedirect.com

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Aggregatibacter actinomycetemcomitans biofilm can be inactivated by methylene blue-mediated photodynamic therapy

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Available online 22 October 2014

KEYWORDS
Antimicrobial photodynamic inactivation;
Antimicrobial photodynamic chemotherapy;
Periodontitis;
Periodontal disease;
Photosensitizer;
Red laser

Summary
Background: The purpose of this study was to evaluate the antibacterial effects of photodynamic action of methylene blue (MB) against *Aggregatibacter actinomycetemcomitans* organized on biofilm.
Methods: After the biofilm growth in 96 flat-bottom well plate, the following groups were used: control group, untreated by either laser or photosensitizer (PS); MB group or dark toxicity group, which was exposed to MB alone (100 μM) for 1 min (pre-irradiation time); laser group, irradiated with laser for 5 min in the absence of PS and three antimicrobial photodynamic inactivation (APDI) groups, with three exposure times of 1, 3 and 5 min of irradiation, corresponding to fluences of 15, 45, and 75 J/cm^2 respectively. The results were compared to the control group for statistical proposes. Scanning electronic microscope analysis was used to access structural changes in biofilm.
Results: Red laser alone and MB alone were not able to inactivate bacterial biofilm. APDI groups showed differences when compared to the control group and they were dependent on the exposure time. No statistically significant differences were observed among the APDI groups at 1 and 3 min of irradiation. On the other hand, 5 min of APDI showed 99.85% of bacterial reduction ($p = 0.0004$). In addition, the biofilm loose its structure following 5 min APDI.

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http://dx.doi.org/10.1016/j.pdpdt.2014.10.002
1572-1000/

Conclusions: The results of this study suggest that *A. actinomycetemcomitans* biofilm can be inactivated by MB mediated APDI.

Introduction

Periodontitis is an inflammatory condition that occurs in gingival tissue and it may produce progressive loss of supportive periodontal bone and, in advanced cases, tooth loss. The cause of periodontal disease is related to bacterial accumulation on the teeth surface around the gingival margin and it is not associated with a single microorganism, but rather is a consortium of bacterial species that play a role in the initiation, maintenance, and progression of the disease. Bacterial growth promotes the formation of a microbial community called biofilm, which protect microbial cells against antimicrobial treatment and it also improves the ability of bacteria to produce damage in the host tissue. This biofilm produces toxins that attack host tissue following by the production of inflammatory mediator products. The treatment of periodontal condition is based on the patient's mechanical control of biofilm associated with dental professional procedures, which consist in disrupting biofilm and mineral deposits in subgingival sites by scaling and root planing (SRP) [1].

Antimicrobial photodynamic inactivation (APDI) combines a harmless photoactivatable dye with visible light and it has been studied as an antimicrobial strategy to improve periodontal treatment. A frequently employed photosensitizer (PS) class is the phenothiazinium salt, represented by methylene blue (MB) (λ_{max} = 664 nm) and toluidine blue O (TBO) (λ_{max} = 630 nm). They are amphipathic tricyclic planar molecules that hold one intrinsic quaternary nitrogen atom and have phototoxic efficiency against periodontopathogens [2,3].

The microorganism *Aggregatibacter actinomycetemcomitans* has been appointed as one of the causative agents of periodontal disease and it is a Gram-negative microaerophilic bacterium [4]. Efforts to understand APDI effect have been done, however, predictable results are not usually found because there are a large amount of parameters such as irradiation doses, photosensitizer concentration, and inoculum concentration. Eick et al. [5] demonstrated that APDI could inactivate a large number of periodontopathogenic bacteria in suspension and in organized biofilms. On the other hand, the present study focus on the biofilm structures after the APDI process. In this study a monobacterium specie biofilm model was developed using *A. actinomycetemconitans*. The biofilm was stained by PS and irradiated with red light. The aim of this study was to evaluate APDI irradiation effects on *A. actinomycetemcomitans* biofilm.

Materials and methods

Bacterial strain and inoculum

Aggregatibacter actinomycetemcomitans strain ATCC 29523 was subcultured in a 96-well cell-culture cluster with brain heart infusion (BHI) broth and 10^8 *A. actinomycetemcomitans* per mL were incubated under a 5% CO_2 atmosphere at 37 °C for 5 days. After an incubation period of 48 h, the liquid medium was carefully aspirated from each well and the biofilms were replenished with fresh BHI broth. Then, on day 5 of the development of the biofilm, the medium was removed, and the wells were washed 3 times with PBS, pH 7.2. The PDT was performed next. Catalase and morphologic Gram test were used to confirm species identification.

Photosensitizer and light source

Methylene blue (MB) (Sigma—Aldrich, St Louis, USA) stock solution was prepared in distilled water at a concentration of 10,000 μM, filtered through a sterile 0.22 μm filter membrane and stored for a maximum of 2 weeks at 4 °C in the dark condition. Before the experiments, 100 μM MB solution was prepared by 100-fold dilution of the stock solution in distilled water.

The light source used for the photosensitizer excitation was a diode laser with an emission at $\lambda = 660 \pm 2$ nm (Photon Lase III, DMC, São Carlos, Brazil). The biofilm was irradiated from the top of the flat-bottom microtiter plate. The total output power provided by the device was 100 mW, and the spot area was 0.4 cm^2.

Antimicrobial photodynamic therapy

After the biofilm growth, the following groups were used: control group (L—PS—) untreated by either laser or photosensitizer; MB group (L—PS+) or dark toxicity group, which was exposed to MB alone (100 μM) for 1 min (pre-irradiation time); laser group (L+PS—), irradiated with laser for 5 min in the absence of PS and three APDI groups, with three exposure times of 1 (L1+FS+), 3 (L3+FS+) and 5 min (L5+FS+) of irradiation, corresponding to fluences of 15, 45 and 75 J/cm^2 respectively.

After irradiation procedures, each group was serially diluted to give dilutions of 10^{-1} to 10^{-5} times the original concentrations, and then 10 μL of each dilution were streaked over the surfaces of tryptic soy agar (TSA) plates in duplicate. The plates were incubated at 37 °C for 48 h under a 5—10% CO_2 atmosphere. The bacterial colonies were counted and converted into colony forming units (CFU) for analysis.

Statistics

Comparisons between groups were made by analysis of variance followed by the *t*-test. The significance level was set at 5% ($p < 0.05$) [2,6]. The experiment was repeated 3 times with 4 wells per group.

Scanning electron microscope (SEM) evaluation

Scanning electron microscopy was used to image biofilm structure following APDI. *A. actinomycetemcomitans* ATCC 29523 biofilms were grown on 4 mm × 4 mm samples of bovine dental root. The samples were immersed on BHI inoculum with 10^8 cells/mL in 24-well plate, and it was incubated under a 5% CO_2 atmosphere at 37 °C for 5 days. After an incubation period of 48 h, the liquid medium was carefully aspirated from each well and the samples were replenished with fresh BHI broth. Then, on day 5 of the development of the biofilm, the medium was removed, and the wells were washed 3 times with PBS, pH 7.2. The APDI was performed next. Following irradiation, the specimens were fixed on 2.5% glutaraldehyde for 1 h at 25 °C, subsequently they were secondarily fixed in 1% osmium tetroxide and dehydrated on ethanol in the sequence of 50, 60, 70, 80, 90, and 100%. The SEM used for this study was a Hitachi Analytical Table Top Microscope TM3000 (Hitachi, Tokyo, Japan). Representative SEMs were taken at 1000× and 3000× of magnification, 15 kVA, in the more density biofilm growth area in the specimen. To qualitative assessment of biofilm structure, 3 specimens per group were observed.

Results

Neither the MB 100 μM in the absence of light (MB group) nor the laser light in the absence of MB (laser group) had a statistically significant effect on the viable count of the bacteria in the biofilm. The control group presented average and standard deviation of 8.87 ± 0.34 Log_{10}(cfu/mL) and laser group showed 8.13 ± 0.67 Log_{10}(cfu/mL) ($p > 0.05$). As can be observed, a 100 μM concentration of MB did not show any toxicity for *A. actinomycetemcomitans* cells after previous incubation for 1 min in the dark. The average and standard deviation of bacterial count in Log_{10}(cfu/mL) was 8.47 ± 0.06, and $p > 0.05$ when compared to control group and laser group.

Fig. 1 demonstrates that *A. actinomycetemcomitans* was eradicated in the APDI groups. However, the degree of photoinactivation was dependent upon the time of the irradiation.

APDI groups showed differences when compared to the control group and they were dependent on the exposure time. No statistically significant differences were observed among the APDI groups at 1 and 3 min of irradiation. On the other hand, a significant reduction was observed with 5 min of APDI when compared to control group ($p < 0.05$).

The amount of bacterial killing was 50% in group L1+FS+ (15 J/cm^2) and 57% in group L3+FS+ (45 J/cm^2). However, the best result was obtained in group L5+FS+ (75 J/cm^2), with 99.85% cell death being reached ($p = 0.0004$).

The SEM analysis of the control group showed the presence of a well-organized biofilm formed by bacterial clusters and abundant extracellular matrix (Fig. 2A). *A. actinomycetemcomitans* cells can be seen (white arrows) wrapped by extracellular matrix (black arrows). Following 5 min of laser irradiation in the presence of MB it was observed a severe disruption of biofilm structure (Fig. 2B). The cluster of bacterial cells was shattered and only the bacterial residue (white arrows) remained on the biofilm-damaged matrix (black arrows).

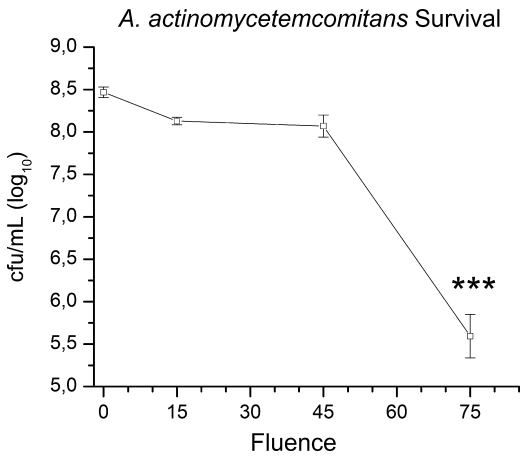

Figure 1 The effect of APDI on *A. actinomycetemcomitans* survival in Log_{10} cfu/ml. Biofilm was incubated with 100 μM MB for 1 min, and irradiated (λ = 660 nm). Data are the means of three independent experiments and the bars are the standard deviation. ***$p = 0.0004$.

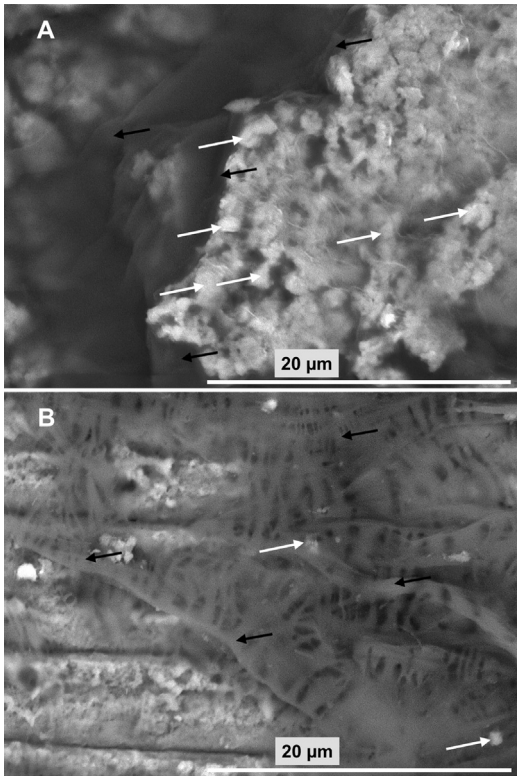

Figure 2 Scanning electron microscopy of *A. actinomycetemcomitans* biofilm is presented in (A) without treatment (control group) and in (B) after 5 min APDI. Biofilms were incubated with 100 μM MB for 1 min and then irradiated for 5 min (λ = 660 nm). The white bars represent 20 μm. White arrows indicate bacterial cells and black arrows indicate extracellular matrix.

Discussion

The results of this study showed that laser irradiation (λ = 660 nm and 100 mW) combined with 100 μM MB reduced *Aggregatibacter actinomycetemcomitans* by up to 99.85% on the group treated with PDT with 5 min of irradiation.

Previous studies evaluated the effectiveness of the adjunctive therapy of PDT in nonsurgical periodontal treatment. Andersen et al. [7] verified significant differences in all investigated clinical parameters in the patients 3 months after treatment with PDT and scaling and root planing (SRP) when compared with SRP alone.

A less significant bacterial reduction was observed by Fontana et al. [8] after PDT treatment in microbial biofilm. They verified that oral bacteria in biofilms are less affected by PDT than bacteria in planktonic phase. They reported that PDT killed 63% of bacteria present in suspension, against 31% in biofilms. However there was no variation in the irradiation time and fluence, which was 30 J/cm^2. Moreover, they investigated the photodynamic effects on biofilms developed with bacteria collected from different subjects. In the present study, we observed a 57% reduction in *A. actinomycetemcomitans* biofilm after 45 J/cm^2 irradiation. Three irradiation times were used and the results showed that bacterial reduction was directly dependent on these conditions, whereas the best results were achieved after longer irradiations.

Goulart et al. [9] tested the efficacy of PDT by using methylene blue and erythrosine (ERY) to inactivate *A. actinomycetemcomitans* in planktonic cultures and biofilm. The results showed that ERY was more efficient at killing bacterial cells of *A. actinomycetemcomitans* in planktonic (75%) and biofilm (77%) culture compared with MB (50% and 54%, respectively). A hand-held photopolymerizer (HHP) was used as light source in all the experiments. The HHP had a continuous output of 350—500 mW of potency from a halogen light isolated from an inner filter that selected the wavelength range of 400—500 nm, which is more appropriate for dyes that have an absorption band in this range, such as ERY, with the absorption spectrum of 536 nm. This wavelength is not well absorbed by MB, which has an absorption band in the red range of the electromagnetic spectrum, between 600 and 660 nm [10], and this can justify the results. A relationship between wavelength and absorption of the dye must be observed in the use of APDI. The cell toxicity only occurs when the absorption band of the photosensitizer is resonant with the emitted radiation [11]. The laser equipment used in the present study has a wavelength of 660 nm, within the range of resonance of MB, permitting APDI to be compatible to the dye [3].

The light wavelength, light intensity, photosensitizer absorbance and exposure time determine the results, since for the photochemical and photophysical effects occur, the photosensitizer has to be efficiently sensitized by the light source [12,13]. In this study, the groups treated with APDI showed alterations compared to the control group and these were dependent on the irradiation time. There was a bacterial reduction in L1+FS+ and L3+FS+ groups, but this reduction was not significant ($p > 0.05$). On the other hand, a significant reduction was observed with 5 min of PDT when compared to control group ($p < 0.05$). This relationship between bacterial reduction and exposure time was previously observed by Prates et al. [12]. They investigated the ability of malachite green combined with a low-power red laser to kill *A. actinomycetemcomitans*. Two groups were treated with APDI, with two exposure times of 3 and 5 min, and energy doses of 5.4 and 9 J/cm^2, respectively. The degree of photoinactivation was dependent upon the irradiation time, with 97.2% reduction of *A. actinomycetemcomitans* on the group tested with 3 min of irradiation and 99.9% on the group irradiated for 5 min.

In the present study, 100 μM MB for 1 min did not present any toxic effects against *A. actinomycetemcomitans* as there was no reduction in the CFU number. MB can be used in surgical procedures at considerable higher concentrations (3292 μM) without being toxic to humans [10]. There are many variables that can influence PDT, such as concentration and preincubation time of the dye, the presence of oxygen, blood or gingival fluid, light wavelength and fluence [14,15]. Some studies have not found any statistically significant differences when APDI was used in addition to conventional treatment. Polansky et al. [16] evaluated APDI for its bacterial potential and clinical effect in the treatment of periodontitis. They observed some clinical and microbial parameters in 3 months and no significant difference was found. In other clinical study, Oliveira et al. [17] investigated TNF-α (tumor necrosis factor-alpha) and RANKL (receptor activator of nuclear factor-kappaB ligand) levels in the gingival crevicular fluid of patients with aggressive periodontitis. Similar results were found on SRP group and APDI group. However, in both studies, they used the photosensitizer HELBO® Blue, which has a concentration of 3192 μM. This concentration is much higher than that used in the present study (100 μM), and this can justify the findings.

The major goal of periodontal therapy is to significantly reduce or eliminate oral microbial biofilms and periodontopathogens. The appropriate therapy for patients with periodontitis includes procedures such as scaling and root planing (SRP); however, the optimal therapy varies considerably with the extent and pattern of attachment loss, local anatomical variations, type of periodontal disease, and therapeutic objective of the treatment. The goal of periodontal therapy is to halt disease progression and stabilize periodontal attachment levels [18]. Our results demonstrated that the use of APDI decreases bacterial content of biofilm following 5 min of irradiation. In addition, the structural disruption of biofilm matrix can make then more sensitive to killing [19].

Due to the large number of variables that can influence APDI, the parameters used in *in vitro* studies may not promote the same bacterial reduction *in vivo*. Therefore, the treatment success involves the optimization of many parameters and the dynamics of the photoreactions must be more clearly understood. Clinical studies are still necessary to determine the effectiveness of PDT in the long term and to confirm the findings of *in vitro* studies.

Acknowledgment

This work was partially supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grant nos. 05/01756-5 and 10/13313-9).

References

[1] Dentino A, Lee S, Mailhot J, Hefti AF. Principles of periodontology. Periodontology 2000 2013;61(February (1)):16—53.

[2] Prates RA, Fuchs BB, Mizuno K, Naqvi Q, Kato IT, Ribeiro MS, et al. Effect of virulence factors on the photodynamic inactivation of *Cryptococcus neoformans*. PLOS ONE 2013;8(1):e54387.

[3] Prates RA, Yamada AM, Suzuki LC, França CM, Cai S, Mayer MP, et al. Histomorphometric and microbiological assessment of photodynamic therapy as an adjuvant treatment for periodontitis: a short-term evaluation of inflammatory periodontal conditions and bacterial reduction in a rat model. Photomed Laser Surg 2011;29(December (12)):835—44.

[4] Henderson B, Ward JM, Ready D. *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*: a triple A* periodontopathogen? Periodontology 2000 2010;54(October (1)):78—105.

[5] Eick S, Markauskaite G, Nietzsche S, Laugisch O, Salvi GE, Sculean A. Effect of photoactivated disinfection with a light-emitting diode on bacterial species and biofilms associated with periodontitis and peri-implantitis. Photodiagnosis Photodyn Ther 2013;10(May (2)):156—67.

[6] Demidova TN, Hamblin MR. Photodynamic therapy targeted to pathogens. Int J Immunopathol Pharmacol 2004;17(September—December (3)):245—54.

[7] Andersen R, Loebel N, Hammond D, Wilson M. Treatment of periodontal disease by photodisinfection compared to scaling and root planing. J Clin Dent 2007;18(2):34—8.

[8] Fontana CR, Abernethy AD, Som S, Ruggiero K, Doucette S, Marcantonio RC, et al. The antibacterial effect of photodynamic therapy in dental plaque-derived biofilms. J Periodontal Res 2009;44(December (6)):751—9.

[9] Goulart RC, Thedei G, Souza SL, Tedesco AC, Ciancaglini P. Comparative study of methylene blue and erythrosine dyes employed in photodynamic therapy for inactivation of planktonic and biofilm-cultivated *Aggregatibacter actinomycetemcomitans*. Photomed Laser Surg 2010;28(August (1)):85—90.

[10] Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). J Antimicrob Chemother 1998;42(July (1)):13—28.

[11] Chan Y, Lai CH. Bactericidal effects of different laser wavelengths on periodontopathic germs in photodynamic therapy. Lasers Med Sci 2003;18(1):51—5.

[12] Prates RA, Yamada Jr AM, Suzuki LC, Eiko Hashimoto MC, Cai S, Gouw-Soares S, et al. Bactericidal effect of malachite green and red laser on *Actinobacillus actinomycetemcomitans*. J Photochem Photobiol B 2007;86(January (1)):70—6.

[13] Prates RA, Silva EG, Yamada Jr AM, Suzuki LC, Paula CR, Ribeiro MS. Light parameters influence cell viability in antifungal photodynamic therapy in a fluence and rate fluence-dependent manner. Laser Phys 2009;19(May (5)):1038—44.

[14] Núñez SC, Garcez AS, Gomes L, Baptista MS, Ribeiro MS. Methylene blue aggregation in the presence of human saliva. Spie Photonics West; 2008. Progress in Biomedical Optics and Imaging 2008.

[15] Soukos NS, Goodson JM. Photodynamic therapy in the control of oral biofilms. Periodontology 2000 2011;55(February (1)):143—66.

[16] Polansky R, Haas M, Heschi A, Wimmer G. Clinical effectiveness of photodynamic therapy in the treatment of periodontitis. J Clin Periodontol 2009;36(July (7)):575—80.

[17] de Oliveira RR, Schwartz-Filho HO, Novaes AB, Garlet GP, de Souza RF, Taba M, et al. Antimicrobial photodynamic therapy in the non-surgical treatment of aggressive periodontitis: cytokine profile in gingival crevicular fluid, preliminary results. J Periodontol 2009;80(January (1)):98—105.

[18] Slots J, Ting M. Systemic antibiotics in the treatment of periodontal disease. Periodontology 2000 2002;28(July (1)):106—76.

[19] Izano EA, Sadovskaya I, Wang H, Vinogradov E, Ragunath C, Ramasubbu N, et al. Poly-N-acetylglucosamine mediates biofilm formation and detergent resistance in *Aggregatibacter actinomycetemcomitans*. Microb Pathog 2008;44(January (1)):52—60.