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Parameters for antimicrobial photodynamic therapy on periodontal pocket—Randomized clinical trial

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ABSTRACT

Background: Antimicrobial photodynamic therapy (aPDT) has been investigated as an adjunctive to periodontal treatment but the dosimetry parameters adopted have discrepancies and represent a challenge to measure efficacy. There is a need to understand the clinical parameters required to obtain antimicrobial effects by using aPDT in periodontal pockets. The aim of this study was to investigate parameters relating to the antimicrobial effects of photodynamic therapy in periodontal pockets.

Material and methods: This randomized controlled clinical trial included 30 patients with chronic periodontitis. Three incisors from each patient were selected and randomized for the experimental procedures. Microbiological evaluations were performed to quantify microorganisms before and after treatments and spectroscopy was used to identify methylene blue in the pocket. A laser source with emission of radiation at wavelength of $\lambda = 660$ nm and output radiant power of 100 mW was used for 1, 3 and 5 min. One hundred μM methylene blue was used in aqueous solution and on surfactant vehicle.

Results: The results demonstrated the absence of any antimicrobial effect with aqueous methylene blue-mediated PDT. On the other hand, methylene blue in the surfactant vehicle produced microbial reduction in the group irradiated for 5 min ($p < 0.05$). Spectroscopy showed that surfactant vehicle decreased the dimer peak signal at 610 nm.

Conclusion: Within the parameters used in this study, PDT mediated by methylene blue in a surfactant vehicle reached significant microbial reduction levels with 5 min of irradiation. The clinical use of PDT may be limited by factors that reduce the antimicrobial effect. Forms of irradiation and stability of the photosensitizers play an important role in clinical aPDT.

1. Introduction

Periodontitis is an infection that produces inflammatory response on the supporting tissues of teeth that leads to alveolar bone reabsorption in response to this microbial challenge. The velocity and severity of bone damage is regulated by the immune-inflammatory response of the host. The purpose of periodontal treatment is to eliminate bacterial deposits by removing the supra- and subgingival calculus and biofilm, and consequently reducing the excessive inflammatory response. Such treatment is performed by mechanical scaling and root planing methods, which often results in significant clinical improvements. However, in cases of advanced infections, the use of antibiotics may be necessary [1]. The use of these agents has shown no improvement in long-term periodontal clinical parameters, and it can cause adverse effects and lead to the development of bacterial resistance [2,3]. Thus, there is growing interest in the development of other forms of

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treatment, and the antimicrobial photodynamic therapy (aPDT) represents an alternative to the use of antimicrobials (antibiotics and disinfectant agents) [4,5].

Antimicrobial PDT is a noninvasive form of treatment and it has been proposed as an adjuvant intervention for periodontitis [6]. It is based on the use of photoactive substances, known as photosensitizers (PS) that bind to the target cell and produces reactive oxygen species, *via* pathway of type I or type II reaction, following light irradiation of a suitable wavelength [7–9]. The photosensitizer is inserted into the periodontal pocket and it binds to microbial biofilm and bacteria [4]. The light-PS interactions will promote the death of microorganisms by the formation of reactive oxygen species (ROS) and to the best of our knowledge the development of bacterial resistance has not been reported.

Blue dyes of the phenothiazinium class are the most used photosensitizers in clinical aPDT for periodontal treatment, among them toluidine blue and methylene blue [9,10]. The phenothiazinium chromophore is a flat tricyclic moiety having a delocalized, permanent positive charge. Both toluidine blue and methylene blue are efficient singlet oxygen producers and exhibit efficient phototoxicity against microorganisms, including Gram-positive and Gram-negative bacteria, and fungi [11].

Previous *in vitro* study conducted by Alvarenga et al, showed that 100 μM methylene blue (MB)-mediated aPDT was able to inactivate *Aggregatibacter actinomycetemcomitans* biofilms following red laser irradiation (λ = 660 nm) for 5 min [12]. However, well-conducted clinical trials that aimed to investigate aPDT showed poor clinical improvements following aPDT [10,13]. Studies have been conducted to evaluate periodontal clinical outcomes provided by aPDT and many parameters of dye concentration and irradiation were reported [14–16]. Clinical trials that commonly use 1 min of light irradiation as well as high photosensitizer concentration (≥1000 mM), often report limited clinical results. Therefore, the light dosimetric parameters for clinical use of aPDT still need investigation. This is an important step in understanding the phenomena of microbial inactivation that should occur in infected periodontal pockets. This study aimed to evaluate antimicrobial effect of aPDT in periodontal pockets following laser irradiation for 1, 3, and 5 min.

2. Materials and methods

This clinical trial was registered on ClinicalTrials.gov (NCT 03262077). It was conducted involving 30 patients with chronic periodontitis. They were recruited from the Odontological Clinic of the Nove de Julho University (UNINOVE, Brazil). The project was approved by the Human Research Ethics Committee of the University (number 1.517.902). Patients who agreed to participate signed a statement of informed consent approved by the Research Ethics Committee of the University.

2.1. Inclusion and exclusion criteria

The patients selected were under periodontal treatment in the Dental Clinic of the UNINOVE University. The inclusion criteria were patients with chronic periodontitis [17], presence of at least 15 teeth and at least 3 different upper incisors with probing depth greater than 4 mm [18,19]. The exclusion criteria comprise current smokers or regular smoking 12 months prior to enrollment; patients with anemia, active cancer, pregnancy, history of antibiotic therapy in the previous six months, history of anti-inflammatory therapy in the previous three months, clotting disorders, and those currently undergoing orthodontic treatment.

2.2. Primary outcome

The primary outcome variable of the study was microbial reduction immediately after PDT.

2.3. Experimental groups and study design

Sixty periodontal sites were selected from twenty patients (n = 60) meeting the inclusion criteria and they were randomly divided into 3 groups. Then, opaque envelopes (randomly containing information about application of light, 1, 3 and 5 min) were labelled with sequential numbers. The researcher responsible for irradiation openned the first envelope and perform the procedure written therein. Each pacient had 3 periodontal pockets with 5 to 7 mm deep in the incisors region. One hundred μM MB in aqueous solution (dye content ≥ 82%, Sigma-Aldrich, MO, USA) was used as photosensitizer (MB groups). For application of MB, a no point needle was inserted until the bottom of the periodontal pocket and PS was injected until its overflow, to guarantee complete filling of the pocket. One min of dark incubation was set to allow photosensitizer diffusion through the bacterial biofilm [20,21]. Thereafter, red laser (Photon Lase III, DMC, São Carlos, Brazil) (660 nm, 100 mW) was used through the oral mucosa, over epithelium. Three irradiation times were established (1, 3 and 5 min), and each incisor was irradiated for one of these times. Following the analisys of the results, another group of 10 patients were selected and they had 30 additional sites (n = 30). At this time, 100 μM methylene blue was used in a 0.25% sodium dodecyl sulfate solution (MBS) [20]. In other words, in MB groups we had 3 different times of irradiation: 1 min (MB1), 3 min (MB3) and 5 min (MB5) and for MBS groups the same 3 different times of irradiation: 1 min (MBS1), 3 min (MBS3) and 5 min (MBS5). Light scattering in the gingival tissue and absorption spectra were collected at all 90 sites.

It is important to note that the selected incisors did not receive the photosensitizer (PS) simultaneously. The approach was adopted to avoid overlap of light irradiation. Each irradiated site covered an area of 0.4 cm^2, which resulted in radiant exposure of 15, 45 and 75 J/cm^2. The irradiation had a constant power energy density of 250 mW/cm^2, output radiant power of 100 mW with a tip diameter of 0335 cm and energy per point were 6, 18, and 30 J, with 1 point per teeth. Since each patient received irradiation under three parameters and in three distinctis periodontal sites, the total energy applied per patient was 54 J.

2.4. Microbiological evaluation

Two collections were performed at each experimental site before irradiation with the laser, and immediately after the irradiation procedures.

The PS was deposited in the periodontal pocket of the first incisor evaluated and after irradiation of this site, the microbiological collection of this tooth was performed. The next incisor evaluated only received PS after the end of the first tooth's microbiological collection. Subgingival biofilm samples were collected from the periodontal pockets of the incisors. The collection was performed with relative isolation using cotton rolls following the removal of the supragingival biofilm with a sterile compress. Sterile absorbent paper points (No. 30) (Tanari Industrial Ltda, São Paulo, Brazil) were inserted into each site for 30 s. The samples were stored in properly identified sterile plastic microtubes, with each paper point being stored in a different microtube and the paper cones were processed for microbial analysis in up to 20 min following it was harvested.

The samples were used to determine the number of CFU (Colony Forming Unit). 1 mL of brain heart infusion broth medium was added to each microtube, followed by vortex homogenization. Aliquots of 10 μL in 5 dilutions were streaked in triplicate on blood agar Petri dishes. The plates were incubated at 37 °C for 48 h under anaerobic conditions to evaluate the total bacterial inactivation. After this period the CFU were counted and converted in survival fraction for analysis. Since the number of CFU/mL change from each periodontal site to another, survival fraction was calculated to normalize the microbial load. Survival fraction data was calculated as the number of CFU/mL after treatment divided by the number of CFU/mL before treatment at the same periodontal site.

2.5. Assessment of photosensitizer spectra in periodontal pockets

The measurement of the MB and MBS spectra in the periodontal pockets was performed prior to laser irradiation. After the pre-irradiation time of 1 min, a spectrometer (USB2000 +, Ocean Optics, Winter Park, USA) was positioned over the periodontal pocket on the oral epithelium, and the UV–vis spectra was recorded.

2.6. Data analysis

The distribution of the data was evaluated (Shapiro-Wilk) and as it resulted in non-normal distribution, the Wilcoxon test was used to compare the groups. Differences between groups were assumed when $p < 0.05$. The data were presented graphically with median and quartiles 1 and 2 of the survival fraction of the microorganisms recovered from the periodontal pocket.

3. Results

It was observed that there was no significant difference in the survival fraction before and after irradiation in the groups treated with 100 μM MB and irradiated for 1, 3 and 5 min ($p < 0.05$)(Fig. 1A).

Likewise, the groups treated with MBS and irradiated for 1 and 3 min did not present a significant reduction in survival fraction ($p < 0.05$). On the other hand, it was possible to observe a significant reduction ($p = 0.0067$) of 1.6 logs in survival fraction following 5 min of irradiation (MBS5) (Fig. 1B).

The ratio of survival fraction before and after treatment was observed on the different irradiation times for MB and MBS groups. The MBS group irradiated for 5 min presented different behavior, with a reduction of 1.6 Log. There was no killing effect on 1 and 3 min of irradiation for MB or MBS groups, and even following 5 min of irradiation there was no bactericidal effect on MB5 group. The MBS1 group had a survival fraction equal to MBS3 ($p > 0.05$). However, the MBS1 and MBS3 groups had a higher survival fraction than MBS5 ($p = 0.0069$ and $p = 0.0051$, respectively).

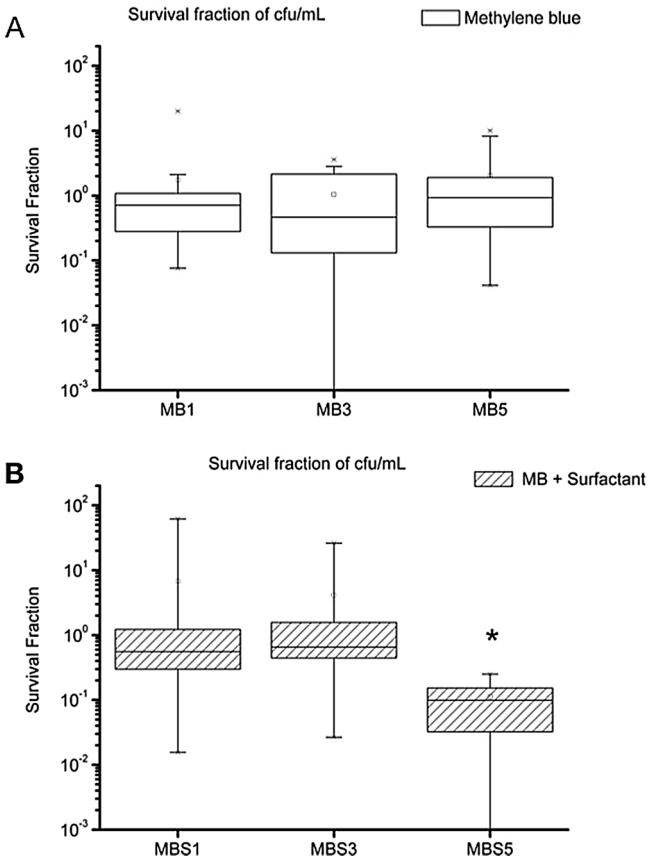

Fig. 1. Survival fraction in CFU/mL of the groups treated with MB (Fig.1 A) and MBS (Fig.1 B) and irradiated for 1, 3 and 5 min. The data were presented in boxes with median and quartiles 1 and 2 of the survival fraction of the microorganisms recovered from the periodontal pocket.

Observing the growth pattern of the bacterial colonies in the Petri dishes, we verified that in MB1, MB3, and MB5 groups, there was no bacterial reduction and the growth pattern of the colonies remained the same before and after irradiation (Fig. 2A).

In the groups treated with MBS vehicle, the pockets irradiated for 1 and 3 min (MBS1 and MBS3, respectively) did not present a significant reduction in the number of CFU/mL. However, there was a change in the growth pattern of the colonies. Although there was bacterial growth after irradiation, it was noticeable that the colonies presented smaller size, possibly due to a delay in their growth (Fig. 2B).

The analysis of the data obtained with the spectrometer indicated that there were absorption bands in the ultraviolet region and two absorption bands in the visible region in MB in water (MB), relative to the dimer (610 nm) and the monomer (660 nm). On the other hand, in MB in the surfactant vehicle (MBS), there was only one absorption peak, approximately at 660 nm (Fig. 3).

4. Discussion

The results of the study showed that PDT mediated by 100 μM MB photosensitizer in the surfactant vehicle and laser irradiation ($\lambda = 660$ nm) had 96% of microbial reduction in the group irradiated for 5 min (MBS5).

The groups treated with 100 μM MB did not present significant bacterial reduction, even after 5 min of irradiation. Alvarenga et al., 2015 conducted a study using aPDT on *Aggregatibacter actinomycetemcomitans* biofilm and reported bacterial reduction of 99.85% in the group treated with aPDT and irradiated for 5 min [12]. They used aPDT mediated by 100 μM MB and red laser ($\lambda = 660$ nm and output power P = 100 mW). Their results indicated that the irradiation time exerts an influence on cell death. Bacterial reduction in the clinical environment can be different to an *in vitro* experiment, due to the large number of variables, such as oxygen tension inside the periodontal pocket, the presence of gingival blood and fluids. Carvalho et al. observed in a clinical study that PDT mediated by MB in aqueous solution did not promote microbial reduction and also did not produce clinical improvement in patients with chronic periodontitis [10].

Even following 5 min of irradiation (MB5) group, no antimicrobial effect was apparent on periodontal pockets. This long irradiation time may impact negatively on the clinical use of aPDT mediated by MB. Thus, a more effective photosensitizer or a better method or vehicle will be important to improve the antimicrobial effect and consequently the clinical outcome provide by this techinique.

The analysis of bacterial growth in the Petri dishes showed that the groups treated with MBS and irradiated for 1 and 3 min showed an alteration in the growth pattern of the colonies, even though there was no microbial reduction. It can be seen that PDT was able to modify the biofilm. A delay in the microbial growth may play an important role on the ability of the host immune defence to act against the microbial content of periontal pocket.

Due to the known dimerization of simple phenothiazinium dyes, which may lead to impaired photosensitizing efficacy, it was decided to include a surfactant vehicle in three of the test groups (MBS1, MBS3, and MBS5) in order to increase the possibility of photochemical action.

Collina et al., 2018 proposed an oral formulation containing MB and sodium dodecyl sulfate (SDS) with the purpose of reducing the MB aggregation. They observed that the oral formulation was an efficient strategy and an increased effectiveness of the therapy was observed in a planktonic *Candida albicans* culture, when compared with no formulation of MB [20]. The incubation time in dark was also evaluated

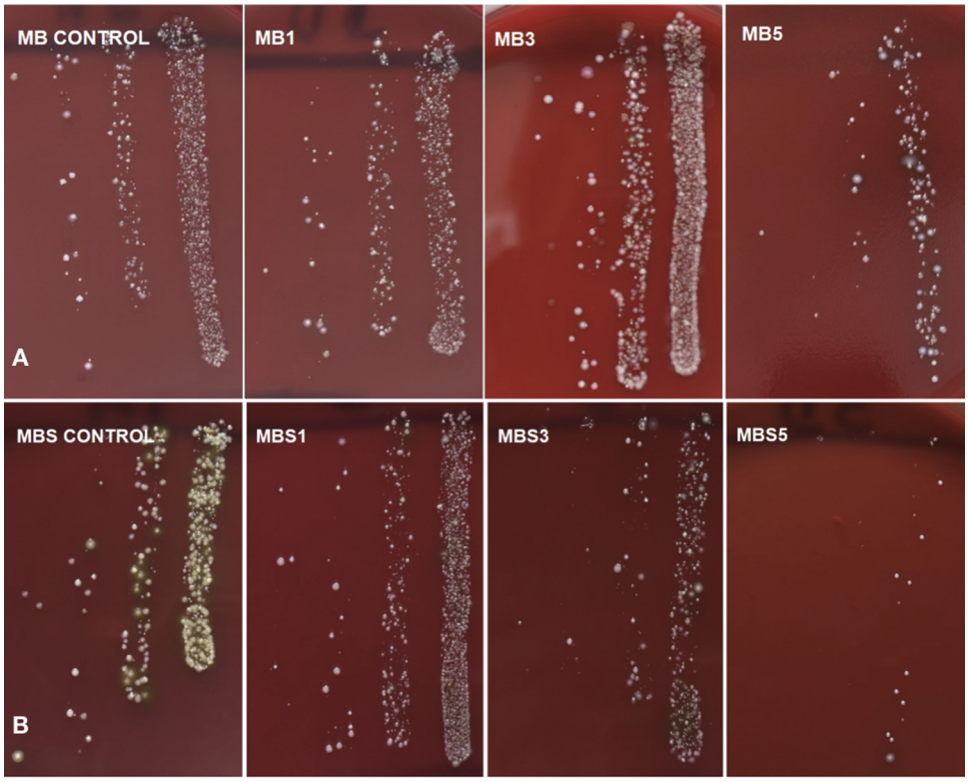

Fig. 2. Growth pattern of bacterial colonies before and after irradiation in the groups treated with 100 μM methylene blue in water solution (MB Fig. 2A) and 100 μM methylene blue + surfactant (MBS Fig. 2B) irradiated for 1, 3 and 5 min. Four tracks were stretched on the agar plate surface corresponding to the concentrations 10^{-1} (on the right) to 10^{-4} (on the left) and bacterial colonies can be observed.

between 1 and 20 min, and it produced no difference between the photodynamic effect and darktoxicity [20]. Bacterial biofilme were analysed using confocal microscopy and after 1 or 5 min of incubation, methylene blue was able to diffuse 400 μm [21]. Previous studies from our group used 1 min of dark incubation before treat bacterial biofilm using PACT [12], however, there was no comparison of another incubation time. On the other hand, studies have been using 5 min of incubation time [4,7,9,10,13] and even more [8]. The irradiation time may impact on the treatment duration in which patients spend on clinical treatment. Thus, more studies are needed to understand the roles and limits of dark incubation in a clinical environment.

MB molecules in the form of dimers exhibit less production of singlet oxygen [22], thus since MB should be less dimerized in this new composition and it could explain the better photodynamic action of the surfactant-associated MB. Spectroscopic analysis shows that there were two absorption bands in methylene blue in water (MB) relative to the dimer (approximately 610 nm) and the monomer (approximately 660 nm), while in the surfactant vehicle (MBS) only one absorption peak was observed at 660 nm, referring to the monomer. This indicates that, in this new composition, MB provided less dimerization and so had greater potential for an improved PDT/antimicrobial effect. More studies are needed to elucidate the mechanism behind these phenomena and it is also worthwhile to evaluate not only the dimer/monomer ratio but also the effective concentration of MB in the microbial biofilm.

It is concluded that methylene blue in surfactant solution promoted a bacterial reduction of 96% after 5 min of irradiation on periodontal pockets. On the other hand, aqueous MB photosensitizer did not produce an antimicrobial effect in the parameters used in this study.

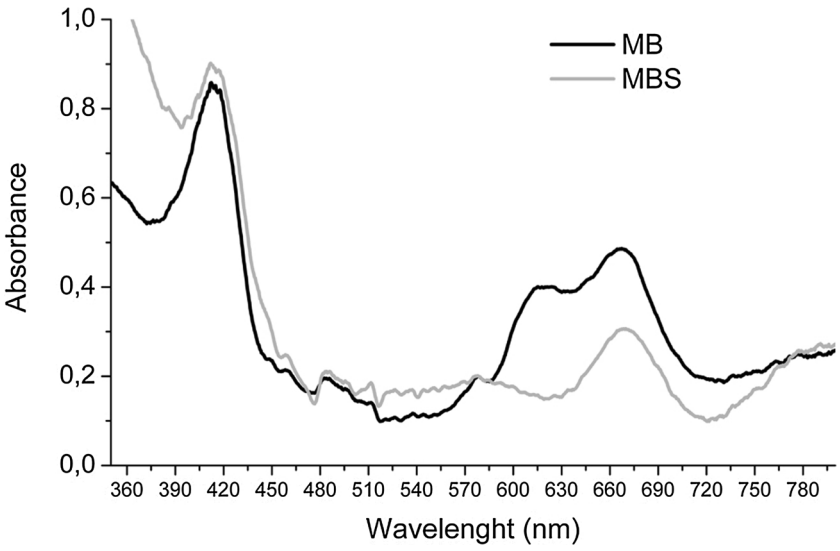

Fig. 3. Absorption spectrum of methylene blue photosensitizer in water vehicle (MB) and surfactant-associated (MBS). The X-axis represents the wavelength in nm and in the Y-axis we have the absorbance. MB showed two absorption bands, one of 600 to 640 nm (relative to the dimer) and another of 660 to 690 nm (relative to the monomer), while the MBS had an absorption band of 650 at 690 nm (relative to the monomer). The data were normalized (at wavelength 578.47 nm) and then the mean gingival spectrum was subtracted from the spectrum of the MB and MBS photosensitizers.

Conflict of interest and source of funding

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The authors have stated explicitly that there are no conflicts of interest in connection with this article.

References

[1] J. Slots, M. Ting, Systemic antibiotics in the treatment of periodontal disease, Periodontology 2000 (28) (2002) 106–176.
[2] J. Slots, Selection of antimicrobial agents in periodontal therapy, J. Periodontal Res. Suppl. 37 (2002) 389–398.
[3] A.D. Haffajee, M. Patel, S.S. Socransky, Microbiological changes associated with four different periodontal therapies for the treatment of chronic periodontitis, Oral Microbiol. Immunol. 23 (2008) 148–157.
[4] R.R. de Oliveira, H.O. Schwartz-Filho, A.B. Novaes Jr, G.P. Garlet, R.F. de Souza, M. Taba Jr, S.L. Scombatti de Souza, F.J. Ribeiro, Antimicrobial photodynamic therapy in the non-surgical treatment of aggressive periodontitis: cytokine profile in gingival crevicular fluid, preliminary results, J. Periodontol. 80 (2009) 98–105.
[5] A.A. Takasaki, A.I. Aoki, K. Mizutani, F. Schwarz, A. Sculean, C.Y. Wang, Application of antimicrobial photodynamic therapy in periodontal and peri-implant diseases, Periodontology 2000 (51) (2009) 109–140.
[6] K. Mizutani, A. Aoki, D. Coluzzi, R. Yukna, C.Y. Wang, V. Pavlic, Y. Izumi, Lasers in minimally invasive periodontal and peri-implant therapy, Periodontology 2000 (71) (2016) 185–212.
[7] S.C. Núñez, A.S. Garcez, L. Gomes, M.S. Baptista, M.S. Ribeiro, Methylene blue aggregation in the presence of human saliva, Progr. Biomed. Opt. Image 6846 (2008) 1–7.
[8] R.A. Prates, E.G. Silva, A.M. Yamada Jr, L.C. Suzuki, C.R. Paula, M.S. Ribeiro, Light parameters influence cell viability in antifungal photodynamic therapy in a fluence and rate fluence-dependent manner, Laser Phys. 19 (2009) 1038–1044.
[9] R.A. Prates, A.M. Yamada Jr, L.C. Suzuki, C.M. França, S. Cai, M.P. Mayer, A.C. Ribeiro, M.S. Ribeiro, Histomorphometric and microbiological assessment of photodynamic therapy as an adjuvant treatment for periodontitis: a short-term evaluation of inflamatory periodontal conditions and bacterial reduction in a rat model, Photomed. Laser Surg. 29 (2011) 835–844.
[10] V.F. Carvalho, P.V. Andrade, M.F. Rodrigues, M.H. Hirata, R.D. Hirata, C.M. Pannuti, G. de Micheli, M.C. Conde, Antimicrobial photodynamic effect to treat residual pockets in periodontal patients: a randomized controlled clinical trial, J. Clin. Periodontol. 42 (2015) 440–447.
[11] M. Wainwright, A. McLean, Rational design of phenothiazinium derivatives and photoantimicrobial drug discovery, Dyes Pigm. 136 (2017) 590–600.
[12] L.H. Alvarenga, R.A. Prates, T.M. Yoshimura, I.T. Kato, L.C. Suzuki, M.S. Ribeiro, L.R. Ferreira, S.A. Pereira, E.F. Martinez, E. Saba-Chujfi, Aggregatibacter actinomycetemcomitans biofilm can be inactivated by methylene blue-mediated photodynamic therapy, Photodiagnosis Photodyn. Ther. 12 (2015) 131–135.
[13] R. Polansky, M. Haas, A. Heschi, G. Wimmer, Clinical effectiveness of photodynamic therapy in the treatment of periodontitis, J. Clin. Periodontol. 36 (2009) 575–580.
[14] J. Lui, E.F. Corbet, L. Jin, Combined photodynamic and low-level laser therapies as an adjunct to nonsurgical treatment of chronic periodontitis, J. Periodontal Res. Suppl. 46 (2011) 89–96.
[15] J. Betsy, C.S. Prasanth, K.V. Baiju, J. Prasanthila, N. Subhash, Efficacy of antimicrobial photodynamic therapy in the management of chronic periodontitis: a randomized controlled clinical trial, J. Clin. Periodontol. 41 (2014) 573–581.
[16] N.C. Castro dos Santos, N.M. Andere, C.F. Araujo, A.C. de Marco, L.M. dos Santos, M.A. Jardini, M.P. Santamaria, Local adjunct effect of antimicrobial photodynamic therapy for the treatment of chronic periodontitis in type 2 diabetics: split-mouth double-blind randomized controlled clinical trial, Lasers Med. Sci. 31 (2016) 1633–1640.
[17] American Academy of Periodontology, Treatment of plaque-induced gingivitis, chronic periodontitis, and other clinical conditions, J. Clin. Periodontol. 72 (2001) 1790–1800.
[18] A. Braun, C. Dehn, F. Krause, S. Jepsen, Short-term clinical effects of adjunctive antimicrobial photodynamic therapy in periodontal treatment: a randomized clinical trial, J. Clin. Periodontol. 35 (2008) 877–884.
[19] G.N. Campos, S.P. Pimentel, F.V. Ribeiro, R.C. Casarin, F.R. Cirano, C.H. Saraceni, M.Z. Casati, The adjunctive effect of photodynamic therapy for residual pockets in single-rooted teeth: a randomized controlled clinical trial, Lasers Med. Sci. 28 (2013) 317–324.
[20] G.A. da Collina, F. Freire, T.P.D.C. Santos, N.G. Sobrinho, S. Aquino, R.A. Prates, D.F.T. da Silva, A.C.R. Tempestini Horliana, C. Pavani, Controlling methylene blue aggregation: a more efficient alternative to treat Candida albicans infections using photodynamic therapy, Photochem. Photobiol. Sci. 10 (2018) 1355–1364.
[21] N.S. Soukos, S.S. Socransky, S.E. Mulholland, S. Lee, A.G. Doukas, Photomechanical drug delivery into bacterial biofilms, Pharm. Res. 17 (2000) 405–409.
[22] M.N. Usacheva, M.C. Teichert, M.A. Biel, The role of the methylene blue and toluidine blue monomers and dimers in the photoinactivation of bacteria, J. Photochem. Photobiol. B 71 (2003) 87–98.