Photodiagnosis and Photodynamic Therapy 28 (2019) 221–225

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

Oral microbiological control by photodynamic action in orthodontic patients

Luiz G.P. Soares[a,b,c], Pedro J.L. Crugeira[a,d], Iago P.F. Nunes[a], Amanda S. Santos[a], Maria C.T. Cangussú[a,e], Paulo F. de Almeida[b,d], Antônio L.B. Pinheiro[a,f,g,*], Fernando A.L. Habib[c]

[a] Center of Biophotonics, School of Dentistry, Federal University of Bahia, Salvador, BA, CEP 40110-150, Brazil
[b] Department of Biointeraction, Institute of Health Science, Federal University of Bahia, Salvador, BA, CEP 40110-100, Brazil
[c] Center for Orthodontics and Facial Orthopedics Prof. José Édimo Soares Martins, School of Dentistry, Federal University of Bahia, Salvador, BA, CEP 40110-150, Brazil
[d] Laboratory of Biotechnology and Ecology of Micro-organisms, Institute of Health Science, Federal University of Bahia, Salvador, BA, CEP 40110-100, Brazil
[e] Epidemiology and Public Health, School of Dentistry, Federal University of Bahia, Salvador, BA, CEP 40110-150, Brazil
[f] National Institute Basic Optics and Applied to Life Science, 400, Trabalhador São-Carlense Ave, Parque Arnold Schimidt, São Carlos, SP, CEP: 13566-590, Brazil
[g] Brasil University Scientific and Techological Institute, 235, Carolina Fonseca St, Itaquera, São Paulo, SP, CEP:08230-030, Brazil

ARTICLE INFO

Keywords:
Orthodontics
Phenothiazine
Photodynamic therapy
Photoinactivation

ABSTRACT

Background: Orthodontics involves diagnosis and treatment of dental and skeletal malocclusions. Orthodontic apparatus may repair these malocclusions but may also impair oral hygiene making patients prone to develop both periodontal diseases and caries. Antimicrobial agents may be used to prevent this.To avoid increased antimicrobial resistance to available drugs, A-PDT (Antimicrobial Photodynamic Therapy) appears as a viable alternative.

Objective: This work aimed to evaluate the efficacy of A-PDT on reducing the number of colony forming units (CFU) through the use of phenothiazine compound (methylene blue + toluidine blue) as a photosensitizer, associated with red LED (λ640 ± 5ηm) irradiation in orthodontic patients.

Methodology: Twenty-one patients consented to participate in the study. Three biofilm collections were performed around the brackets and gums of the inferior central incisors; first before any intervention (Control); second after 5min of pre-irradiation and the last one immediately after AmPDT. Subsequently, a microbiological routine for microorganism growth period were performed and CFU counting after a 24h done.

Results: The data showed that the AmPDT was able to reduce CFU count around 90% when compared to Control group ($p = 0.007$) and also between the A-PDT and Photosensitizer groups ($p = 0.010$). However, there were no differences between the Control and Photosensitizer groups.

Conclusion: A-PDT associated with the use of phenothiazine compounds and red LED was able to significantly reduce the number of CFUs in orthodontic patients.

1. Introduction

Orthodontic treatment is often performed at early ages and represents an improvement of both oral function and esthetics providing a better quality of life for patients. The occlusal disharmony is also capable to cause periodontal diseases, and these conditions may worsen with the use of fixed corrective orthodontic appliances. Biofilm accumulation and oral hygiene difficulty caused by these disposits make maintenance of periodontal health problematic. Usually orthodontic patients show some degree of gingivitis during treatment [1]. Under normal conditions, oral pathogens are controlled or even eliminated by the bacteriostatic and bactericidal properties of saliva [2].

Different microbial species found in the biofilm are responsible for the development of caries, induction and maintenance of gingival inflammation, among other conditions. The inflammatory process induced by the accumulated biofilm may cause gingival pockets, loss of periodontal insertion, bone destruction, pathological migration and, finally, possible tooth loss [2].

Oral biofilm has been considered an ecosystem of continuous changes, varying in composition around different places of the mouth.

* Corresponding author at: Center of Biophotonics, Federal University of Bahia, 62, Araújo Pinho Ave, Canela, Salvador, BA, 40110-150, Brazil.
E-mail addresses: luizgpinheiro@gmail.com (L.G.P. Soares), pedrocrugeira@yahoo.com.br (P.J.L. Crugeira), iago_fortuna@hotmail.com (I.P.F. Nunes), amanda_santos@live.com (A.S. Santos), cangussu@ufba.br (M.C.T. Cangussú), pfa@ufba.br (P.F. de Almeida), albp@ufba.br (A.L.B. Pinheiro), fhabib@terra.com.br (F.A.L. Habib).

https://doi.org/10.1016/j.pdpdt.2019.08.002
Received 28 May 2019; Received in revised form 15 July 2019; Accepted 2 August 2019
Available online 05 August 2019
1572-1000/

It is estimated that more than 800 microbial species are able to colonize the oral cavity and each individual can carry around of 150–200 species [3]. The establishment and maintenance of an infectious process depends on a complex interaction between the virulence factors of pathogenic microorganisms and the defense mechanisms of the host [4].

When reports of light-absorbing and fluorescence properties of the light emerged it become clear that light can influence cellular metabolism as well as on causing damage to biological systems in specific situations. One of those phenomenon is called photodynamic effect, being described as a process in which light, after being absorbed by a certain substance accumulated in the target cells, lead to ROS and singlet oxygen production inducing damage through a phototoxic reaction, usually via oxidative damage [4–7].

Currently, photodynamic antimicrobial therapy (A-PDT) is an option for treating oral infections caused by biofilm accumulation, being able to kill a wide range of pathogens and disorganize the biofilm. Initially it was used in vitro [8–11] and later it was used in several clinical situations such as root canal infections, periodontal treatment [12–14] and against oral biofilms [15,16].

The photosensitizers (PS) choice should consider the target cell, once gram-positive bacterium has structural differences in relation to the gram-negative and consequently have different mechanisms of interaction with the drugs. The effectiveness of the PS depends on the relation between its ionic charge (positive or negative) and that presented by the cellular structures. Phenothiazine derivatives presents some characteristics that make them capable of interacting with both positive and negative molecules, so they are characterized as hydrophilic and hydrophobic and capable to be used against a wide variety of microorganisms [4].

It was hypothesized that AmPDT could help the microbial control on orthodontic patients. Therefore, the aim of this study was to evaluate the efficacy of A-PDT using a phenothiazine compound (Methylene Blue + Toluidine Blue, 1: 1, 12.5μg) as a photosensitizing agent associated with red LED irradiation (λ640 ± 5 ηm, CW, 110mW, 294s, 30J/cm^2) against microbial consortium of oral biofilms of orthodontic patients.

2. Methods

2.1. Ethical aspects and sampling

This cross-over clinical study was approved by the Ethics Committee of the School of Dentistry of the Federal University (No. 2,857,839). The sample size was calculated assuming a level of significance of 95%, 90% of power, with an estimated difference of 40% between the groups. The minimum sample size defined was defined in 19 individuals. An increase of 10% was assumed with the aim of homogenizing the groups. Thus, 21 individuals submitted to orthodontic treatment at the Center for Orthodontics and Facial Orthopedics Prof. José Édimo Soares Martins, Faculty of Dentistry, UFBA were selected. Patients that agreed to participate the study were asked to sign the Informed Consent Form (ICF).

2.2. Biofilm collection

Three biofilm collections were performed in the gingival region and around the brackets of 41 and 42 teeth with sterile swabs. The first was carried out before any procedure to determine the bacterial load present in the patient's oral cavity. The second was done after the rinsing with FS and the last after irradiation with red LED.

For AmPDT, 20ml of the photosensitizing solution (Methylene Blue + Toluidine Blue, 1: 1, 12.5μg/mL, Laboratory Fórmula, Salvador, Bahia, Brazil) was delivered to the patient and requested to do mouthwash for 5min (pre-irradiation time) to allow contact of the solution with oral biofilm. After expelling the solution, the irradiation protocol was applied with a Red LED (MMOptics, São Carlos, São Paulo, Brazil, λ640 ± 5ηm, CW, 110mW, 294ss., 30J/cm^2). All procedures followed the standards regulation for infection control as well as eye protection by using glasses with specific optical density for wavelength used.

2.3. Microbiological analysis

The collected material was transferred to test tubes containing 4ml TSB culture broth (Tryptic Soy Broth, Merck Darmstadt, Hessen, Germany). Then, the solution was homogenized by vortex device (Genie 2, Scientific Industries, New York, USA) and thereafter; successive decimal dilutions (10^{-2} to 10^{-5}) were carried out in PBS (Phosphate Buffered Saline, Merck, Darmstadt, Germany). Finally, 100μL of the inoculum was taken to Petri dishes with BHI Agar (Brain Heart Infusion, Merck Darmstadt, Hessen, Germany) in triplicate for each dilution through Drigalski loop. After incubation for 24-h in incubator (TE - 392 / I, Tecnal, Piracicaba, São Paulo, Brazil) at 37°C, colony forming units (CFU) were quantified by direct counting.

2.3.1. Gram stain

The Gram staining technique was used to characterize the bacterial consortium collected in the patients´ biofilm. This allowed identify through optical microscope (Olympus CX 40, Shinjuku, Tokyo, Japan) bacterial species into two groups: gram-positive and gram-negative.

3. Results

3.1. Gram stain

Samples from all patients, at the three collection moments, were stained using the Gram technique and it was possible to differentiate both gram-negative and gram-positive microorganisms in all of them.

3.2. CFU count

The CFU counts for each patient can be seen in Table 1. After descriptive analysis was carried out, the following measures of central tendency and variability (mean and standard deviation) were obtained to verify the effectiveness of antimicrobial photodynamic therapy in the colony counts reduction: Control group = 21514000 ± 3694869436,948,694; Photosensitizing Group = 20,111,111 ± 30,243,013; and TFD group = 7,288,121 ± (Fig.1).

ANOVA general linear model was applied to determine if the means

Table 1
CFU count for each patient in the biofilm collection.

Patient	Control	Photosensitizer	AmPDT
1	7.73E + 05	2.77E + 05	3.33E + 04
2	6.87E + 05	2.17E + 05	2.27E + 05
3	9.53E + 04	1.15E + 05	3.33E + 03
4	1.01E + 05	8.03E + 04	6.67E + 04
5	5.87E + 06	6.63E + 06	4.40E + 06
6	2.74E + 07	1.48E + 07	7.80E + 06
7	9.17E + 06	9.40E + 06	4.60E + 06
8	3.97E + 06	1.42E + 07	1.67E + 05
9	3.63E + 07	2.73E + 07	1.11E + 07
10	2.37E + 06	3.83E + 05	3.83E + 03
11	1.59E + 06	8.87E + 05	2.83E + 05
12	2.33E + 05	6.97E + 05	2.33E + 04
13	3.07E + 07	2.53E + 07	3.67E + 06
14	1.30E + 04	1.10E + 04	3.33E + 01
15	1.24E + 06	9.60E + 06	1.30E + 07
16	3.15E + 07	2.26E + 07	9.83E + 06
17	4.34E + 07	1.71E + 07	5.17E + 06
18	4.33E + 06	5.03E + 06	3.33E + 03
19	9.67E + 07	7.40E + 07	6.67E + 03
20	1.29E + 08	9.97E + 07	4.80E + 07
21	1.22E + 08	9,40E + 07	4.47E + 07

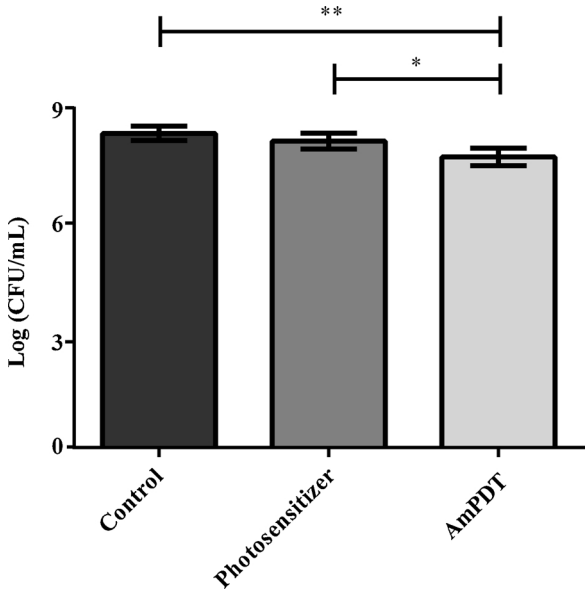

Fig. 1. Representation of the ANOVA and Tukey analyzes between the Control, Photosensitizer and AmPDT groups. Comparison of the exponential reduction of the microbial load after the addition of the photosensitizer and the treatment with AmPDT. * = $p < 0.05$, ** = $p < 0.01$.

of two or more collection moments were different. When verifying the differences between the groups in the Covariance Analysis and Multiple Regression analysis, no statistically significant difference was observed between the initial collection and after the photosensitizer application ($p = 0.787$). However, statistically significant differences were observed when AmPDT Group and Control Group ($p = 0.007$) where compared and when Photosensitizing Group and the AmPDT Group ($p = 0.010$) where compared. The model adjustment of the model was analyzed from the likelihood ratio, which obtained a variance of 4.98%, indicating that it was considered good.

4. Discussion

The development of resistant multidrug strains caused a search of new approaches in substitution to the conventional use of antibiotics whose effect is decreasing day by day [17–19]. However, it is necessary to consider the difficulty of treating bacterial biofilm-forming consortia, *in vivo,* as the main problems with conventional antimicrobial drugs are both their unique target and mode of action. Most of microorganisms can develop advanced resistance mechanisms for a single attack point [3,4,19].

Thus, the A-PDT has proved itself to be both viable and effective, obtaining satisfactory results in several clinical situations of difficult solution [8–21]. The photodynamic effect may induce damage to biomolecules that will lead to loss of biological functionality, leading to cell inactivation. The dynamics of substance distribution is one of the main advantages of A-PDT [22]. Photosensitizers act either via singlet oxygen or ROS production, which do not exhibit a specific target, reacting rapidly with a variety of substrates such as cholesterol, lipid membrane layers, amino acid residues as well as on nucleic acid bases of the DNA, particularly guanine and thymine [22–24].

These molecules may be triplet type (type I reaction) or singlet (type II reaction) and they damage microbial cells by impairing membrane lipids, proteins, nucleic acids and other cellular components. Singlet oxygen causes greater damage to molecules due to their higher reactivity (oxidation capacity), which justifies the use of photosensitizers capable of generating large amounts of this molecule when performing this therapy. Toluidine Blue produces larger amounts of this type of molecule (type I reaction-short half-life time), when compared to methylene blue, which produces free radicals such as superoxide, peroxides and hydroxyl radicals (Long Half-life time) [22–26].

For the photodynamic effect to have the highest possible yield and efficacy, the choice of the photosensitizing substance is of paramount importance. Thus, the photosensitizer should be kinetic and thermodynamically stable molecule of rapid synthesis, high yield and low cost. In addition, it shall present favorable pharmacokinetics and present low toxicity in the dark and consequently not causing cell death without activation. The application of the irradiation protocol (amount of energy delivered) as well as the concentration of the photosensitizing agent in the target cells, represent great challenges for the efficacy of the AmPDT, being necessary to adjust these parameters to achieve results like those observed *in vitro* [6–22,24–27].

The pre-irradiation time is an important factor on AmPTD. In the present study a pre-irradiation time of five minutes was used for sensitization of the biofilm. Longer times could not be practical in clinical pratice due to the difficulty to keep the drug in the mouth [16,26].

Sensitization sites and the effects of photodynamic inactivation of microbial species are different for each class of photosensitizing agents. Toluidine Blue has greater solubility in hydrophobic regions of the membrane, consequently it concentrates on a larger scale within the cell, while Methylene Blue binds with the cell membrane [10,15,23,25,28,30]. In this way, although its action depends on the type of target cell, in general the first causes damage to the nucleic acids and the second acts on the cell membrane, causing increased permeability [23,25].

Another important factor for the efficacy of the AmPDT is the choice of the light source as well as the wavelength to be used for the photoactivation. Typically, these wavelengths are within the visible and near-infrared spectrum (λ600-900nm) [26]. Wavelengths above λ850nm due to their low energetic levels, reduces the efficacy of the photosensitizer. The choice of the wavelength should be the closest to absorption peak of the photosensitizer [6,7,25]. On the present study the red LED device emitted at the λ 640 ± 5nm [16,23,27], placed between the absorption peaks of Methylene Blue (λ664nm) and Toluidine Blue (λ626nm) [25]. The use of inadequate wavelength will reduce or even not photoactivate the photosensitizer thus making the AmPDT not efficacious.

This study showed that AmPDT was effective in significantly ($p = 0.007$) reducing the number of microorganisms as seen on the UFC counts in patients undergoing active orthodontic treatment. The direct count of colonies has the advantage of observing viable cells, while other methods do not distinguish between dead and living microorganisms. The percentage of reduction observed in the present study was ˜90%. Similar results have been observed in other studies using phenothiazine derivatives as photosensitizing agents, but the rate of microorganism death was not superior to 70% [8–16,23,28–30]. That could be explained by the combination of Methylene Blue and Toluidine Blue (1:1) used on this study as their present different sites of accumulation in the cell being the compound able to sensitize a wide range of cells types. The combination of the two compounds in orthodontics was never reported.

The irradiation protocol applied in this study was chosen based on an *in vitro* study using red LED associated with phenothiazine derivatives, Methylene blue and Toluidine blue, where the concentration of 12.5μg/ml achieved the highest lethality when combined with the energy density of 12J/cm^2 [10]. On this study, the energy density was adjusted to 30J/cm^2, due to large energy loss being observed when scanning process is used. This parameter represents an important limitation for clinical trials due to the large variation found in several studies using other photosensitizers [10,29,30].

As Gram-positive and Gram-negative bacteria present differences in relation to the susceptibility to AmPDT [15,16,18] it was necessary the identification of the microorganisms present in the samples, so Gram staining was performed, and both Gram-positive and Gram-negative microorganisms were observed. The results found showed that the use

of the photosensitizer at low-concentration (12.5μg/ml) was efficacious on killing most of these microorganisms after activation by LED light.

A significant reduction in the number of bacteria (˜90%) was detected when compared to the initial load of microorganism observed in each patient. On the other hand, the sole use of the photosensitizer did not cause cell death in a statistically significant way (P = 0.787) [15–17,22–26]. These findings are in agreement with results observed in a previous study [31] which showed, by SEM, that the AmPDT causes morphological alterations in the biofilm formed by both Gram-positive and Gram-negative bacteria leading to greater reduction in the number of microorganisms and this, when associated to the disorganization of the extracellular matrix of the biofilm (antimicrobial resistance factor), the bacteria that were not killed by the AmPDT, were exposed to conventional antibiotics and/or oral fluids as isolated cells and no longer as biofilm, facilitating its inactivation.

It should be noted that the protocol proposed in this study, different from conventional pharmacologic protocols using antibiotics, is unable of producing local and/or systemic effects of microbial resistance. Therefore, it can be repeated safely during clinical care, increasing the efficacy of therapy [31,32]. Another important factor is the synergism of the immune system acting against the infection. In addition, the use of antibiotics may represent a good alternative associated with AmPDT, because of it causes the disorganization of biofilms impairing some mechanisms of resistance and thus leaving the remaining microorganisms more susceptible to antimicrobials [9,11,15,16,24,30,31].

Thus, it is crucial to reinforce oral hygiene instructions through routine follow-up in patients undergoing fixed and removable orthodontic treatment. The small amount of studies involving orthodontic patients and AmPDT, as reported in a systematic review work recently published [33], it is difficult to prove the efficacy of AmPDT to control the oral microbiota of these patients. However, it is believed that the AmPTD protocol used in this study, in conjunction with maintenance of oral hygiene and routine dental checkups, is reliable method to minimize the risk of periodontal diseases in these patients.

Finally, new studies should be conducted in the search for even more effective AmPDT protocol to achieve even higher percentages of microbial death in the control of oral biofilms, avoiding bacterial resistance and the occurrence of infections in patients undergoing orthodontic treatment.

5. Conclusion

It was concluded that AmPDT associated with the use of phenothiazine compound and red LED was able to significantly reduce the number of CFUs in orthodontic patients.

Compliance with ethical standards

The authors of the present investigation state that they have no competing conflict of interest to declare. Author (ALBP) received Productivity Fellowship from the Brazilian National Council for Scientific and Technological Development (CNPq) (No.304279/2018-8). The author (PJLG) received a PhD grant from the Bahia State Research Support Foundation) FAPESB (BOL0777/2016)and authors (IPFN and ASS) received a MSci grant from the Bahia State Research Support Foundation – FAPESB.

Declaration of Competing Interest

None.

References

[1] S. Re, G. Corrente, R. Abundo, Cardaropoli d orthodontic treatment in periodontally compromised patients: 12-Year report, Int. J. Periodontics Restorative Dent. 20 (2) (2000) 30–39, https://doi.org/10.1111/j.1600-051X.1981.tb02039.x.

[2] M.M. Ong, W. Hom-Lay, Periodontic and orthodontic treatment in adults, Am. J. Orthod. Dentofacial Orthop. 122 (4) (2002) 420–428 PMID: 12411890.

[3] R.P. Darveau, A. Tanner, Page RC the microbial challenge in periodontitis, Periodontology 14 (1997) 12–32 PMID: 9567964.

[4] P. Meisel, T. Kocher, Photodynamic therapy for periodontal diseases: State of the art, J. Photochem. Photobiol. B: Biol. 79 (2005) 159–170, https://doi.org/10.1016/j.jphotobiol.2004.11.023.

[5] J.D. Spikes, R. Livingston, Biological systems, in: L. Augenstein, R. Mason, M. Zelle (Eds.), The Molecular Biology of Photodynamic Action: Sensitized Photo-Autooxidations, Advances in Radiation Biology, 3 edn., Academic Press, New York, 1967, pp. 29–121, , https://doi.org/10.15171/jims.2017.25.

[6] Blum HF roots of photobiology, Photochem. Photobiol. 26 (1977) 447–448.

[7] T.I. Karu, L.V. Pyatibrat, S.V. Moskvin, Elementary processes in cells after light absorption do not depend on the degree of polarization: implications for the mechanisms of laser phototherapy, Photomed. Laser Surg. 26 (2) (2008) 77–82, https://doi.org/10.1089/pho.2007.2134.

[8] M.M. Gois, C. Kurachi, E.J.B. Santana, E.G.O. Mima, D.M.P. Spolidorio, J.E.P. Pelino, Bagnato VS Susceptibility of Staphylococcus aureus to porphyrin mediated photodynamic antimicrobial chemotherapy: an in vitro study, Lasers Med. Sci. 25 (2009) 391–395, https://doi.org/10.1007/s10103-009-0705-0.

[9] V. Bagnato, V. Panhóca, M. Geralde, T. Corrêa, M. Carvalho, C. Souza, Enhancement of the photodynamic therapy effect on Streptococcus mutans biofilm, J Phys Sci Appl. 4 (2014) 107–114, https://doi.org/10.1117/12.2286980.

[10] O. SCPS, J.S.C. Monteiro, G.M. Pires-Santos, F.J.P. Sampaio, A.P. Soares, L.G.P. Soares, A.L.B. Pinheiro, LED antimicrobial photodynamic therapy with phenothiazinium dye against Staphylococcus aureus: An in vitro study, J. Photochem. Photobiol. B 175 (2017) 46–50, https://doi.org/10.3906/sag-1803-44.

[11] L. Beytollahi, M. Pourhajibagher, N. Chiniforush, R. Ghorbanzadeh, R. Raoofian, B. Pourakbari, Bahador A the efficacy of photodynamic and photothermal therapy on biofilm formation of Streptococcus mutans: an in vitro study, Photodiagnosis Photodyn. Ther. 17 (2017) 56–60, https://doi.org/10.1016/j.pdpdt.2016.10.006.1572-1000.

[12] A.S. Garcez, S.C. Nunez, M.R. Hamblin, Ribeiro MS Antimicrobial effects of photodynamic therapy on patients with necrotic pulps and periapical lesion, J. Endod. 34 (2008) 138–142, https://doi.org/10.1016/j.joen.2007.10.020.

[13] A.S. Garcez, M.S. Ribeiro, G.P. Tegos, S.C. Nunez, A.O. Jorge, Hamblin MR Antimicrobial photodynamic therapy combined with conventional endodontic treatment to eliminate root canal biofilm infection, Lasers Surg. Med. 39 (2007) 59–66, https://doi.org/10.1002/lsm.20415.

[14] F. Foschi, C.R. Fontana, K. Ruggiero, R. Riahi, A. Vera, A.G. Doukas, T.C. Pagonis, R. Kent, P.P. Stashenko, Soukos NS Photodynamic inactivation of Enterococcus faecalis in dental root canals in vitro, Lasers Surg. Med. 39 (2007) 782–787, https://doi.org/10.1002/lsm.20579.

[15] L. Misba, S. Zaidi, A.U. Khan, A comparison of antibacterial and antibiofilm efficacy of phenothiazinium dyes between Gram positive and Gram-negative bacterial biofilm, Photodiagnosis Photodyn. Ther. 18 (2017) 24–33 PMID: 30405728.

[16] A.C. Fumes, P.D.S. Telles, S.A.M. Corona, Borsatto MC Effect of AmPDT on Streptococcus mutans and Candida albicans present in the dental biofilm: systematic review, Photodiagnosis Photodyn. Ther. 21 (2018) 363–366, https://doi.org/10.1007/s10103-017-2336-1.

[17] C.M. Cassidy, R.F. Donnelly, J.S. Elborn, N.D. Magee, Tunney MM Photodynamic antimicrobial chemotherapy (AMPDT) in combination with antibiotics for treatment of Burkholderia cepacia complex infection, J. Photochem. Photobiol. B 106 (2012) 95–100, https://doi.org/10.1016/j.jphotobiol.2011.10.010.

[18] H.M. Tang, M.R. Hamblin, C.M. Yow, A comparative in vitro photoinactivation study of clinical isolates of multidrug-resistant pathogens, J. Infect. Chemother. 13 (2007) 87–91, https://doi.org/10.1007/s10156-006-0501-8.

[19] M. Ferres, A.D. Haffajee, K. Allard, S. Som, J.M. Goodson, S.S. Socransky, Antibiotic resistance of subgingival species during and after antibiotic therapy, J. Clin. Periodontol. 29 (2002) 724–735 PMID: 12390569.

[20] M.B. Fonseca, P.O. Junior, R.C. Pallota, H.F. Filho, O.V. Denardin, A. Rapoport, R.A. Dedivitis, J.F. Veronezi, W.J. Genovese, A.L. Ricardo, Photodynamic therapy for root canals infected with Enterococcus faecalis, Photomed. Laser Surg. 26 (2008) 209–213, https://doi.org/10.1089/pho.2007.2124.

[21] A. Silva Garcez, S.C. Nunez, L. JL, A.O. Jorge, M.S. Ribeiro, Efficiency of NaOCl and laser-assisted photosensitization on the reduction of Enterococcus faecalis in vitro, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 102 (2006) e93–e98, https://doi.org/10.1016/j.tripleo.2006.02.015.

[22] D.A. Caminos, M.B. Spesia, E.N. Durantini, Photodynamic inactivation of *Escherichia coli* by novel meso-substitstuted porphyrins by 4-3-N, N,N-trimethyl-ammonium-propoxy-phenyl and 4-trifluoromethylphenil groups, Photochem. Photobiol. Sci. 5 (2006) 56–65, https://doi.org/10.1039/b513511g.

[23] L. Misba, S. Kulshrestha, A.U. Khan, Antibiofilm action of a toluidine blue O-silver nanoparticle conjugate on Streptococcus mutans: a mechanism of type I photodynamic therapy, Biofouling 32 (2016) 313–328, https://doi.org/10.1080/08927014.2016.1141899.

[24] L. Misba, S. Zaidi, A.U. Khan, Efficacy of photodynamic therapy against Streptococcus mutans biofilm: role of singlet oxygen, J. Photochem. Photobiol. B, Biol. 183 (2018) 16–21, https://doi.org/10.1016/j.jphotobiol.2018.04.024.

[25] T.N. Demidova, Hamblin MR Effect of cell-photosensitizer binding and cell density on microbial photoinactivation, Antimicrob. Agents Chemother. 49 (2005) 2329–2335, https://doi.org/10.1128/AAC.49.6.2329-2335.2005.

[26] M. Wainwright, Photodynamic antimicrobial chemotherapy (PACT), J. Antimicrob. Chemother. 42 (1998) 13–28, https://doi.org/10.1093/jac/42.1.13.

[27] L. Brancaleon, H. Moseley, Laser e non-laser light sources for Photodynamic Therapy, Lasers Med. Sci. 1 (2002) 173–186, https://doi.org/10.1007/

s101030200027.
[28] G. Sridharan, A.A. Shankar, Toluidine blue: a review of its chemistry and clinical utility, J. Oral Maxillofac. Pathol. 16 (2) (2012) 251–255, https://doi.org/10.4103/0973-029X.99081.
[29] T.C. Huang, C.J. Chen, S.J. Ding, C.C. Chen, Antimicrobial efficacy of methylene blue-mediated photodynamic therapy on titanium alloy surfaces in vitro, Photodiagnosis Photodyn. Ther. 25 (2019) 7–16, https://doi.org/10.1016/j.pdpdt.2018.11.008.
[30] M.A. Nemezio, S.S.S. Farias, M.C. Borsattoa, C.P. Aires, Coronac SAM Effect of methylene blue-induced photodynamic therapy on a Streptococcus mutans biofilm model, Photodiagnosis Photodyn. Ther. 20 (2017) 234–237, https://doi.org/10.1016/j.pdpdt.2017.10.025.
[31] M.R. Ronqui, C. TMSFA, L.M. Freitas, E.T. Miranda, C.R. Fontana, Synergistic antimicrobial effect of photodynamic therapy and ciprofloxacin, J. Photochem. Photobiol. B, Biol. 158 (2016) 122–129, https://doi.org/10.1016/j.jphotobiol.2016.02.036.
[32] M.A. Paschoal, C.M.Z. Moura, F. Jeremias, J.F. Souza, V.S. Bagnato, J.S.M. Giusti, L. Santos-Pinto, Longitudinal effect of curcumin-photodynamic antimicrobial chemotherapy in adolescents during fixed orthodontic treatment: a single-blind randomized clinical trial study, Lasers Med. Sci. 30 (2015) 2059–2065, https://doi.org/10.1007/s10103-014-1700-7.
[33] D. Al-Shammery, D. Michelogiannakis, Z.U. Ahmed, H.B. Ahmed, P.E. Rossouw, G.E. Romanos, F. Javed, Scope of antimicrobial photodynamic therapy in Orthodontics and related research: a review, Photodiagnosis Photodyn. Ther. 25 (2019) 456–459, https://doi.org/10.1016/j.pdpdt.2019.02.011.