Published online: 2019-09-25

Original Article

Comparative evaluation of photodynamic therapy using LASER or light emitting diode on cariogenic bacteria: An *in vitro* study

Liliana Guimarães Oliveira Ricatto[1], Luis Augusto Lopato Conrado[2], Cecilia Pedroso Turssi[1], Fabiana Mantovani Gomes França[1], Roberta Tarkany Basting[1], Flávia Lucisano Botelho Amaral[1]

Correspondence: Dr. Flávia Lucisano Botelho Amaral
Email: flbamaral@gmail.com

[1]Department of Restorative Dentistry, São Leopoldo Mandic Institute and Research Center, Campinas, São Paulo, Brazil,
[2]Department of Biomedical Engineering, Unicastelo, São José dos Campos, São Paulo, Brazil

ABSTRACT

Objective: The aim of this study was to evaluate *in vitro* the effect of photodynamic therapy (PDT) using LASER or light emitting diode (LED) on cariogenic bacteria (*Streptococcus mutans* [SM] *and Lactobacillus casei* [LC]) in bovine dentin. **Materials and Methods:** Twenty five fragments of dentin were contaminated with SM and LC strands and divided into five experimental groups according to the therapy they received (n = 5): C – control (no treatment), SCLED – no dye/LED application (94 J/cm^2), SCLASER – no dye/LASER application (94 J/cm^2), CCLED – dye/LED application (94 J/cm^2) and CCLASER – dye/LASER application (94 J/cm^2). The dye used was methylene blue at 10 mM. Dentin scrapes were harvested from each fragment and prepared for counts of colony forming units (CFU)/mL. The data were analyzed using Kruskal–Wallis, followed by Student–Newman–Keuls (α =0.05). **Results:** Regarding SM, groups CCLASER and CCLED showed a significant reduction in CFU/mL, which was statistically superior to the SCLASER, SCLED and C groups. Regarding LC, the groups CCLASER and CCLED caused a significant reduction in CFU/mL when compared with SCLASER, which showed intermediate values. SCLED and C had a lesser effect on reducing CFU/mL, where the former showed values similar to those of SCLASER. **Conclusions:** In conclusion, PDT combined with LASER or LED and methylene blue had a significant antimicrobial effect on cariogenic bacteria in the dentin.

Key words: *Lactobacillus casei*, photodynamic therapy, semi-conductor lasers, *Streptococcus mutans*

INTRODUCTION

Photodynamic therapy, also known as PDT, combines the use of nontoxic photosensitizing dyes with a visible light of the appropriate wavelength.[1] Photosensitive substances absorb energy from light and become activated, producing highly reactive oxygen species, which results in cell damage and cell death.[2] PDT has been used in many situations, including cutaneous lesions, burns, skin cancer, leishmaniosis, etc.[3-7]

In dentistry, PDT has been investigated for the treatment of oral infections, such as caries, pulpitis, periodontal disease, mucosal and endodontic infections.[8-13] In the specific case of caries, PDT has shown promising results in inactivating cariogenic microorganisms[14] in the biofilm[9,15,16] or carious dentine.[17,18] Due to PDT-induced decontamination, one may speculate that post-PDT caries removal may be performed conservatively.[19] In addition, PDT can be regarded as selective that is, neither the photosensitizer nor the light shows bactericidal properties when used separately.

How to cite this article: Ricatto LG, Conrado LA, Turssi CP, Franca FM, Basting RT, Amaral FL. Comparative evaluation of photodynamic therapy using LASER or light emitting diode on cariogenic bacteria: An *in vitro* study. Eur J Dent 2014;8:509-14.

Copyright © 2014 Dental Investigations Society. **DOI:** 10.4103/1305-7456.143634

Consequently, antimicrobial activity is achieved by combining the dye with light simultaneously, thus not disturbing the flora at distant sites.[20,21] Another important aspect of this approach is its atraumatic nature, which could be indicated especially for patients with special needs and children.[1,16]

There are several types of photosensitizing agents, light sources and protocols, which are currently been investigated in terms of compositions, light-absorbing properties, etc. Such vast array of options tends to hinder the establishment of defined parameters for the use of PDT to eliminate cariogenic bacteria. Light source devices can be equipped with a halogen light,[11,22,23] light emitting diode (LED),[1,9,17,18,24] laser diode[15,19,25] and HeNe.[9]

Light emitting diode devices have the advantage of their applicability on PDT, since when compared with low-intensity lasers, they also produce light at a specific wavelength, however, within a wider electromagnetic spectrum range and at a lower cost, which makes it accessible.[1,9,16] Nevertheless, despite numerous reports on either technique, comparisons between the two light sources are scarce in the literature. Therefore, the aim of this study was to compare the antimicrobial effect of PDT using diode laser and LED combined with methylene blue dye.

MATERIALS AND METHODS

Ethical issues

This study was approved by the Ethics Committee in Animal Research (CEUA) of the Sao Leopoldo Mandic Dental School and Postgraduate Research Center, registration number 2012/0378.

Study design

The method used in this study was based on Lima *et al*.[17] This *in vitro* investigation was composed of 25 experimental units randomly divided into five experimental groups, according to the therapy used on bovine dentin (n = 5): C – control (no treatment); SCLED – LED application (94 J/cm^2) without a dye; SCLASER – LASER application (94 J/cm^2) without a dye; CCLED – LED application (94 J/cm^2) with the dye; CCLASER – LED application (94 J/cm^2) with the dye.

The quantitative response variable to treatment was the count of colony forming units (CFU)/mL.

Preparation of the dentin specimens

Fragments of dentin measuring 5 × 5 × 2 mm were prepared from bovine incisors using a flexible high concentration diamond disk (104 mm diameter × 0.3 mm thick), series 15 HC (Buehler Ltd., Lake Bluff, Illinois, USA) mounted on a precision saw (Isomet 1000 Precision Diamond Saw, Buehler Ltd., Lake Bluff, Illinois, USA). Two sections were carried out 5 mm apart to standardize the fragments. All 50 fragments were polished under water cooling (Politriz Aropol 2V, Arotec, São Paulo, SP, Brazil) using a sequential grains of aluminum oxide sandpaper (Imperial Wetordry, 3M, Sumaré, SP, Brazil) – number 400, number 600 and number 1200 - so that the final depth of the specimen was standardized at 2 mm.

Protocol of contamination

Activation of the strands of Streptococcus mutans and Lactobacillus casei

Streptococcus mutans (SM) and *Lactobacillus casei* (LC) strands were cultured in brain heart infusion (BHI) for 24 h and 48 h at 36 ± 1°C, following activation. The microorganisms were then gram tested, suspended in 2 mL sterile saline to obtain quantities of viable colonies.

Strand activation was performed according to the following procedure:

- Hydration of the primary culture: Standard strands were purchased from the American Type Culture Collection (ATCC), with a certificate of origin obtained from the André Tosello Foundation. From the lyophilized ATCC or the collection cultures, stationary-phase cultures were prepared following the instructions provided with the certificate: Disinfection of the upper aspect of the ampoule, identification of the mid-point of the cotton plug using a file or a diamond-tip pen, removal of the upper aspect of the broken ampoule using a sterile forceps, disposal of the fragments in disinfectant solution. Using a sterile Pasteur pipette, 1 mL of the recommended medium was added to the culture, the suspension was gently homogenized, left to rest for a few minutes and transferred into tubes containing the specific medium for each microorganism. The samples were then incubated for the recommended amount of time for each microorganism. Whenever growth was not detected, the suspensions were left to incubate for up to twice the recommended time, before assuming that the culture was not viable. The primary culture was preserved so as to maintain its morphological, physiological and genetic features, as well as its viability during the storage period
- Preparation of the secondary culture: A tube was removed from the frozen primary culture

stock for reactivation. It was defrosted in ice and subsequently transferred to a test tube containing 5 mL of growth medium (specific for each microorganism), which was incubated and activated.

Bacterial inoculation

The teeth were autoclaved for 15 min at 121°C, dried using absorbent paper and divided into two groups (*n* = 5), according to the type of bacteria to be inoculated:

- The teeth were placed in receptacles containing 1.5 × 108 CFU/mL of SM suspension (BHI medium)
- The remaining teeth were placed in receptacles containing 10 µL of 1.4 × 107 CFU/mL of LC suspension (MRS medium), followed by incubation at 37°C and CO_2.

Photosensitizing agent and light

The photosensitizing agent used was methylene blue at 10 mM, filtered through a sterile 20-µm mesh membrane filter and stored in the dark. For the experiments, 100 µM (100 × dilution) aliquots were prepared using distilled water (Batista, accepted in 2011).

Irradiation protocol

According to the method proposed by Lima *et al*.[17] 94 J/cm² of energy was the most effective parameter in PDT for carious dentin. Therefore, the calculation of the irradiation needed to achieve 94 J/cm² for LASER and LED was performed using the following formula:

$$DE\,(J/cm^2) = \frac{P\,(W) \times T\,(S)}{A}$$

Where: DE = Energy density; *P* = Power; T = Irradiation time, A = Area of the device tip.

Table 1 shows the data used to calculate irradiation time. Irradiation was carried out from a distance of 2 cm.

Microbiological analysis

Dentin samples were collected from the fragments using a microbrush embedded in saline solution. The material collected was placed in Eppendorf tubes containing 1000 µL of BHI. Two dilutions were made from the original suspension to place the samples in. Ten microliter from each dilution was inoculated into a specific medium in duplicates. SM and LC growth was determined by counts of colony forming units in viable plates of medium:

- SM: MSB agar (Mitis salivarius agar);
- LC: MRS agar (de Man, Rogosa, Sharpe).

Statistical analysis

In order to check for data distribution error, exploratory analysis was performed on SPSS 20.0 (IBM, Chicago, IL, EUA), which demonstrated that the data did not conform to a normal distribution, thus not fulfilling the requirements for analysis of variance. No transformation was possible to adjust the Gauss curve, consequently; nonparametric tests were selected, namely Kruskal–Wallis for two variables (CFU/mL of SM and of LC), followed by Student–Newman–Keuls for multiple comparisons. The significance level adopted was 5%.

RESULTS

Kruskal–Wallis test demonstrated no significant difference ($P < 0.05$) between the SM groups in terms of CFU/mL [Table 2]. The Student–Newman–Keuls test revealed that the use of methylene blue alone, that is, without LED or LASER, led to a significant reduction in CFU/mL of SM when compared to the control group.

Kruskal–Wallis test demonstrated a significant difference between the LC groups ($P < 0.05$) [Table 3]. The Student–Newman–Keuls test revealed that the use of methylene blue alone, that is, without LED or LASER, significantly reduced the CFU/mL of LC when compared with the control and the remaining

Table 1: Data relating to the equipment tested and the time calculated for irradiation

Equipment and data techniques	Wavelength (nm)	Potential (mW)	Cross-sectional area of the tip (cm²)	Time calculated, based on the energy density of ~94 J/cm² (s)
LED Bios therapy II Bios medical equipment (Bios Equipamentos Médicos-São José dos Campos, SP, Brazil)	630±20	300 (0.3 W)	0.3848	120
LASER Whitening lase (DMC Equipamentos Ltd., São Carlos, SP, Brazil)	660	30 (0.03 W)	0.028	88

LED: Light emitting diode

Table 2: Median, minimum and maximum values of CFU/mL for the group contaminated with *S. mutans*

Group	Median	Minimum value	Maximum value
Control	650[b]	13,850	150
No dye+LED	550[b]	1050	50
No dye+LASER	900[b]	23,700	50
Dye+LED	0[a]	0	0.5
Dye+LASER	2[a]	0	7.5

Distinct letters to the side of the median indicate a significant difference between the experimental groups. Significance level 5%. LED: Light emitting diode, CFU: Colony forming unit, *S. mutans: Streptococcus mutans*

Table 3: Median, minimum and maximum values for the CFU/mL for the group contaminated with *L. casei*

Group	Median	Minimum value	Maximum value
Control	12,050[c]	26,550	5100
No dye+LED	1850[b,c]	5500	100
No dye+LASER	1300[b]	2300	50
Dye+LED	3.5[a]	38	0
Dye+LASER	9a	35.5	0

Distinct letters to the side of the median indicate a significant difference between the experimental groups. Significance level 5%. LED: Light emitting diode, CFU: Colony forming unit, *L. casei: Lactobacillus casei*

groups. The control group had the highest CFU/mL of LC, which was statistically similar to the no dye/LED (SCLED) group. The group where LASER alone was used without the dye showed intermediate CFU/mL values, which were significantly lower than the control group and similar to the group where LED was used alone without the dye (SCLED).

DISCUSSION

Photodynamic therapy is characterized by the use of light to activate a photosensitive agent in the presence of oxygen, resulting in reactive species *in situ*, the oxygen singlet, which can induce cell death.[26] It has been considered a promising alternative to the classic treatment of dental caries. *In vitro*[9,11,16,23-25] and *in situ*[16,17] studies have demonstrated the sensitivity of bacteria such as SM and LC to this treatment. Although studies have demonstrated that the combination of light and a dye is an effective approach to inactivate microbials, some variables still influence the outcome, such as the nature and concentration of the dye, the cariogenic microorganism species, the light source, as well as the duration and dose of exposure to light. The effect of these factors on the success of PDT has been the target of much investigation that seeks to make it a feasible method to control various infections in clinical practice.[14,15,17,23-25,27,28]

The results of this study have demonstrated a significant reduction in SM and LC bacteria when the dentin was treated with methylene blue and irradiated either with a laser diode or LED. These results corroborate those by Zanin *et al*.,[9] who evaluated the antimicrobial effect of toluidine blue (TBO) combined with HeNe and LED on biofilm-organized SM. In their study, a 99% reduction in microorganisms was achieved with combined HeNe laser and LED. Similarly, Paulino *et al*.[22] stated that any source of light with appropriate spectral characteristic could be used in PDT, such as tungsten or halogen bulbs, laser or LED.

This study was based on the method by Lima *et al*.,[17] which evaluated two energy densities and demonstrated that 94 J/cm^2 was the most efficient at significantly reducing bacterial count. They evaluated two energy levels (47 and 94 J/cm^2) and found a significant reduction in the bacterial count for SM (3.08 and 4.16, respectively), whilst for LC counts, 3.24 and 4.66, respectively. The control, which was treated with 94 J/cm^2, was also effective in eliminating all oral bacteria studied. Similarly, Baptista *et al*.[1] reported an *in vivo* study, in which they created dental carious lesions in an animal model and evaluated the reduction in microaerophilic bacterial count using PDT combined with methylene blue at 100 μM and red LED ($\lambda = 640 \pm 30$ nm), at 240 mW and 86 J/cm^2 for 3 min.

It is likely that the statistical similarity between the LED and LASER groups when combined with methylene blue may have been due to the energy level used, which was the same for both (approximately 94 J/cm^2). In order to achieve that level, 88 s of laser diode and 120 of LED were used, since the devices are different in terms of tip size and power. Such values are important, since the use of laser diode reduces the amount of bacteria effectively with reduction of the clinical time needed for bacterial inactivation. Nevertheless, the cost of laser diode devices is higher than that of LED, which would make the latter more popular.

This study also showed that when light was applied without a photosensitizer (groups SCLED and SCLASER), bacterial reduction was significantly lower than in the groups for which both light and photosensitizer were combined (groups CCLED and CCLASER). This occurred because oral bacteria generally do not absorb visible light at a certain wavelength range as observed by Zanin *et al*.[9]

Other photosensitizers have been used in research, such as erythrosine,[11,23,29] TBO[9,16,18,29] and methylene blue.[19,29,30] The photosensitizer methylene blue belongs to the phenothiazine family, for which the light absorption range oscillates between 610 and 670 nm.[20] Longo and Azevedo[31] also demonstrated that PDT combined with methylene blue using laser significantly decreased bacterial load in samples of carious dentin. According to Rolim *et al*.[29] the use of methylene blue promotes the formation of oxygen singlet, which is the reactive species responsible for bacterial death, at a rate 1.3 times higher than TBO.

In this study, fragments of dentin were irradiated directly with the light source (LED). Further *in vivo* studies are needed, since cariogenic oral bacteria are present in cavities of different depths. Zanin *et al*.[10] have reported that the bacteria present in tooth decay may be less susceptible to PDT due to the limited penetration of the photosensitizing agent or even the difficulty of light propagation through dentin. Teixeira *et al*.[16] reported that the higher thickness of the cariogenic biofilm *in situ* may have been responsible for reduced effectiveness of PDT. Guglielmi Cde *et al*.[19] however, obtained positive results in an *in vivo* study in deep caries. Similarly to the present study, most *in vitro* investigations evaluate caries-related bacteria isolatedly that is, not growing together within the biofilm. It is known that in a natural oral ecosystem, SM, LC and other bacteria can interact in such a way as to influence each other's metabolism.[32] It seems that biofilm-grown bacteria are less susceptible to antimicrobial agents than their planktonic counterparts.[33] Therefore, *in vitro* results must be interpreted with caution. It is paramount that results obtained from *in vitro* studies are confirmed clinically and hence that this therapy can become an alternative to conventional treatment of carious lesions.

CONCLUSION

Regardless of the light source used, either LED or LASER, PDT was effective in reducing SM and LC in dentin.

REFERENCES

1. Baptista A, Kato IT, Prates RA, Suzuki LC, Raele MP, Freitas AZ, *et al.* Antimicrobial photodynamic therapy as a strategy to arrest enamel demineralization: A short-term study on incipient caries in a rat model. Photochem Photobiol 2012;88:584-9.
2. Wilson BC, Patterson MS. The physics, biophysics and technology of photodynamic therapy. Phys Med Biol 2008;53:R61-109.
3. Biel MA. Photodynamic therapy in head and neck cancer. Curr Oncol Rep 2002;4:87-96.
4. Plaetzer K, Krammer B, Berlanda J, Berr F, Kiesslich T. Photophysics and photochemistry of photodynamic therapy: Fundamental aspects. Lasers Med Sci 2009;24:259-68.
5. Hamblin MR, Hasan T. Photodynamic therapy: A new antimicrobial approach to infectious disease? Photochem Photobiol Sci 2004;3:436-50.
6. Kubler AC. Photodynamic therapy. Med Laser Appl 2005;20:37-45.
7. Konopka K, Goslinski T. Photodynamic therapy in dentistry. J Dent Res 2007;86:694-707.
8. Komerik N, MacRobert AJ. Photodynamic therapy as an alternative antimicrobial modality for oral infections. J Environ Pathol Toxicol Oncol 2006;25:487-504.
9. Zanin IC, Gonçalves RB, Junior AB, Hope CK, Pratten J. Susceptibility of *Streptococcus mutans* biofilms to photodynamic therapy: An *in vitro* study. J Antimicrob Chemother 2005;56:324-30.
10. Zanin IC, Lobo MM, Rodrigues LK, Pimenta LA, Höfling JF, Gonçalves RB. Photosensitization of *in vitro* biofilms by toluidine blue O combined with a light-emitting diode. Eur J Oral Sci 2006;114:64-9.
11. Wood S, Metcalf D, Devine D, Robinson C. Erythrosine is a potential photosensitizer for the photodynamic therapy of oral plaque biofilms. J Antimicrob Chemother 2006;57:680-4.
12. de Oliveira BP, Aguiar CM, Camara AC. Photodynamic therapy in combating the causative microorganisms from endodontic infections. Eur J Dent 2014;8:424-30.
13. Yildirim C, Karaarslan ES, Ozsevik S, Zer Y, Sari T, Usumez A. Antimicrobial efficiency of photodynamic therapy with different irradiation durations. Eur J Dent 2013;7:469-73.
14. Nagata JY, Hioka N, Kimura E, Batistela VR, Terada RS, Graciano AX, *et al*. Antibacterial photodynamic therapy for dental caries: Evaluation of the photosensitizers used and light source properties. Photodiagnosis Photodyn Ther 2012;9:122-31.
15. Mang TS, Tayal DP, Baier R. Photodynamic therapy as an alternative treatment for disinfection of bacteria in oral biofilms. Lasers Surg Med 2012;44:588-96.
16. Teixeira AH, Pereira ES, Rodrigues LK, Saxena D, Duarte S, Zanin IC. Effect of photodynamic antimicrobial chemotherapy on *in vitro* and *in situ* biofilms. Caries Res 2012;46:549-54.
17. Lima JP, Sampaio de Melo MA, Borges FM, Teixeira AH, Steiner-Oliveira C, Nobre Dos Santos M, *et al*. Evaluation of the antimicrobial effect of photodynamic antimicrobial therapy in an *in situ* model of dentine caries. Eur J Oral Sci 2009;117:568-74.
18. de Melo MA, Rolim JP, Zanin IC, Barros EB, da-Costa EF, Rodrigues LK. Characterization of antimicrobial photodynamic therapy-treated *Streptococci mutans*: An atomic force microscopy study. Photomed Laser Surg 2013;31:105-9.
19. Guglielmi Cde A, Simionato MR, Ramalho KM, Imparato JC, Pinheiro SL, Luz MA. Clinical use of photodynamic antimicrobial chemotherapy for the treatment of deep carious lesions. J Biomed Opt 2011;16:088003.
20. Dobson J, Wilson M. Sensitization of oral bacteria in biofilms to killing by light from a low-power laser. Arch Oral Biol 1992;37:883-7.
21. Okamoto H, Iwase T, Morioka T. Dye-mediated bactericidal effect of He-Ne laser irradiation on oral microorganisms. Lasers Surg Med 1992;12:450-8.
22. Paulino TP, Magalhães PP, Thedei G Jr, Tedesco AC, Ciancaglini P. Use of visible light-based photodynamic therapy to bacterial photoinactivation. Biochem Mol Biol Educ 2005;33:46-9.
23. Lee YH, Park HW, Lee JH, Seo HW, Lee SY. The photodynamic therapy on *Streptococcus mutans* biofilms using erythrosine and dental halogen curing unit. Int J Oral Sci 2012;4:196-201.
24. Araújo NC, Fontana CR, Bagnato VS, Gerbi ME. Photodynamic antimicrobial therapy of curcumin in biofilms and carious dentine. Lasers Med Sci 2014;29:629-35.
25. Vahabi S, Fekrazad R, Ayremlou S, Taheri S, Zangeneh N. The effect of antimicrobial photodynamic therapy with radachlorin and toluidine blue on *Streptococcus mutans*: An *in vitro* study. J Dent (Tehran) 2011;8:48-54.
26. Nogueira AC, Graciano AX, Nagata JY, Fujimaki M, Terada RS, Bento AC, *et al.* Photosensitizer and light diffusion through dentin in photodynamic therapy. J Biomed Opt 2013;18:55004.
27. Gursoy H, Ozcakir-Tomruk C, Tanalp J, Yilmaz S. Photodynamic therapy in dentistry: A literature review. Clin Oral Investig 2013;17:1113-25.
28. Pereira CA, Costa AC, Carreira CM, Junqueira JC, Jorge AO. Photodynamic inactivation of *Streptococcus mutans* and *Streptococcus sanguinis* biofilms *in vitro*. Lasers Med Sci 2013;28:859-64.

29. Rolim JP, de-Melo MA, Guedes SF, Albuquerque-Filho FB, de Souza JR, Nogueira NA, *et al*. The antimicrobial activity of photodynamic therapy against *Streptococcus mutans* using different photosensitizers. J Photochem Photobiol B 2012;106:40-6.
30. Garcez AS, Núñez SC, Azambuja N Jr, Fregnani ER, Rodriguez HM, Hamblin MR, *et al*. Effects of photodynamic therapy on Gram-positive and Gram-negative bacterial biofilms by bioluminescence imaging and scanning electron microscopic analysis. Photomed Laser Surg 2013;31:519-25.
31. Longo JP, Azevedo RB. Effect of photodynamic therapy mediated by methylene blue in cariogenic bacteria. J Dent Clin Res 2010;6:249-57.
32. Bowden GH, Hamilton IR. Competition between *Streptococcus mutans* and *Lactobacillus casei* in mixed continuous culture. Oral Microbiol Immunol 1989;4:57-64.
33. Wilson M. Susceptibility of oral bacterial biofilms to antimicrobial agents. J Med Microbiol 1996;44:79-87.