http://dx.doi.org/10.1590/1677-3225v13n1a11

Original Article

Braz J Oral Sci.
January | March 2014 - Volume 13, Number 1

# *In Vitro* antimicrobial photoinactivation with methylene blue in different microorganisms

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## Abstract

**Aim:** To evaluate the *in vitro* antimicrobial effects of photodynamic therapy (PDT). **Methods:** The microorganism indicators were: *Candida albicans*, *Pseudomonas aeruginosa*,*Enterococcus faecalis* and *Staphylococcus aureus*. A microbial pool was prepared ($10^8$ cells/mL), from which aliquots were transferred to culture plates for carrying out the PDT using methylene blue (50 μM) and low-power laser (660 nm, 100 mW and 9 J).The effect of methylene blue alone, low power laser and the absence of treatments were evaluated. Then, aliquots of 1 μL were plated in a media culture, the number of colony forming units (CFU/mL) was obtained and the data submitted to the F test (ANOVA) with Tamhane's comparisons. **Results:**The laser radiation in the presence of methylene blue was able to eliminate 74.90% of *C. albicans*, 72.41% of *P. aeruginosa*, 96.44% of *E. faecalis* and 95.42% of *S. aureus*, thus statistically significant differences were found among the groups ($p$<0.001). **Conclusions:** PDT was effective in reducing the number of viable cells in the studiedmicroorganisms, especially *E. faecalis* and *S. aureus*.

**Keywords:** endodontics; *Enterococcus faecalis;* methylene blue; microbiology; photodynamic therapy.

## Introduction

Microorganisms play an essential role in the development and maintenance of pathologies that affect the pulp and the periapical region[1], and their removal during the biomechanical preparation is crucial to the success of endodontic treatment[2].

*Pseudomonas aeruginosa* and *Staphylococcus aureus* have been commonly associated with persistent infections[3-5].Special attention has been given to *Enterococcus faecalis*, a tough Gram-positive bacterium, which has a much higher incidence in cases of endodontic treatment failure[6-7]. This microorganism has the property of survival in extremely alkaline pH environments, with scarce nutrients, invading and growing within dentinal tubules, colonizing the root canal and reinfecting the root-filled teeth[8-9].

Fungi are occasionally found in the primary infection of root canals, but occur more frequently in teeth obturated with lesions refractory to treatment. *Candida albicans* is the most prevalent fungal species, a microorganism that has affinity for dentin and is resistant to some intracanal medications, for example, those based on calcium hydroxide[10].

**Received for publication**: January 28, 2014
**Accepted:** March 20, 2014

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The antibacterial activity of low power lasers associated with a photosensitizer has been studied as adjuvant treatment together with conventional endodontic therapy[11]. Photodynamic therapy (PDT) assumes that the interaction of light with an appropriate wavelength, when associated with a nontoxic photosensitizing dye in the presence of oxygen, results in free radicals of high cytotoxicity, such as superoxides and singlet oxygen. These highly reactive species can cause serious damage to microorganisms via irreversible oxidation of cellular components[12].

However, this treatment presents other challenges regarding its susceptibility to different microorganisms, according to their physiology[13-14].Therefore, it is still necessary to set specific parameters so that PDT can be used for maximum effectiveness in removing microorganisms that cause endodontic infections.

The aim of this study was to contribute to other studies that seek to clarify the effects of antimicrobial PDT, evaluating the effects of *in vitro* photosensitization of methylene blue by laser irradiation in suspensions containing various species of microorganisms.

## Materials and methods

### Microorganisms and preparation of microbial suspensions

The microorganisms used in the study were obtained from the Department of Microbiology and Antibiotics of the Federal University of Pernambuco, one yeast and three bacterial strains: *Candida albicans* (ATCC 10231), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853) and *Enterococcus faecalis* (ATCC 6057) previously cultured in Agar Nutrient (Difco, Detroit, MI, USA). Four microbial suspensions of 3 mL each were formed in test tubes, in which microorganism indicators were diluted using sterile saline (0.9% NaCl). The suspensions of the microorganisms had the optical density adjusted spectrophotometrically to approximately 1.0 x $10^8$ colonyforming units (CFU) $mL^{-1}$(equivalent to 1.0 McFarland scale)[5,15]. From each microbial suspension, 2 mL was removed and a mixture with the four microorganisms was prepared (microbial pool).

### Description of experimental groups

Aliquots of 200µLwere removed from the microbial pool and transferred to culture plates with 24 wells each. Experimental groups were formed as follows (n = 10): Group L-P-: positive control (microbial pool); Group L + P-: formed by the microbial pool that received the isolated action of the laser; Group L-P +: microbial pool that received 20µl of the photosensitizer for two minutes, and Group L + P +: microbial pool that received 20µl of the photosensitizer for two minutes and then laser irradiation.

### Laser and photosensitizer

The photosensitizer used in the study was a solution of methylene blue 50µM (Chimiolux®; Hypofarma, Belo Horizonte, MG, Brazil). The light source came from a low power laser (Equipment Whitening Lase II, DMC equipment Ltd) with a wavelength of 660 nm, 100 mW, at an irradiation time of 3 min. This resulted in an energy dose of 9 J for each sample.

### Photosensitization *in vitro*

Irradiation of samples was performed under aseptic conditions in a laminar flow hood (A/B3 CASS II; AIR TECH, Tokyo, Japan). Throughout the experiment, all the samples were handled in the dark. A bulkhead was made using an opaque black paper sheet with a central hole with a diameter similar to the wells, to prevent the same well from being irradiated more than once. A burette clamp was used in order to standardize the distance of 3 cm between the tip of the laser and the bottom of each well on the plate.

To evaluate the antimicrobial treatment, aliquots of 1µLwere obtained from each well and plated in Agar Sabouraud (Difco) growth medium and in Blood Agar (Difco). After incubation for 48 h at 37°C in a bacteriological incubator, the CFU/mL was counted through observation of the morphology of the colonies. All experiments were conducted in triplicate.

### Statistical Analysis

For data analysis, statistical measures were obtained using the average and standard deviation of the colonies count (in CFU/mL). Calculation of percentage (descriptive statistics) was made using the F test (ANOVA), with comparisons using Tamhane's inferential statistics technique. The hypothesis verification of equal variances was performed using Levene's F test with $p < 0.001$ considered as statistically significant. The statistical program used was SPSS (Statistical Package for Social Sciences) version 15 (SPSS Inc., Chicago, IL, USA).

## Results

The microbial effectiveness of the group treated with laser in the presence of the photosensitizer (L + P +) in all the microorganisms tested showed the lowest average value of CFU/mLwith significant difference between the groups ($p < 0.001$) (Table 1).

Table 2 shows the percentage of reduction in CFU/mL observed for the L + P + group compared to the L-P-. Among the evaluated microorganisms, *P. aeruginosa* was the most resistant to PDT, followed by *C. albicans*, *S. aureus* and *E. faecalis*.

Figure 1 shows the average and the standard deviation of CFU/mL obtained for the various microorganisms studied in each experimental group. In the group L + P-, *C. albicans* and *P. aeruginosa* showed a reduction in the number of CFU/mL, similar to L + P +; whereas in group L + P +, *E. faecalis* and *S. aureus* showed a significant reduction compared to L + P-.The CFU/mL number in L-P + was similar to the group L-P-. When the groups L + P- andL-P + were compared, a significant decrease of the microbial growth in all the studied microorganisms was observed.

**Table 1.** Average and standard deviation of the logarithm of colony-forming units per milliliter (CFU/mL) for the following groups (n = 10): L + P- = group treated only with laser, L- P + = group treated only with photosensitizer, L + P + = group irradiated with laser in the presence of photosensitizer, L-P- = positive control - group which has not been treated with laser or photosensitizer.

| SPECIES | Log (10) CFU/mL | | | | |
|---|---|---|---|---|---|
| | L+P- | L-P+ | L+P+ | L-P- | *P*-value |
| *C. albicans* | 9.66 ± 0.21 (A) | 10.02 ± 0.42 (B) | 9.51 ± 0.49 (A) | 10.21 ± 0.32 (B) | $p^{(1)}$< 0.001* |
| *P. aeruginosa* | 10.10 ± 0.19 (A) | 10.70 ± 0.02 (B) | 10.09 ± 0.24 (A) | 10.69 ± 0.02 (B) | $p^{(1)}$< 0.001* |
| *E. faecalis* | 9.39 ± 0.17 (A) | 10.70 ± 0.02 (B) | 7.30 ± 3.73 (C) | 10.69 ± 0.02 (B) | $p^{(1)}$< 0.001* |
| *S. aureus* | 10.18 ± 0.18 (A) | 10.69 ± 0.02 (B) | 9.14 ± 0.46 (C) | 10.69 ± 0.02 (B) | $p^{(1)}$< 0.001* |

(*): Significant difference at level of 5.0%.
(1) By F test (ANOVA).
Note: If all the letters in parentheses are different, it shows a significant difference between the corresponding groups, using Tamhane'scomparison method.

**Table 2.** Percentage of reduction, expressed in the average and standard deviation of the values (CFU/mL) in the cell viability of the microorganisms exposed to the laser in the presence of a photosensitizer (L + P +) compared to positive control (L-P-).

| SPECIES | L+P+ | L-P- | REDUCTION IN CFU/mL (%) |
|---|---|---|---|
| *C. albicans* | 50.06 ± 37.04 | 199.50 ± 133.89 | 74.90% |
| *P. aeruginosa* | 131.85 ± 44.56 | 492.10 ± 24.03 | 72.41% |
| *E. faecalis* | 17.43 ± 24.96 | 489.20 ± 23.51 | 96.44% |
| *S. aureus* | 22.43 ± 24.22 | 490.20 ± 22.57 | 95.42% |

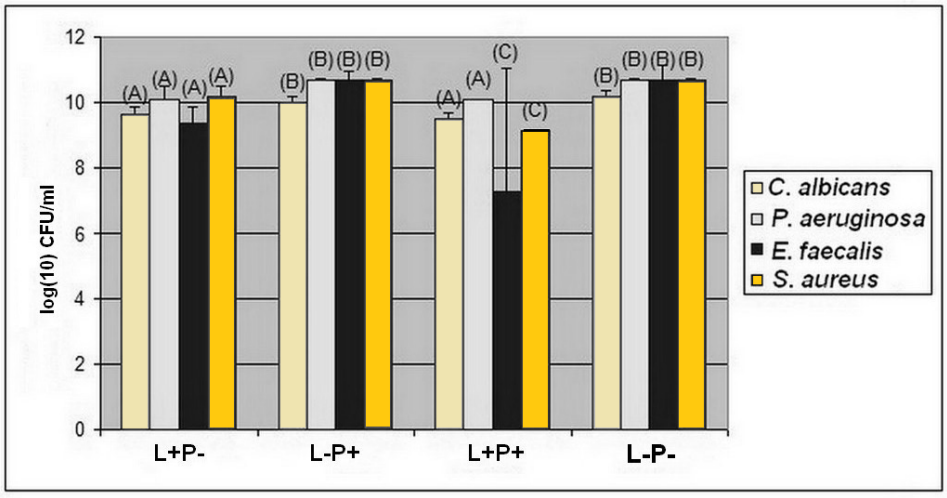


**Fig. 1.** Average and standard deviation obtained for *C. albicans*, *P. aeruginosa*, *E. faecalis* and *S. aureus* in all experimental groups (n=10): L+P- = group treated only with laser, L- P+ = group treated only with photosensitizer, L+P+ = group irradiated with laser in the presence of photosensitizer, L-P- = positive control.
A, B and C: statistically significant difference (test F (ANOVA): $p$<0.001)

## Discussion

The application of PDT, as an adjuvant treatment, has been indicated in endodontics, seeking to help the conventional therapy in eradicating the resistant pathogens of the root canal[16-19].

Microbial agents are considered the main etiological factors to the progression and perpetuation of pulp and periradicular inflammatory diseases[20].The pathogens used in the present study were selected because of their clinical importance and association with endodontic infection[21].

Various dyes have been used to perform PDT, such as toluidine blue and methylene blue[14]. The latter had its chemical properties tested in several studies that proved its antimicrobial efficacy, which motivated the choice for using this product in the present study[22-24].

The results obtained in this study demonstrated that when methylene blue was used alone, there was no significant reduction in the number of CFU/mL for all studied species. This result indicates that the concentration and the amount used in the present study showed no cytotoxic effect on the test microorganisms, corroborating the findings of Pupo et al.[25] (2011) and Miyabe et al.[26] (2011) who used only methylene blue at 100 mg/mL in *C. albicans* and at 3 mM in

*S. aureus* respectively. These results, however, are different from those of Foschi et al.[27] (2007), who reported a 19.5% reduction in viability of *E. faecalis* when 6.25 mg/mL of methylene blue was used without photosensitization in extracted single-rooted teeth.

Regarding the laser effects in the absence of a photosensitizer, *P. aeruginosa* and *C. albicans* showed a reduction in the number of CFU/mL similar to the group treated with PDT, differing from the findings of Queiroga et al.[28] (2011),who found no reduction in cell viability of *C. albicans* after their exposure to the laser in the parameters of 60 J/cm$^2$, 120 J/cm$^2$ and 180 J/cm$^2$.

Thus, in comparison with other groups, PDT behaved better in microbial reduction using methylene blue with a concentration of 50 μM at 660 nm, 100 mW and 9J, corroborating other studies that showed that the use of the laser associated with a photosensitizer is effective against various microorganisms[16,29-31].

Microbial reduction by photodynamic effect faces various challenges when used against Gram-positive bacteria, Gram-negative and fungi. *E. faecalis* was the microorganism with the highest reduced percentage of CFU/mL (96.44%), followed by *S. aureus* (95.42%), *C. albicans* (74.90%) and *P. aeruginosa* (72.41%).

In general, the literature shows that Gram-positive bacteria are more susceptible to the action of PDT compared to Gram-negative bacteria. This is due to differences in the physiology of these microorganisms, since Gram-positive bacteria have a relatively porous outer membrane formed by a thicker layer of peptidoglycan and lipoteichoic acid[14]. This feature allowsa greater diffusion of the photosensitizer within the microbial cells,sincethey can be eliminated by various types of dye and lower doses of radiation, which explains the greater susceptibility of *E. faecalis* and *S. aureus*to PDT in this study.

On the other hand, the outer membrane of Gram-negative bacteria (*Pseudomonas aeruginosa*) is thinner and complex, being formed by a heterogeneous composition of proteins with porin function, lipopolysaccharides and lipoproteins that act as an effective barrier to limit the penetration of various substances[14].

Regarding fungi, besides their nuclear membrane and increased cellular volume, they possess a cell wall composed of a thick layer of beta glucan and chitin, which promotes an intermediate permeability barrier between the Gram-positive and Gram-negative bacteria[32].

Variables such as exposure time and laser energy density, type and dye concentration influence the number of microorganisms affected by PDT[12]. In this study, a reduction in the number of CFU/mL *C. albicans* to 74.90% was achieved. On the other hand, de Souza et al.[33] (2006) obtained a reduction of CFU/mL in a suspension of *C. albicans* to 88.6% when 0.1 mg/mL of methylene blue, 685 nm of laser light and an energy dose of 28 J/cm$^2$ was used. The differences in results between these studies may be attributed to the dye concentration or to parameters used for laser irradiation.

In summary, despite the PDT not reducing the microorganisms completely, the results obtained lead to the conclusion that the treatment was able to promote the reduction of microbial cell viability using the selected parameters.

## Acknowledgements

This study was supported by grants from Pernambuco State Foundation for Science and Technology- FACEPE (BIC-0874-4.02/10) and CNPq - Brazil. The English version of this study has been revised by Sidney Pratt, Canadian, BA, MAT (The Johns Hopkins University), RSAdip (TEFL).

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