# Photodynamic therapy application in endodontic aerobic microorganisms and facultative anaerobic

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The aim of this study was to evaluate the possible antimicrobial action of photodynamic therapy (PDT) in the endodontic microbiota. This work was divided into two stages: the first, consisting of an immature group composed of a pool of microbial strains with four days of life; and a second group, formed by mature strains with four weeks of life, previously grown in BHI. After this period, 200μL aliquots were removed and transferred to culture dishes for both treatments. Negative control group (C): BHI; Positive control group (C+): microbial pool; Group L+F+: microbial pool receiving the action of blue methylene (0.01%) for two minutes and low-power laser at λ=660nm, 100mW and 9J for three minutes; Group F+: formed by the microbial pool receiving the dye action for two minutes; and Group L+: formed by the microbial pool receiving the laser action at λ=660nm, 100mW and 9J for three minutes. Then, the samples were seeded in culture media, the Petri dishes incubated at 37° C ± for 48 hours, the counting of CFU/mL was performed. The data were submitted to the test F (ANOVA) by Tamanhe's comparisons. The results showed that PDT was more effective in reducing cell viability of both immature and mature microorganisms when compared to the other groups ($p < 0.001$). In relation to group C+ and L+ F+ there was a further decrease in CFU/mL in the strains with four weeks microorganisms: *C. albicans* (71.46%), *E. faecalis* (83.75%) and *S. aureus* (68.98%), but no decrease was observed in *P. aeruginosa*. Thus, it was found that PDT used in these parameters was able to decrease the growth of majority of pathogens that were studied, being ineffective against *P. aeruginosa*.

**Keywords:** Endodontic; Therapy photodynamic; Root canal treatment

## 1. Introduction

Due to the large increase in the number of resistant bacteria, many researchers have been looking for new therapeutic agents able to combat theses pathogens. Among them, the antimicrobial ability of low power lasers associated with a photosensitizer has been investigated in Endodontics as an auxiliar treatment in cases of persistent microorganisms to conventional endodontic therapy [1], seeking the guarantee of maximum disinfection of the root canal system [2].

This antimicrobial action of low power lasers began to be studied in recent decades, when the photodynamic therapy (PDT), initially proposed to cancer treatment, became to be studied in dentistry in order to treat infectious diseases [3]. It assumes that the light interaction with appropriated wavelength, associated to a photosensitize agent and oxygen results in free radicals with high cytotoxicity, such as singlet and superoxide, which are able to promote the induction of cellular metabolic invariability, causing the death of microorganisms [4-8].

Despite of technologic and scientific advances in Endodontic, there are many cases resulting in failures due to the capacity of microorganisms to adapt to environmental changes by different mechanisms, such as biofilm, physiological changes, changes in genetic materials and the creation of cell subpopulations [9,10].

As in the biomechanical preparation of root canals, the reduction or elimination of microorganisms is achieved through the mechanical action of endodontic instruments and physico-chemical and antimicrobial properties of irrigating solution [11-13], and the access into root canals is limited by the complexity of the internal anatomy, microorganism can remain viable within dentinal tubules, and when they find a favorable environment, can proliferate and reinfect the root canal [14,11].

Therefore, the PDT has been used after the chemical-mechanical preparation as an auxiliary method to eliminate the most resistant pathogens [15,16], and it has an advantage to be selective, do not promote bacterial resistance, is easily applied [17]; and several studies have shown that the dose required to kill bacteria is less than the dose to cause damage to keratinocytes and fibroblasts [18- 21].

As there is permanent scientific interest to make antimicrobial action more effective, faster, more practical to use and better biological behavior, it is necessary to seek and test new substances and new treatments aiming to solve those cases of microbial resistance in order to increase the success of treatments. Thus, the objective of this study was to evaluate the possible antimicrobial actions of phototherapy on an endodontic microbiota.

## 2. Material and Methods

### 2.1 Microorganisms and preparation of microbial suspensions

The microorganisms used were three bacteria and one fungus: *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Enterococcus faecalis* (ATCC 194433) and *Candida albicans* (ATCC 10231).

Procedures were performed in a laminar flow hood (Air Tech - Cass II A/B3, Tokyo - Japan), using sterilized materials and instruments. Four microbial suspensions were grown in test tubes with 3ml each, using BHI, in which indicator microorganisms were added and incubated in bacterial glass Kiln at ± 37ºC for 4 days and 4 weeks until the moment that the increase of the suspension reached the optical density (O.D.) of 0,8 +/- 0,01, which is equivalent to a concentration close to 1,0 x $10^8$ CFU/mL (colony-forming unity per milliliter), confirmed by espectophotometer. From each microbial suspension, 1mL was removed by automatic micropipette and a mix of four microorganisms (microbial pool) was prepared.

### 2.2 Photosensitizers and laser

The photosensitizer selected was the methylene blue 0.01% solution (Chimiolux® - Hyrofarma, Belo Horizonte, Brazil), and the light source used was the low power laser equipment (Whitening Lase II, DMC equipment Ltd.) with a wavelength of 660 nm, 100 MW at the irradiation time of 3 minutes, resulting in an energy dose of 9 J for each sample.

### 2.3 Experimental Groups

Five groups were formed (n = 10): Group 1 negative control (C-): composed of BHI; Group 2 Positive control (C +): formed by the microbial pool; Group 3 (L+ F+): microbial pool that received the action of methylene blue for two minutes and low-power laser; Group 4 (F+): microbial pool and methylene blue for two minutes; Group (L+): microbial pool that received the isolated low-power laser action.

#### 2.3.1 Experiment 1: immature Group, composed by strains with four days of life

After the composition of microbial pool and the obtaining of the suspension at a concentration of 1.0 x $10^8$ CFU/ mL, the suspension was incubated at ± 37ºC for 4 days in bacteriological incubator in order to carry out the treatment according to the description of experimental groups. The amount of 0.2 mL was taken from each suspension and transferred to culture plates with 6 wells of 35 mm in diameter (Falcon plate type) to assess the action of photodynamic therapy in the inactivation of the immature biofilm.

#### 2.3.2 Experiment 2: Mature group, formed by strains with four weeks of life

After the formation of microbial pool and the obtaining of the suspension at a concentration of 1.0 x $10^8$ CFU/ mL, the suspension was incubated at ± 37ºC for 4 weeks in bacteriological incubator in order to carry out the treatment according to the description of experimental groups. Then, 0.2 mL was removed from each suspension and transferred to culture plates with 6 wells of 35 mm in diameter (Falcon plate type), aiming to evaluate the effect of photodynamic therapy in the inactivation of the mature biofilm.

### 2.4 Statistical Analysis

For data analysis, the following statistical measures were obtained: mean and standard deviation and percentage calculation (descriptive statistics techniques) and F (ANOVA) test with Tamanhe's comparisons (inferential statistical techniques) was performed. To verify the hypothesis of equal variances, the F Levine test was performed. It is noteworthy that the logarithm transformation in cell number plus 1 (one) was used for the analysis of ANOVA (log10 (N ° 1 + cells)). The margin of error used in the decision of the statistical tests was 5.0%. The statistical software used for data entry and to obtain the statistical calculations was SPSS (Statistical Package for Social Sciences) version 15.

## 3. Results

Microbial growth was observed in all the positive controls before and after the treatments, while the negative controls did not show any growth. The data showed that the group L+F+ was more efficient to reduce cells variability in immature microorganisms as well as in matures, when compared with the other groups($p<0.001$) (Table 1).

Regarding Group C+ and L+F+ was observed a decrease of CFU/mL in the strains with 4 weeks of life: *Candida albicans* (71.46%), *Enterococcus faecalis* (83.75%) and *Staphylococcus aureus* (68.98%), however, it did not show a significant cytotoxic effect for *Pseudomonas aeruginosa* by the methodology applied (Table 2).

 

**Table 1** Number of cells logarithm statistics + 1(by mL in the concentration $10^8$) according to the studied group L+= irradiated by laser; F+ =photosensitize; L+F+ =irradiated by laser with photosensitize C+ = positive control.

| Species | Evaluation | Group | | | | p Values |
|---|---|---|---|---|---|---|
| | | L+ mean ± SD | F+ mean ± SD | L + F+ mean ± SD | C + mean ± SD | |
| ***C. albicans*** | 4 days | 10.75 ± 0.04 (A, b) | 10.79 ± 0.10 (AB, b) | 10.75 ± 0.02 (A, b) | 10.86 ± 0.08 (B, b) | **pII(1) = 0.002*** |
| | 4 weeks | 9.83 ± 0.21 (AB, c) | 9.75 ± 0.36 (AB, a) | 9.47 ± 0.35 (A, a) | 9.98 ± 0.48 (B, a) | **pI(1) = 0.026*** |
| | **p Values** | **pI(1) < 0.001*** | **pI(1) < 0.001*** | **pI(1) < 0.001*** | **pII(1) < 0.001*** | |
| ***P. aeruginosas*** | 4 days | 10.73 ± 0.07 (AC, b) | 10.80 ± 0.05 (B, b) | 10.76 ± 0.02 (AB, b) | 10.70 ± 0.02 (C, a) | **pI(1) < 0.001*** |
| | 4 weeks | 10.74 ± 0.03 (A, b) | 10.51 ± 0.18 (B, c) | 10.75 ± 0.03 (A, b) | 10.75 ± 0.03 (A, b) | **pI(1) < 0.001*** |
| | **p Values** | **pI(1) < 0.001*** | **pI(1) < 0.001*** | **pI(1) < 0.001*** | **pII(1) < 0.001*** | |
| ***E. faecalis*** | 4 days | 10.75 ± 0.06 (AB, b) | 10.78 ± 0.07 (A, a) | 10.75 ± 0.02 (AB, b) | 10.69 ± 0.02 (B, a) | **pI(1) = 0.001*** |
| | 4 weeks | 10.10 ± 0.15 (A, c) | 9.89 ± 0.35 (A, b) | 9.85 ± 0.32 (A, ab) | 10.73 ± 0.03 (B, b) | **pI(1) < 0.001*** |
| | **p Values** | **pI(1) < 0.001*** | **pI(1) < 0.001*** | **pI(1) = 0.003*** | **pII(1) < 0.001*** | |
| ***S. aureus*** | 4 days | 10.71 ± 0.06 (AC, b) | 10.81 ± 0.05 (B, b) | 10.74 ± 0.01 (A, b) | 10.69 ± 0.02 (C, a) | **pI(1) < 0.001*** |
| | 4 weeks | 10.38 ± 0.15 (A, c) | 9.44 ± 0.24 (B, c) | 10.22 ± 0.17 (A, c) | 10.76 ± 0.02 (C, b) | **pI(1) < 0.001*** |
| | **p Values** | **pI(1) < 0.001*** | **pI(1) < 0.001*** | **pI(1) < 0.001*** | **pII(1) < 0.001*** | |

(*) Significant difference at 5% level

(1): by F test (ANOVA).

Note: If all capital letters in brackets are different, it proves a significant difference among the groups by paired comparinson by tukey(i) or tamhane's T2(ii). However, if all lower case letters in the brackets are different, it proves difference among the time of evaluation corresponding by paired comparisons tukey(i) or tamhane's t2(ii).

**Table 2** Mean of the number of CFU/mL and percentage of decrease in each group compared to the positive control.

| Microorganisms | Evaluation | Grupos | | % of decrease (CFU/mL) among groups |
|---|---|---|---|---|
| | | L+ F+ mean | C+ mean | |
| ***C. albicans*** | 4 days | 568.80 | 729.20 | **22.00** |
| | 4 weeks | 36.50 | 127.90 | **71.46** |
| ***P. aeruginosas*** | 4 days | 572.40 | 502.60 | **-13.89** |
| | 4 weeks | 567.50 | 561.80 | **-1.01** |
| ***E. faecalis*** | 4 days | 558.50 | 489.20 | **-14.17** |
| | 4 weeks | 88.10 | 542.20 | **83.75** |
| ***S. aureus*** | 4 days | 554.80 | 490.20 | **-13.18** |
| | 4 weeks | 178.60 | 575.70 | **68.98** |

## 4. Discussion

The microbial reduction by photodynamic effect, although effective in several species, faces different challenges when used against Gram-positive bacteria, Gram-negative bacteria, bacterial spores and fungi [22]. In general, Gram-positive bacteria can be eliminated by various photosensitizers at lower doses of irradiation than Gram-negative bacteria, sporulating bacteria and fungi. This is due to structural differences in the cell membranes of these organisms, as well as the laser parameters, pre-irradiation time and photosensitizer concentrations [23].

The photosensitization of bacteria is related to the photosensitizer charge. Because it has characteristics such as the positive charge, low molecular weight and hydrophilicity, methylene blue is capable of interacting with anionic lipopolysaccharide macromolecules and penetrating the outer membrane of Gram-negative bacteria [24]. Regarding to fungi, these microorganisms have a cell wall constituted by a thick layer of β-glucan and chitin, which promotes an intermediate permeability barrier between Gram-positive and Gram-negative bacteria [25].

In most scientific studies, the most commonly used photosensitizers are dyes: toluidine blue or methylene blue [26-28]. It is observed that, when the photosensitizer or laser isolated are used, antibacterial effect is not observed, but when the association between the photosensitizer and the laser occurs, there is a significant decrease in the number of colony-forming units [29]. In this study, *E. faecalis*, *P. aeruginosa*, *S. aureus* and *C. albicans* microorganisms were chosen because they have clinical significance in endodontic failures [30, 31], which is in accordance with other studies [32-34].

The results obtained in this study revealed that PDT was effective in microbial reduction using methylene blue at a concentration of 0.01% associated with the laser at 660 nm and 9J parameters, collaborating with other studies, which also concluded that this treatment is an effective antimicrobial adjuvant in conventional endodontic procedures, eliminating a percentage above 90% of the bacterial biofilm [29, 35, 36]. According to Fimple et al. [37] increasing the concentration of methylene blue and light energy fluence (J/cm$^2$), there is an increase in the antibacterial ability of PDT.

Many studies both *in vivo* and *in vitro* have shown that PDT efficiency as means of disinfection of the root canals, in particular against *E. faecalis* [36, 38-41]. What corroborates with the results observed in this study, which found a reduction of 83.75% of CFU/mL for this microorganism both mature and immature. However, Siddiqui, Awan, Javed [26] concluded in their literature surveys that the efficacy of PDT is still questionable on *E. faecalis* from root canals.

It is worth emphasizing that in the conclusions of Silva et al. [42], PDT was indifferent to the results of colony-forming units of *E. faecalis* colonies when using intracanal medication with 2% chlorhexidine gel associated with polymyxin B after chemical-mechanical instrumentation when compared to the group that PDT was not performed. However, it has been effective in eliminating this microorganism when the medication into the canals was not used.

Oliveira, Aguiar, Câmara [43], in their literature surveys, concluded that PDT is an important auxiliary tool in endodontic treatment, but draws attention to the fact that different microorganisms, depending on their physiology, have different susceptibilities to this therapy. This conclusion explains the absence of the cytotoxic effect on *P. aeruginosa* observed in this study.

Faced with the microbial diversity associated with each clinical endodontic [17], it is still necessary to find a suitable agent that is able to inactivate, if not all, but most species present in the root canal. Also emphasizing the need to search for an ideal photosensitizer, as well as the most suitable laser, because it does not yet exist in current literature any standardization, using different photosensitizers, irradiation doses, and light sources with different wavelengths [35,39,44], and to establish the photosensitizers that better penetrate the root canal system, the activity of free radicals and energy dose [41].

## 5. Conclusion

According to the methodology used and the results obtained, it is concluded that the protocol used for PDT, which had the photosensitizing agent methylene blue was effective against cell viability of microorganisms *C. albicans*, *E. faecalis* and *S. aureus,* but a satisfactory action to eliminate *P. aeruginosa* was not observed, what leads to the searching and development of new protocols for this microorganism in order to obtain a better antimicrobial effect.

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