

# Enhancement of Antimicrobial Action of Photodynamic Therapy in the Presence of Hydrogen Peroxide

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This study aimed to compare the effects of antimicrobial photodynamic therapy in the presence of hydrogen peroxide under microorganisms from infected root canals: *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosas*, and *Enterococcus faecalis*. It was prepared a pool microbial, and aliquots of 200 $\mu$ L were transferred to culture dishes for both treatments, divided into three experimental groups (n = 10): Group L-P- H<sub>2</sub>O<sub>2</sub><sup>-</sup>: microbial pool, L+ P+ H<sub>2</sub>O<sub>2</sub><sup>-</sup>: microbial pool that received the action of low power laser ( $\lambda$  = 660nm, 100mW and 9J) in the presence of methylene blue (0.01%) and group L+P +H<sub>2</sub>O<sub>2</sub><sup>+</sup>: pool formed by microbial action who received photodynamic therapy associated hydrogen peroxide to 1 M. To evaluate the antimicrobial treatments, the samples were grown in culture media and incubated in Petri dishes bacteriological incubator for 48h, and then the counting of the colonies and the data submitted to the F test (ANOVA) with comparisons Tamhane. The results showed that photodynamic therapy in combination with hydrogen peroxide produced a greater reduction of microbial microorganisms tested, was significantly higher for *E. faecalis* and *P. aeruginosas* (p <0.05). Thus, it was concluded that the combination of photosensitizer with hydrogen peroxide increased the efficiency of this treatment.

**Keywords** Endodontics; Photodynamic Therapy; Methylene Blue; Hydrogen Peroxide.

## 1. Introduction

In endodontics the inflamed pulp removal and disposal of viable microorganisms of the root canal system are of supreme importance during endodontic therapy [1]. In the phase of the biomechanical preparation of root canal system is considered one of the most important steps of treatment, it aims to reduce the microorganisms, their products and substrates; properly modeling the root canal for the treatment success be achieved [2].

Traditionally, microbial reduction or elimination is achieved through the mechanical action of endodontic instruments and the physicochemical and antimicrobial properties of the irrigating solutions [3-5]. As the access to the root canal is limited and the internal anatomy is complex, microorganisms may remain inside the dentinal tubules, and when they find a favorable environment, they can proliferate and reinfect the root canal system [6, 7].

Photodynamic therapy (PDT) has been the subject of research in dentistry due to its analgesic, healing and antimicrobial properties [1]. It has been presented as an alternative to combat resistant pathogens [8-14], shown to be a viable alternative to solve the problem of microbial resistance [15-18], which has been exacerbated mainly by the use and overprescribing of antibiotics inducing selection of resistant strains [19].

Its mechanism of action happens when the photosensitizer absorbs photons of light source and pass their electrons to an excited state forming molecules short-lived and highly reactive, such as singlet oxygen, superoxide and other free radicals with high toxicity, that can cause serious damage to microorganisms via irreversible oxidation of cellular components, causing damage to the cell membrane, the cell nucleus and mitochondrias [20, 21]. Among the photosensitizing agents, the methylene blue has been widely used in antimicrobial PDT. It is a phenothiazine that has a linear, tricyclic and heteroaromatic structure, soluble in water or alcohol, and for being cationic, it has high reactivity and ability to react with almost any substrate [22].

As the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is a substance known worldwide for its antiseptic and antimicrobial properties [23, 24], and is commonly used in dentistry due to its strong oxidizing property that can kill microorganisms by DNA damage [25, 26]. It is what we aimed when comparing the antimicrobial effect of PDT in the presence and absence of hydrogen peroxide in different microorganisms.

## 2. Material and Methods

### 2.1. Microorganisms and preparation of microbial suspensions

The microorganisms used were three bacteria and one fungus: *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 6057) and *Candida albicans* (ATCC 10231) which had been grown on Nutrient Agar (Difco, Detroit, USA). The microbial suspensions were formed in test tubes with 3ml each, in which microorganisms indicators were diluted using sterile saline solution (0.9% NaCl) until the moment when they reached a concentration close to 1.0 x 10<sup>8</sup> CFU/ml (colony forming unit per milliliter) in a spectrophotometer. From

each microbial suspension, 2 mL was removed and a mixture with the four microorganisms was prepared what was called the microbial *pool*.

## 2.2. Photosensitizers, laser and hydrogen peroxide

The photosensitizer selected was the solution methylene blue 0.01% (Chimiolux ® - Hyrofarma, Belo Horizonte, Brazil), the solution of hydrogen peroxide was manipulated to 1M (Fiel Farma, Recife, 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. Description of the experimental groups and photosensitization

Aliquots of 200µL microbial pool were removed and transferred to culture plates with 24 wells each which were divided into three groups (n = 10): Group L-P- H<sub>2</sub>O<sub>2</sub>-: pool microbial, Group L+P+H<sub>2</sub>O<sub>2</sub>-: pool microbial receiving 20µL of photosensitizer for 2 minutes before the laser exposure, and Group L+P+H<sub>2</sub>O<sub>2</sub>+: formed by microbial pool that received 20µL of photosensitizer for 2 minutes, followed by 20µL of hydrogen peroxide for 10 minutes and after laser exposure.

The irradiation of the samples was performed under aseptic conditions in a laminar flow hood (AIR TECH - CASS II A/B3, Tokyo - Japan). Throughout the experiment the samples were handled in the dark. One plate was made using black opaque paper having a central hole of a diameter similar to the well to prevent the same well was irradiated more than once. It was also used a clamp burette, which served to attach the tip of the laser to maintain a pattern distance of 3cm between the laser tip and the plate.

To evaluate the antimicrobial action of the treatments were obtained aliquots of 1µL of each well and transferred to Petri dishes with culture media: Sabouraud broth Agar (Difco, Detroit, USA) and Blood Agar (Difco, Detroit, USA). The plates were incubated at an average temperature of 37°C for 48h. After this period, it was evaluated the presence or absence of microbial growth and colony counts performed. All experiments were conducted in triplicate.

## 2.4. Statistical Analysis

To analyze the data was obtained statistical measures: average and standard deviation of bacterial colonies (in CFU/mL) and calculating percentage using the test F (ANOVA) with Tamhane comparisons. The verification of the hypothesis of equality of variances was performed using Levene's F test. The statistical program used was SPSS (Statistical Package for the Social Sciences) version 15.

## 3. Results

The results of this study showed that the laser irradiated group in the presence of a photosensitizer associated with hydrogen peroxide (L+P+H<sub>2</sub>O<sub>2</sub>+) caused a reduction in CFU/mL compared to other groups in all tested microorganisms (Table 1). However, it was observed that *C. albicans* was the more resistant to PDT associated with hydrogen peroxide according to the parameters defined in this work for the laser and a photosensitizer (Table 2).

**Table 1** Average and standard deviation of CFU/mL (Log) of experimental groups.

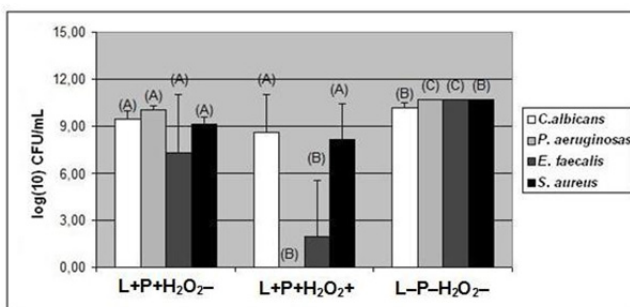
ESPÉCIES	log (10) UFC/ml		
	L+P+H <sub>2</sub> O <sub>2</sub> -	L+P+H <sub>2</sub> O <sub>2</sub> +	L-P-H <sub>2</sub> O <sub>2</sub> -
<i>C. albicans</i>	9.51 ± 0.49 <sup>(A)</sup>	8.64 ± 2.40 <sup>(A)</sup>	10.21 ± 0.32 <sup>(B)</sup>
<i>P. aeruginosas</i>	10.09 ± 0.24 <sup>(A)</sup>	0.00 ± 0.00 <sup>(B)</sup>	10.69 ± 0.02 <sup>(C)</sup>
<i>E. faecalis</i>	7.30 ± 3.73 <sup>(A)</sup>	1.95 ± 3.60 <sup>(B)</sup>	10.69 ± 0.02 <sup>(C)</sup>
<i>S. aureus</i>	9.14 ± 0.46 <sup>(A)</sup>	8.17 ± 2.29 <sup>(A)</sup>	10.69 ± 0.02 <sup>(B)</sup>

A, B e C: statistically significant difference teste F (ANOVA): p<0.05)

**Table 2** Percentage of reduction in CFU/mL of PDT associated with hydrogen peroxide in the control group.

ESPÉCIE	L+P+H <sub>2</sub> O <sub>2</sub> +	L-P-H <sub>2</sub> O <sub>2</sub> -	REDUÇÃO EM UFC/mL (%)
<i>C. albicans</i>	29.90	199.50	85.01%
<i>P. aeruginosas</i>	0.00	492.10	100.00%
<i>E. faecalis</i>	0.63	489.20	99.87%
<i>S. aureus</i>	11.43	490.20	97.67%

When comparing the group treated with hydrogen peroxide with the group treated with photodynamic therapy, we observed that the reduction was significantly greater for *E. faecalis* and *P. aeruginosa* ( $p < 0.05$ ), whereas for the latter, the number of colony forming units reached zero (Fig 1).



**Fig. 1** Average and standard deviation of the log<sub>10</sub> CFU/mL obtained for *C. albicans*, *P. aeruginosa*, *E. faecalis* and *S. aureus* under the different experimental groups. A, B and C: statistically significant difference (Test F (ANOVA)  $p < 0.05$ ).

#### 4. Discussion

Microorganisms play a key role in the etiology and maintenance of endodontic infections [27], so the pathogens used in this study were selected because of their clinical significance and association with persistent endodontic infections [28]. It were selected two Gram+ bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*), one Gram- bacteria (*Pseudomonas aeruginosa*) and one fungus (*Candida albicans*) [5, 29-31].

Among the procedures involved in the control of endodontic infections, the instrumentation and irrigation are important for eliminating the microorganisms in the root canal system [3, 32, 33], the PDT is indicated as a coadjuvant to endodontic treatment in an attempt to remove pathogens persistent to the chemical-surgical preparation [10, 34,35].

The photodynamic effect associated with methylene blue has been described as effective in various species of microorganisms, however there are differences in susceptibility between bacteria Gram+ and Gram- and among the fungi, this is explained by the difference in organization between them; Gram+ bacteria has in their cytoplasmic membrane, a relatively porous layer of peptidoglycan that allows the photosensitizer penetrates. On the other hand, the Gram- bacteria has, besides the cytoplasmic membrane, an outer membrane that forms a functional and physical barrier between the cell and the environment to protect these bacteria from the action of PDT [36].

In several studies it has been observed that there is no consensus regarding the optimal concentration of methylene blue, protocols and types of laser irradiation. Miyabe et al. [37] evaluated the effect of PDT in clinical isolates of staphylococci and observed a reduction of 6,27 log<sub>10</sub> CFU/ mL after use of methylene blue in a concentration of 3 mM associated with the GaAlAs laser (660nm, 35mW and 10J), corroborating the present study and that of Pereira et al. [38] who achieved a decrease of 1,00 and 1,47 log<sub>10</sub> CFU/mL respectively on the viability of *C. albicans* and *S. aureus*, after application of methylene blue at a concentration of 0.1 mg/mL, and laser irradiation InGaAlP (660 nm).

Through the data obtained in this study it was observed that there was an increase in microbial reduction for all microorganisms tested when combined with PDT with H<sub>2</sub>O<sub>2</sub>, collaborating with the findings of Garcez [39] who obtained a decrease in CFU/mL for *S. aureus*, *P. aeruginosa* and *C. albicans* respectively 99.79%, 98.64% and 98.92% after the use of 60μM of methylene blue associated with 1M H<sub>2</sub>O<sub>2</sub>, validating the hypothesis proposed that with the increase of O<sub>2</sub> dissolved in the solution explain the higher values of microbial reduction, due to a process of energy transfer between the excited photosensitizer and triplet oxygen.

Among the microorganisms tested *P. aeruginosa* showed the most significant reduction of CFU/mL, possibly due to the previous contact of hydrogen peroxide in the pre-irradiation period, causing a greater sensibilization in their outer membrane, allowing better penetration of the photosensitizer, however this finding differs from those described in the literature which affirm that Gram+ bacteria continue to suffer a greater reduction than Gram- bacteria and fungi after the photodynamic effect [40, 41].

Given the conditions used and within the limitations of this *in vitro* study, it can be concluded that the addition of hydrogen peroxide enhanced the efficiency of PDT, lead to enhanced uptake of the photosensitizer by the microorganism, and that the mechanisms involved in this process are the consequences of a photobiochemistry synergistic reaction and increase in cellular permeability.

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