Antimicrobial action of photodynamic therapy, in human root canals contaminated with Enterococcus faecalis: *in vitro* study

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ABSTRACT

Introduction: Although we have promising methods for cleaning and disinfecting in Endodontics, none of them have yet been able to completely eliminate microorganisms present in the root canal system. The advent of laser therapies, associated with specific dyes, has shown promising results in reducing root canal microbiota. **Objective:** The aim of this study was to evaluate the antimicrobial action of photodynamic therapy (PDT) associated with methylene blue photosensitizer, as complementary to conventional chemical-mechanical debridement in human teeth infected with Enterococcus faecalis in vitro. **Methods:** A total of 60 human single-rooted permanent teeth were prepared, infected with Enterococcus faecalis and divided into three groups of 20 each. G1 and G2: negative and positive controls, using the same specimens; G3: irrigation with 1% sodium hypochlorite (NaOCl) + 17% ethylenediaminetetracetic acid (EDTA); G4: irrigation with 1% NaOCl + 17% EDTA + PDT. Absorbent paper points were used to collect the material present in root canals, transferred to blood agar plates and cultured at 37 °C for 48 hours. In the negative control group, no samples showed contamination. **Results:** Groups 2, 3 and 4 differed statistically from each other in relation to the mean of bacterial growth, with the lowest growth occurring in G4. **Conclusion:** Photodynamic therapy, when used as a complementary procedure to endodontic therapy, was effective against Enterococcus faecalis, thus suggesting its clinical applicability.

Keywords: Photochemotherapy. Methylene Blue. Enterococcus faecalis.

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Introduction

Endodontic treatment consists of mechanical instrumentation of the root canal system, followed by chemical debridement and filling, thus allowing for maintainance or restoration of periapical tissues health, with pathogenic bacteria elimination.[1,2,3] Mechanical debridement associated with irrigation removes infectious microorganisms, but even after thorough irrigation with sodium hypochlorite, residual bacteria can be detected in root canals.[1]

Therefore, control and elimination of infection is a major challenge in Endodontics. In cases in which the root canal system was recolonized by residual bacteria, even after adequate instrumentation, treatment becomes even more complicated.[4-7] This process is mainly caused by Enterococcus faecalis, a Gram-positive aerobic microorganism.[8] However, none of the techniques, nor even the combinations currently used, are able to eliminate bacteria from root canals, thus impelling the need to investigate new technologies and more efficient methods of disinfecting root canals.[9-12]

Photodynamic therapy (PDT) comprises the association of a photoactive dye (photosensitizer) which, when exposed to light with a specific wavelength and in the presence of oxygen, is activated, therefore producing, highly reactive energy. This energy drastically and instantly reduces bacteria present in the root canal system, and most important, causes no resistance to them nor side effects when used properly. Therefore it is minimally invasive and toxic. Thus, in order to obtain adequate disinfection of the root canals and to significantly increase endodontic success, PDT can be of great help during antimicrobial procedures along with conventional disinfection procedures.[13,14,15]

The present study aimed to investigate the in vitro antibacterial effect of photodynamic therapy in extracted human teeth infected with Enterococcus faecalis, as complementary procedure to conventional chemical-mechanical debridement.

Material and methods

This study was submitted and approved by localo research ethics committee under #596,107,16,9,0000,8040.

A total of 60 extracted human permanent single-rooted teeth were used. They were provided by a university teeth bank, and previously stored in saline solution until the beginning of the experiment.

With a Carborundum™ disc (Dentorium© Products Co. Inc. - The Technicians Choice) mounted on a straight and low-speed handpiece (Kavo Kerr© Dental Excellence - Joinville, SC, Brazil), the roots were sectioned and reduced to 11 mm. Afterwards, teeth were reimersed in saline solution in order to avoid dehydration.

To have working length and patency determined, in addition to ensuring no barrier in the root canal, #10 file (Dentsply-Maillefer™, Ballalgues, Switzerland) was used. For modeling, Protaper Universal™ rotary system (Dentsply-Maillefer™, Ballalgues, Switzerland) was used up to instrument F4, following the manufacturer's recommendations. Root canals were irrigated in between instrument changes with 3 ml of 1% sodium hypochlorite solution. Final irrigation was performed with 5 ml of 1% sodium hypochlorite, followed by 1 ml of 17% EDTA solution for three minutes, and finally 2 ml of sodium hypochlorite solution. With the objective of avoiding unwanted contamination in the subsequent stages of the study, total root coverage with composite resin was performed. All apical foramina were obliterated with composite resin to avoid leakage of irrigating solutions during the next steps. Teeth were then sterilized by autoclaving (Sercon™ model HF-Copyright Sercon chamber and Steris Corporation - Mogi das Cruzes, SP, Brazil) for 63 minutes at 134 °C.

Teeth were divided into four groups of 20 specimens each:

» G1 and G2: negative controls (no contamination) and positive (contamination and no treatment) - the same specimens were used for both groups;
» G3: 1% sodium hypochlorite (NaOCl) + 17% ethylenediaminetetraacetic acid (EDTA)
» G4: NaOCl a 1% + EDTA a 17% + TPD

Culture and preparation of Enterococcus faecalis suspension

In a laminar flow hood, standard strains of E. faecalis inoculated into BHI broth and incubated at 37 °C for 24 hours were used for replication. After this period, replicate occurred; again, the period of 24 hours was waited, thus obtaining a dilution factor of 1/10. For effective assurance of contamination only by En-

terococcus faecalis, blood Agar plates were contaminated with this sample and incubated at 37 °C for 48 hours (Fig 1). After this period, Gram staining (Fig 2) was performed for analysis under microscope (Fig 3).

Group 1 - Negative control

The autoclaved specimens were not inoculated with E. faecalis microorganism, receiving only irrigation of the root canal with 5 ml of 1% NaOCl followed by 17% EDTA for three minutes and 5 ml of 1% NaOCl. To certify absence of any microorganism, sterile Protaper Universal F4™ absorbent paper points (Dentsply™, Konstanz, Germany) were inserted into root canals which were then forwarded for culture in sterile tubes containing Broth Heart Infusion (BHI) (Kasvi Imp. And Dist. Of Products for Laboratories Ltda, Curitiba, PR, Brazil), in order to have turbidity checked (Fig 4). After 24 hours, incubated at 37 °C, the points were transferred from the tubes to blood agar plates (5 ml of defibrinated sheep's blood for each 100 ml of base medium) for 24 hours at 37 °C to have the effectiveness of sterilization confirmed (Fig 5).

Group 2 - Positive control

After the end of the experiment with the Negative Control Group, the same teeth were used for the Positive Control Group, as follows: each sterilized specimen was individually placed in 20 previously sterilized test tubes (Fig 6). Teeth were then contaminated with an isolated solution of Enterococcus faecalis 1/10 in BHI broth, inserted with a 5 ml pipette, until they were submerged and incubated at 37 °C for 24 hours. After this period, 1 ml of broth was added and further 24 hours were used. The process hat was repeated one more time, totaling contamination in 72 hours, thus allowing for biofilm formation (Fig 7).

After contamination, root canals were irrigated with 5 ml of saline solution, dried with sterile absorbent paper points and transferred to tubes with pure BHI broth previously prepared and sterilized (Fig 8), grown at 37 °C for 24 hours. After this period, they were transferred to blood agar plates cultured at 37 °C for 48 hours (Fig 9) to have the presence of E. faecalis in the canals determined; therefore, certifying the effectiveness of the inoculation method chosen.

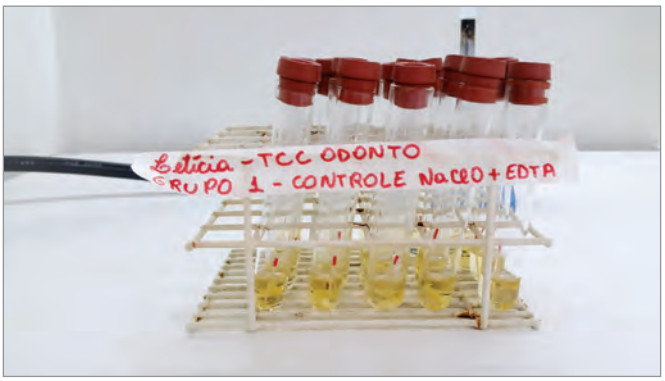

Figure 1. Absorbent paper points submerged in BHI broth after Group 1 procedures, being crystalline, thus evincing absence of bacterial contamination by E. faecalis.

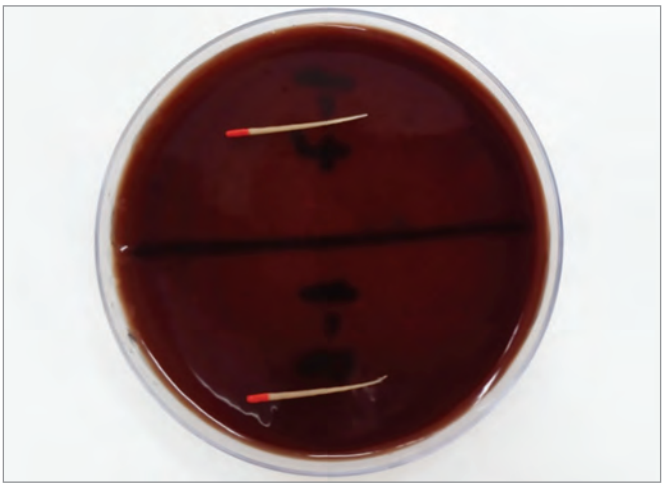

Figure 2. Blood agar plate presented without evident bacterial colonization, with cones that were removed from the tubes with BHI broth after Group 1 procedures.

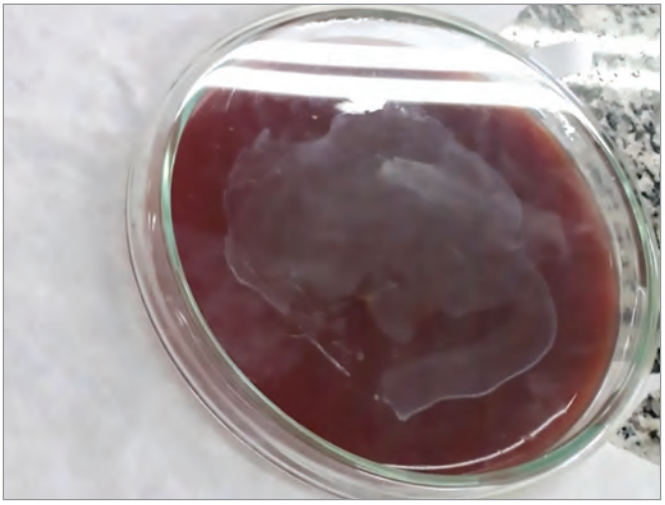

Figure 3. Cultivation of E. faecalis on Petri dish with blood agar.

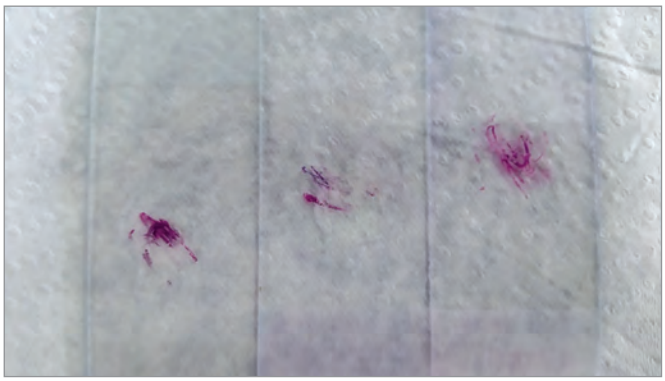

Figure 4. Gram staining of Enterococcus faecalis.

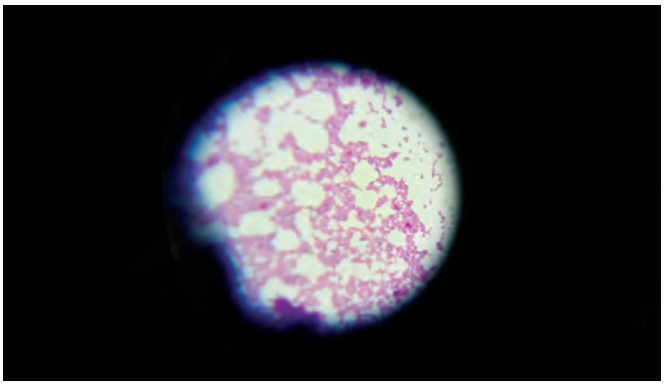

Figure 5. Microscope analysis of Gram stain of Enterococcus faecalis.

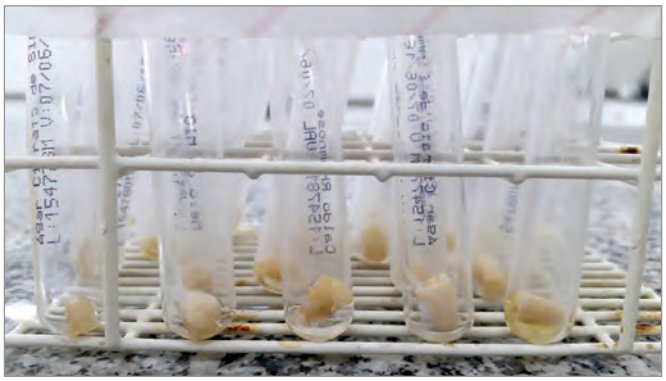

Figure 6. Teeth placement in test tubes for further contamination.

Figure 7. Teeth submerged in isolated solution of Enterococcus faecalis 1/10 in BHI broth, after totaling 72 hours of culture, to enable biofilm formation.

Contamination of Groups 3 and 4 specimens with Enterococcus faecalis

Each specimen was individually placed in 40 previously sterilized test tubes. Afterwards, teeth were contaminated with E. faecalis 1/10 solution in BHI broth, inserted into the tubes with a 5 ml pipette, until teeth were submerged. Teeth were incubated at 37 °C for 24 hours, after which they received 1 ml of broth for 24 hours. The process was repeated once more, totaling contamination within 72 hours, and allowing for biofilm formation. After contamination, root canals were irrigated with 5 ml of saline solution and dried with sterile absorbent paper points. Subsequently, absorbent paper points were transferred to tubes with pure BHI broth previously prepared and

Figure 8. Absorbent paper points after being inserted into tubes after contamination and cultivated for 24 hours at 37 °C, being turbid, thus proving the presence of E. faecalis.

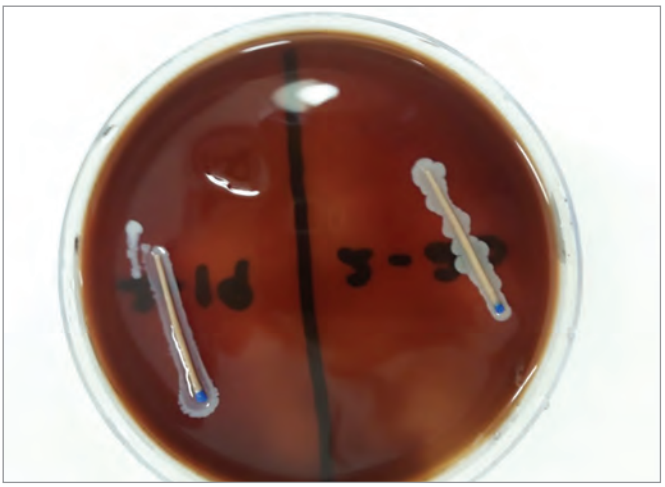

Figure 9. Evidence of bacterial colonization on blood agar plate of E. faecalis after completion of the procedures performed in Group 2 (control).

sterilized to be cultured for 24 hours at 37 °C and then to blood agar plates, thus determining the presence of E. faecalis in the canals.

Group 3

Root canals contaminated with E. faecalis were irrigated with 10 ml of 1% sodium hypochlorite, followed by 17% EDTA for three minutes and 5 ml of 1% sodium hypochlorite (Fig 10). They were dried with absorbent paper points.

The points were placed into tubes with BHI broth at 37 °C for 24 hours to have turbidity checked. Then, they were placed in blood agar plates and incubated at 37 °C for 48 hours.

Group 4

Root canals contaminated with E. faecalis microorganism were irrigated with 10 ml of 1% sodium hypochlorite, followed by 17% EDTA for three minutes and 5 ml of 1% sodium hypochlorite.

Root canals were then dried with sterile absorbent paper points and filled with 0.01% methylene blue Chimiolux 10 © (Chimiolux 10 - DMC Import and Export Equipment Ltd., São Carlos, SP, Brazil) (Fig 11) to reflux for five minutes. The irradiation source of the red light diode laser, of which Clean Line © (Brite Laser Max - Clean Line - Copyright, Taubaté, SP, Brazil), (Figure 13) with wavelength of 662 nm, 50 mW, 15 J was positioned at the entrance of the root canal (Fig 12), triggered for 300 seconds, as indicated by the study carried out by Komine C, Tsujimoto Y.[16]

Afterwards, root canals were dried with sterile absorbent paper points. The points were placed into tubes with BHI broth and routed to the oven at 37 °C for 24 hours to have turbidity checked. Soon after the points were placed in blood agar plates and incubated at 37 °C for 48 hours.

Analysis of colony forming units - UFC

Samples from Groups 2, 3 and 4 were analyzed for the number of colony forming units per milliliter (CFU / ml).

Test tubes were used with 10 ml saline solution with 100 µl of Fucsin stain (Fucsina Fenicada de Gram - Newprov - laboratory products, Pinhais, PR, Brazil). In the other sterile tubes, 100 µl of this solution and 10 µl of Groups 2, 3 and 4 samples were placed separately, totaling 60 portions, as each group had 20 samples. They were transferred with the aid of a pipette to the Neubauer chamber (Olympus CX-21LEDS2 5 - 6V 0.5 ª T2 SN - Olympus Corporation, Tokyo, Japan) (Fig 14). In order to count the bacteria present, only bacteria that were present in diplococcus or more were counted. The counting of all samples was performed by the same operator (Fig 15), as follows: counting the four central squares of the central quadrant, the value obtained multiplied by four and the result again multiplied by four, and finally multiplied by the number of quadrants which, in this case, are five. Therefore, the value obtained was placed in the following formula:

$$\text{Number of cells/ml} \quad \frac{\text{total number of cells}}{\text{number of counted quadrants}} \quad \text{xdilution fator x } 10000$$

With the effect of increasing reliability and standardization, all stages of this work were performed by the same operator.

Statistical analysis

Data were tabulated and submitted to statistical analysis of comparison among groups using SPSS software 20.0 IBM STATISTICS.

Figure 10. Evidence of how steps of this study were carried out inside the laminar flow.

Figure 11. Insertion of 0.01% methylene blue photosensitizer Chimiolux 10 inside the root canal, which was inside a sterile container.

Figure 12. Red Line Diode laser irradiation from Clean Line© brand.

Figure 13. Diode laser, Clean Line© brand.

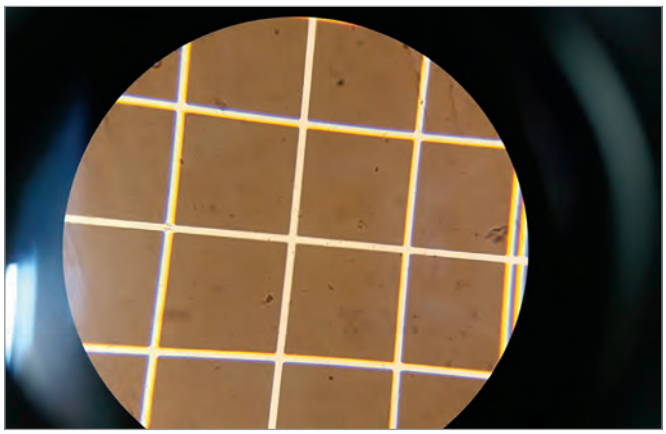

Figure 14. Microscopic view of the sample with E. faecalis in the Neubauer chamber for counting of UFC.

Figure 15. Completion of procedures for accomplishing the counting of colony forming units.

Table 1. Distribution of bacterial growth averages in different treatment groups.

Group	Bacterial growth			p*
	Mean x 10^5	Standard deviation x 10^5	Medium x 10^5	
2	13.816	2.877	13.28	0.000
3	3.552	0.543	3.520	
4	1.544	0.869	1.680	

* Kuskal - Wallis. Significance level of 5%.

Results

In Group 1, there was no bacterial growth on blood agar plates, confirming absence of Enterococcus faecalis. This group was not submitted to statistics because it was only a base group for the beginning of the experiment.

In the other groups, there was a statistically significant difference compared to the mean of bacterial growth among groups, with the lowest bacterial growth observed in Group 4, followed by Group 3, and finally Group 2, according to Table 1.

Discussion

No method of root preparation, irrigation, intracanal medication or even associations, are fully effective in eliminating microorganisms from the root canal system.[9,12,21,22,23] The present study also confirms this reality, since microbial colonization was not eliminated in any of the groups evaluated.

Difficulty in eliminating the greatest number of possible bacteria from the root canal creates the need for new techniques and methods to be investigated, aiming at a more effective endodontic treatment, therefore avoiding bacterial recolonization of the root canal system.[13]

Photodynamic therapy (PDT) is efficient at significantly reducing the viability of bacteria present in the root canal,[18,24,25] but should always be used as complementary procedure for disinfection.[20,26-29]. Studies show that alone, that is, without the aid of irrigating agents, it is not as effective, thus having its use as a substitute for traditional procedures contraindicated.[13,20,27,30,31]

PDT potential of action demonstrates greater effectiveness when associated with irrigation with sodium hypochlorite and EDTA, effectively reducing the most resistant bacterias, such as E. faecalis.[13,18,19,20,27] However, it proves unable to eradicate microorganisms present in the root canal system,[27] corroborating the results of this study.

The present study found superior effectiveness of photodynamic therapy as a complementary procedure in reducing microorganisms of the root canal system when compared to irrigation with 1% sodium hypochlorite and EDTA, thus differing from Yildirim C, Karaarslan ES, et al.[24] The authors claim that PDT would be as effective as conventional irrigation with 5% NaOCl in relation to its antimicrobial action against Enterococcus faecalis. It is possible that this discrepancy is due to difference in sodium hypochlorite concentrations used.

In this study, no optic fiber diffusers were used due to the selection of only short roots without crowns and straights. In view of these characteristics, distribution of light along the canal was facilitated, a technique supported by a study by Garcez AS, Fregnani ER, et al.[26] As follows: coupling long fiber optic tips to laser to be tested on roots with crowns led to bacterial reduction of 99.997%, whereas in another group in which the roots had WL of 13 mm and did not present a crown, the tip was not used and reduction reached 99%, similar results. In order to know if the use of the optic fiber diffuser is relevant or not, it is important to analyze the characteristics of the root canal in question. Therefore, the aim should be to increase the uniformity and distribution of light along the canal, thus allowing for better irradiation near the apex and focusing on obtaining the best possible results of photodynamic endodontic therapy.[26]

Nunes MR, Mello I et al [32] clarify that the optic fiber tip is not a determinant factor in the elimination of bacteria, but the choice of the ideal photosensitizer is. Taking as an example, methylene blue that can be photoactivated at a distance, avoiding direct contact with light, that is, the use of fiber diffusers is dispensable.[32] The 0.01% methylene blue Chimiolux 10 was selected as a photosensitizing product for the present study, as it showed this favorable characteristic, as described in the work of Usacheva MN, Teichert MC, et al,[33] and its greater efficiency in reducing the amount of Gram-positive bacteria because it was used in this research Enterococcus faecalis.

To determine the concentration, time of action and irradiation period of methylene blue used in this study, research by Komine C, Tsujimoto Y,[16] was consulted which suggested the ideal concentration to be used in PDT being from 0.001% to 0.01%, and proposed an action time of 300 seconds followed by irradiation for another 300 seconds.

Several types of lasers have been used in dental procedures, especially in Endodontics, which demonstrate some efficacy in eradication of E. faecalis.[17,27] In this study, low power laser was used because it is biocompatible with human tissues, in addition to having antimicrobial effect.It also has the potential to speed up periapical tissue repair and reduce post-instrumentation discomfort.[23] Unlike high-power lasers, which offer risks and should be used with caution, mainly because they cause increase of temperature in hard and soft tissues, causing thermal lesions in the periodontal tissues. Additionaly, they do not have antimicrobial effects.[17,29,34]

PDT is advantageous in root canals with curvature, apical deltas and in cases of secondary infections because with the aid of tips and photosensitive material they can penetrate the dentinal tubules and approach bacterial elimination. However, it is a high-cost therapy and requires greater clinical time from the dental surgeon. Each case must be analyzed as to the need to perform this treatment, always taking into consideration the cost-benefit both for the patient and the clinician.[13,20,31]

We suggest new research on the subject be carried out with the purpose of establishing an ideal, standardized, safe and feasible protocol for the use of photodynamic therapy in the dental clinic.

Conclusion

Photodynamic therapy, when associated with methylene blue photosensitizer and conventional root canal system endodontic procedures, was effective against Enterococcus faecalis in vitro, which was confirmed in this study by the quantitative decrease of these bacteria. Therefore, new studies are suggested to support the clinical applicability of PTD as A complementary procedure to conventional chemical-mechanical debridement.

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