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ORIGINAL ARTICLE

The Effectiveness of Chelating Solutions and Photodynamic Therapy in Inactivating Bacterial Lipopolysaccharides During Endodontic Therapy

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ABSTRACT

Objective: The purpose of this study was to evaluate the effects of different endodontic treatments – chelation and antimicrobial photodynamic therapy (aPDT), on the reduction of endotoxin levels in root canals.

Methods: Eighty human single-rooted teeth had their crowns sectioned, and their root canals were subsequently prepared. All samples and materials were sterilized using Cobalt-60 irradiation. Subsequently, 10 µL of fresh lipopolysaccharide (LPS) was inoculated into the root canals and incubated for 3 days. On the fourth day, experimental treatments were applied to the root canals according to the groups (n=10): [1] pyrogen-free water, [2] 0.005% methylene blue, [3] diode LASER and [4] 0.005% methylene blue + diode LASER (PDT), [5] 2.5% sodium hypochlorite, [6] 17% trisodium ethylenediaminetetraacetic acid (EDTA), [7] 10% tetrasodium EDTA, [8] 18% etidronate (HEBP). The exposure time for each solution and light irradiation was 5 minutes. The samples collected after treatment were analyzed using the Limulus Amebocyte Lysate test to quantify endotoxins. The data obtained were subjected to Kruskal-Wallis analysis followed by Dunn's test with a significance level of 5%.

Results: All treatments demonstrated efficacy in reducing endotoxin levels in root canals compared to the use of pyrogen-free water (control). A statistically significant reduction was observed in the groups treated with 17% EDTA and 18% HEBP compared to the control group.

Conclusion: Short-term application (5 minutes) of 17% EDTA and 18% HEBP chelating solutions significantly reduced LPS in root canals and may be effective adjuncts in endodontic therapy.

Keywords: Ethylenediaminetetraacetic acid, endodontics, lipopolysaccharide, phototherapy

HIGHLIGHTS

- The different approaches used — PDT with 0.005% methylene blue, diode laser, 2.5% NaOCl, 17% trisodium EDTA, 10% tetrasodium EDTA, and 18% HEBP — exhibited an effect on endotoxin inactivation.
- The contact of methylene blue with LPS was sufficient to reduce its detection in the solution, even without diode laser irradiation.
- Among the irrigants tested, 17% EDTA and 18% HEBP solutions performed superiorly, significantly reducing LPS levels.

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INTRODUCTION

The primary objective of endodontic treatment in cases of pulp necrosis and infection is to reduce the microbial and endotoxin load within the root canals to levels that allow periapical tissue repair (1). However, anatomical complex-

ities can contribute to the persistence of microorganisms and their virulence factors, potentially leading to clinical symptoms and periapical bone resorption, which may compromise the overall prognosis of the treatment (2, 3).

Lipopolysaccharide (LPS), also known as endotoxins (1), is a component of the outer membrane of Gram-negative bacteria and is released during bacterial multiplication or lysis (4). LPS stimulates the release of various chemical mediators and pro-inflammatory cytokines (5). Additionally, it has a strong ability to diffuse through dentin (6), where it can trigger a range of tissue changes, including inflammatory responses, bone resorption, and intense pain (3, 7, 8).

The removal of LPS is particularly challenging due to its strong affinity for mineralised tissues such as cementum and bone (9). Although commonly used irrigants, such as sodium hypochlorite, enhance canal decontamination, they do not inactivate LPS (10–13). Trisodium ethylenediaminetetraacetic acid (EDTA) may indirectly support LPS inactivation, possibly by altering the mineral environment through chelation (14), as lipid A, the primary structural component of LPS responsible for its toxic effects (2), is calcium-bound.

Additionally, novel high-pH chelating solutions have demonstrated promising clinical outcomes, including effective *smear layer* removal with minimal dentin damage. One such solution is etidronate (HEBP), a bisphosphonate compound (15). Therefore, investigating the potential of these high-pH chelating agents for LPS inactivation would be of significant value.

Antimicrobial photodynamic therapy (aPDT) has demonstrated efficacy in eliminating a wide range of microorganisms, including bacteria, viruses, and fungi, by generating singlet oxygen, a cytotoxic species produced when a photosensitising compound is activated by light of a specific wavelength (16–20). The most commonly used photosensitisers in aPDT are toluidine blue and methylene blue, both phenothiazine-based dyes (21). These compounds can penetrate bacterial cell walls due to their cationic charge and have been shown to bind to LPS in Gram-negative bacteria (22).

To date, there is no available evidence regarding the ability of HEBP or tetrasodium EDTA to reduce or inactivate bacterial LPS. In contrast, previous studies have shown that both trisodium EDTA and photodynamic therapy (PDT) possess antimicrobial activity, with the potential to damage bacterial structures and, consequently, indirectly contribute to LPS neutralisation. In this context, the present study aimed to evaluate the effectiveness of different chelating solutions, as well as PDT, in eliminating bacterial LPS. The null hypothesis is that the treatments' ability to eliminate LPS will not differ.

MATERIALS AND METHODS

This study was approved by the local ethics committee (#28171519000005417). An effect size of 0.54, an alpha level (α) of 0.05, and a power of 0.95 (G*Power 3.1 for Macintosh; Heinrich Heine University of Düsseldorf, Germany) were used to calculate the sample size, indicating that each of the eight experimental groups should consist of ten specimens (23).

Sample Selection and Preparation

Eighty freshly extracted single-rooted teeth were selected based on mesiodistal and buccolingual radiographs. The specimens had fully developed roots with single root canals and were free from root resorption or canal calcification.

The crowns were sectioned using a low-speed diamond disc to standardise the specimen length to 14 mm. The canals were explored with #10 and #15 K-files until the tip of the instrument was visible; the working length (WL) was then established as 1 mm short of this measurement. Root canal preparation was performed to the WL using Reciproc 25.07 and 40.04 files (VDW GmbH, Munich, Germany), with 2.5% sodium hypochlorite (NaOCl) used throughout the instrumentation. Following preparation, canals were filled with 17% EDTA for 3 minutes. All specimens then underwent ultrasonic baths for 15 minutes in each of the following solutions: 2.5% NaOCl, 17% EDTA, and pyrogen-free water.

The apical region was sealed with light-cured composite resin, followed by application of two layers of epoxy adhesive on the external root surfaces (24). The samples were then fixed in cell culture plates using chemically activated acrylic resin. Finally, all materials used in the experiment were sterilised with Cobalt-60 gamma radiation (IPEN, São Paulo, Brazil) (25), then the specimens were subjected to contamination.

Endotoxin Inoculation

Oliveira et al. (11) described the inoculation of *Escherichia coli* (*E. coli*) 055:B5 endotoxin. The procedure was repeated three times, with a one-day interval between each inoculation.

Treatment Groups

Twenty-four hours later, the specimens were divided into eight groups (N=10): Group 1: Apyrogenic saline solution (pH≅6) (positive control); Group 2: Chimiolux Methylene Blue 0.005%; Group 3: 660nm Diode LASER; Group 4: PDT; Group 5: 2.5% NaOCl (pH≅11.8); Group 6: 17% Trisodium EDTA (pH≅7.3); Group 7: 10% Tetrasodium EDTA (pH≅12.2); Group 8: 18% HEBP (pH≅10.8). An additional negative control group (non-contaminated specimens) was included to confirm the absence of LPS and validate the sterility of the experimental protocol.

Irrigation was performed with 3 mL of each tested solution for 5 minutes using 1 mL insulin-type syringes with 13 mm×0.45 mm needles (length×diameter) ((Fig 1). In Groups 3 and 4, irradiation was applied for 5 minutes using an optical fiber attached to the laser device (Fig. 2), employing up-and-down and spiral movements within the root canal. After treatment, all remaining content was aspirated, and the root canals were irrigated with 3 mL of apyrogenic water to remove any residual irrigating solution, totaling an irrigation volume of 6 mL per group. Each specimen was then filled with apyrogenic water, and a new pyrogen-free syringe and needle were used to agitate, aspirate, and store the solution in microtubes. This procedure was repeated until 1 mL of solution was collected for each sample. The collected material was initially stored at -20°C for 24 hours, then transferred to -80°C refrigeration until further analysis.

Figure 1. The tested solutions were applied to the root canals using apyrogenic insulin syringes and needles.

Quantification of LPS

LPS quantification was performed using the Limulus Amebocyte Lysate (LAL) kinetic chromogenic assay, following the protocols described by Oliveira et al. (11) and Maekawa et al. (24).

Statistical Analysis

Data normality was assessed prior to analysis. Group comparisons were performed using the Kruskal–Wallis test, followed by Dunn's post hoc test. Statistical analyses were conducted using GraphPad Prism version 8.0, with the significance level set at $p<0.05$.

RESULTS

All treated groups demonstrated efficiency in reducing LPS levels in the main root canal. The contact of methylene blue with LPS alone was sufficient to reduce its detection in solution, even without diode laser irradiation. Furthermore, no significant difference was found between the treated groups and the positive control ($p>0.05$). These findings suggest that the endotoxin was either degraded or underwent structural changes significant enough to interfere with its reactivity in the LAL assay.

Among the irrigants, the 17% EDTA and 18% HEBP solutions demonstrated superior performance, significantly reducing endotoxin levels compared to the pyrogen-free water group ($p<0.05$) (Table 1).

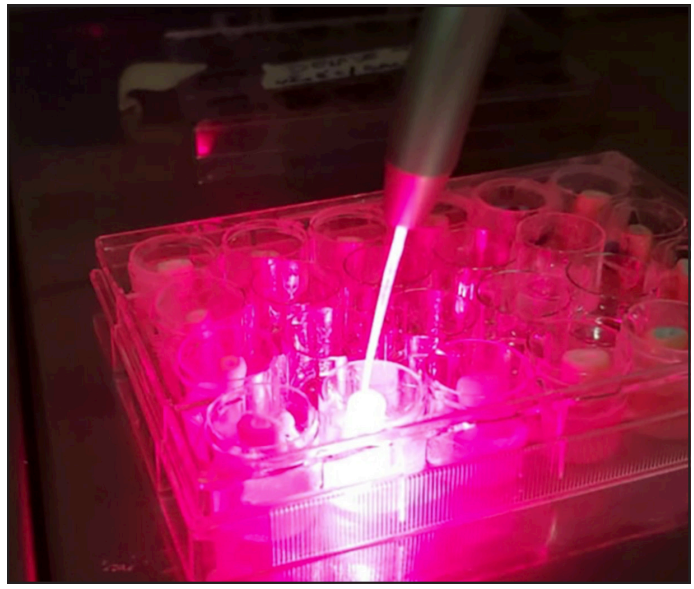

Figure 2. . Irradiation of the root canals using an optic fiber attached to the LASER device.

DISCUSSION

The present study used *E. coli* endotoxin, which is commonly employed in *in vitro* studies evaluating LPS quantification (1, 7, 25, 26). LPS contains a component known as lipid A, responsible for its biological activity, and its structure is highly conserved among various Gram-negative bacterial species (1).

PDT has been shown to be a potent antimicrobial strategy in endodontics (27, 28), with the ability to inactivate endotoxins, as demonstrated in previous studies (20, 29, 30). However, similar results were not observed in the present study regarding LPS inactivation by PDT. Specifically, specimens treated with methylene blue and diode laser irradiation showed no significant difference compared to the apyrogenic saline solution group. This discrepancy with previous findings may be attributed to the choice of photosensitising agent. Notably, studies reporting greater endotoxin inactivation employed Toluidine Blue O rather than methylene blue. Additionally, Rabello et al. (31) observed that although PDT, when used as an adjunct to endodontic treatment, improved root canal decontamination, it did not result in satisfactory endotoxin elimination.

The photosensitising agent is activated upon irradiation with light of an appropriate wavelength, producing highly reactive oxygen-derived cytotoxic species (32, 33). Phenothiazine dyes are capable of binding to and penetrating the outer layer of LPS, potentially altering the structure of lipid A, which carries a strong negative charge (22). In the present study, methylene blue was selected as the photosensitising agent; however, it did not interact with LPS strongly enough to produce a significant reduction compared to the control group.

To exert its antimicrobial effect, PDT requires a light source within the visible spectrum. In this study, a 660 nm red light emitted by a low-energy diode laser was used. Due to its coherence properties, laser light can be guided into the root canal using an optical fiber (34). However, unlike high-ener-

TABLE 1. Median (me), minimum and maximum (min-max) values for remaining endotoxin (EU/mL) from root canals for each experimental group

Endotoxin remain (EU/mL)							
Apyrogenic saline solution	**Methylene blue**	**Laser**	**PDT**	**2.5% NaOCl**	**17% EDTA**	**10% EDTA**	**18% HEBP**
2.00 (0.87–10.33)[A]	1.21 (0.47–9.40)*	3.56 (0.48–5.46)*	1.32 (0.18–9.41)*	1.00 (0.31–5.81)*	0.38 (0.06–2.15)[B]	1.19 (0.11–3.42)*	0.90 (0.00–1.54)[B]

*: No statistically significant differences. Comparison by Kruskal-Wallis and Dunn post hoc tests ($p<0.05$). Different superscript capital letters in a column represent significant differences between groups. PDT: Photodynamic therapy, NaOCl: Sodium hypochlorite, EDTA: Ethylenediaminetetraacetic acid, HEBP: Etidronate

gy or ablative lasers, diode laser light alone is not capable of achieving effective disinfection (32). Consistently, this study observed low levels of endotoxin inactivation in the group treated solely with diode laser light, with no significant difference compared to the control group. Similarly, Giannelli et al. (35) reported that diode laser irradiation alone was ineffective in inactivating endotoxins when evaluating the disinfection of titanium implants contaminated with LPS using different laser types.

Some studies (36, 37) used absorbent paper points to collect endotoxins from the canal; however, this method has limitations, as it cannot access endotoxins located in the dentinal tubules and irregularities of the root canal system. In the present study, endotoxin collection was performed by filling the samples with pyrogen-free water using a new needle, which allowed a larger amount of substrate to be agitated and aspirated for analysis. Additionally, the needle was passed along the dentinal walls to collect contaminated dentine scrapings.

To date, the literature indicates that calcium hydroxide remains the most effective and widely used substance for neutralising bacterial LPS (1, 11, 25, 38), primarily through the hydrolysis of Lipid A, which leads to the formation of fatty acids (13). However, ongoing research aims to identify alternative treatments to calcium hydroxide that can effectively and permanently neutralise LPS within root canal systems.

Due to its ability to dissolve organic tissues and its strong antimicrobial activity, NaOCl is the primary irrigating solution used in endodontic treatment (23). In the present study, a 2.5% concentration was used; however, it did not effectively reduce the endotoxin levels present in the root canal. This finding is consistent with previous studies (12, 13, 23, 39, 40), which reported that even at higher concentrations, sodium hypochlorite exhibited low or no efficacy in neutralizing LPS.

Trisodium EDTA removes the *smear layer* formed during chemomechanical decontamination, resulting in improved cleaning and sealing of dentinal tubules by filling materials (41). In the present study, EDTA effectively reduced LPS levels in the root canal compared to samples treated with pyrogen-free water. This reduction can be attributed to EDTA's ability to bind to the endotoxin structure (14), thereby enhancing its disaggregation (42).

HEBP, like EDTA, has a strong capacity to remove the *smear layer* while causing less damage to dentine (15). According to the present results, only the 18% HEBP and 17% EDTA solutions significantly reduced endotoxin levels compared to the control group. This effect is likely attributable to the ability of both chelating agents to promote Lipid A hydrolysis—whether through their alkaline pH, concentration, or chelating action—thus inactivating LPS.

A 10% Tetrasodium EDTA solution was expected to yield favorable results due to its high pH (12.2). However, although it showed greater efficacy than the 2.5% NaOCl group, it did not demonstrate a significant difference when compared to the apyrogenic saline solution group.

Both EDTA and HEBP cause damage to the cell wall of Gram-negative bacteria by chelating calcium ion (Ca^{2+}), which increases the permeability of the bacterial cell membrane to substances and creates "cracks" in the LPS layer (43). The interaction of these chelating agents with this endotoxin prevents it from forming aggregated structures, thereby facilitating its removal during irrigation and consequently reducing its ability to bind to host immune cells and its proinflammatory activity (36).

This study presents limitations inherent to its *in vitro* nature, which must be considered when interpreting the results. Although the experimental model allows for strict control of variables and standardised procedures, it does not accurately reproduce real clinical conditions. Furthermore, a single irrigation time of 5 minutes was used for all tested substances; however, longer irrigation times with potent chelating agents, such as those used in this study, may weaken the root dentin (44). Therefore, additional studies are necessary to complement these findings and validate their clinical applicability.

CONCLUSION

Among the various protocols tested, the chelating solutions of 17% trisodium EDTA and 18% HEBP were effective in reducing LPS levels in the root canal. In contrast, PDT using a 660 nm diode laser combined with 0.005% methylene blue did not demonstrate comparable effectiveness. Further studies evaluating variations in contact time and concentration of these solutions are necessary to optimise LPS elimination during endodontic treatment.

Disclosures

Ethics Committee Approval: The study was approved by the Bauru School of Dentistry - University of São Paulo Ethics Committee (no: #28171519000005417, date: 17/01/2021).

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