

UNIVERSIDADE DE SÃO PAULO  
FACULDADE DE ODONTOLOGIA DE BAURU

DENISE FERRACIOLI ODA

**Effect of PDT, and new chelating solutions on  
inactivation of bacterial LPS**

**Efeito da PDT, e de novas soluções quelantes na  
inativação do LPS bacteriano**

BAURU

2020



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Tese constituída por artigo apresentada à Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutor em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Endodontia.

Orientadora: Profa. Dra. Flaviana Bombarda de Andrade

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# FOLHA DE APROVAÇÃO





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## DEDICATÓRIA

*Dedico este trabalho aos meus amados pais, Nelson e Rosana, verdadeiros responsáveis por toda conquista que já alcancei.*

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[...Você nunca sabe que resultados virão de sua ação.  
Mas se você não fizer nada, não existirão...]

***Mahatma Gandhi***

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## RESUMO

**OBJETIVO:** Nas necropulpectomias, além da eliminação de micro-organismos e neutralização de lipopolissacarídeos (LPS), presentes no sistema de canais radiculares, é fundamental, uma vez que esta endotoxina está altamente relacionada aos casos de insucesso e de sintomatologia dolorosa. Este trabalho avaliou a capacidade da Terapia fotodinâmica (PDT) e de diferentes irrigantes, para inativar o LPS bacteriano. **MATERIAIS E MÉTODOS:** Foram utilizados 80 dentes humanos unirradiculados, analisados previamente por imagens radiográficas para confirmação de canal único. A porção coronária foi removida para a obtenção de 14mm de segmento radicular, cujos canais foram devidamente preparados. As amostras foram posicionadas verticalmente em placas de cultura de 24 poços e fixados com resina acrílica. As amostras e todos os materiais a serem utilizados na pesquisa foram esterilizados, previamente, em radiação Gama cobalto 60. No interior da cabine de fluxo laminar, 10µL de LPS de *Escherichia coli* foram inoculados no canal de cada amostra, e mantidas durante 24 horas em estufa bacteriológica. O procedimento de inoculação foi repetido mais 2 vezes e então as amostras foram divididas nos seguintes grupos (n=10): [G1] hipoclorito de sódio a 2,5%; [G2] água apirogênica; [G3] EDTA trissódico a 17%; [G4] EDTA tetrassódico a 10%; [G5] Etidronato a 18%; [G6] Azul de metileno a 5%; [G7] LASER de diodo e [G8] PDT (azul de metileno + LASER de diodo). O tempo de ação das soluções irrigadoras, do corante e da irradiação, foi de 5 minutos para todos os grupos. Após o tratamento, os canais foram lavados e preenchidos com 5mL de água apirogênica, onde a mesma foi agitada e aspirada por uma agulha/seringa apirogênica. O conteúdo aspirado de cada espécime foi mantido em refrigeração a -80°C até posterior análise pelo método Limulus Amebocyte Lysate. Os dados obtidos não passaram no teste de normalidade Shapiro Wilks, sendo então submetido ao teste de Kruskal Wallis seguido de Dunn. **RESULTADOS:** Os dados obtidos apresentaram redução significativa apenas nos grupos G3 (p = 0,0452) e G 5 (p = 0,0097) em comparação ao grupo controle G2. A mediana (Mínima/Máxima) da porcentagem de endotoxina remanescente foram: [G1] = 1.005 (0.3100/5.810); [G2] = 2.010 (0.8700/10.33); [G3] = 0.3800 (0.060 /2.150); [G4] = 1.195 (0.110/3,420); [G5] = 0.9000 (0.0/1.540); [G6] = 1.320 (0.1800/9.410); [G7] = 1.210 (0.4700/9.400); [G8] = 3.560 (0.4800/5.460). **CONCLUSÃO:** A PDT não apresentou eficácia em neutralizar

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o LPS, e dentre as soluções avaliadas, o EDTA trissódico a 17% e o etidronato a 18, foram eficazes em inativar a endotoxina bacteriana.

**Palavras-chaves:** Endotoxinas. EDTA. Etidronato. Fotoquimioterapia.

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## ABSTRACT

**INTRODUCTION:** In the necropulpectomies, in addition to the elimination of microorganisms and to neutralization of lipopolysaccharides (LPS), present in root canal system, it is fundamental, since that endotoxin is highly related to unsuccessful cases with painful symptomatology. **OBJECTIVE:** The present study evaluated the capacity of Photodynamic Therapy (PDT) and the different irrigants, to inactivate the bacterial LPS. **MATERIALS AND METHODS:** Eighty single-rooted human teeth were used, previously analyzed by radiographic images to confirm the single canal. The coronary portion was removed to obtain 14mm segment of the root, where the canals were properly prepared. The samples were placed vertically in 24-well plates and fixed with acrylic resin. The samples and all the materials used in the study were previously sterilized with Cobalt-60 Gamma radiation. Inside the laminar flow cabinet, 10 $\mu$ L of LPS from *Escherichia coli* were inoculated inside in root canal of each sample and kept for 24 hours in a bacteriological oven. The inoculation procedure was repeated twice more and then the samples were divided into the following groups (n = 10): [G1] sodium hypochlorite at 2.5%; [G2] pyrogen-free water; [G3] trisodium EDTA at 17%; [G4] tetrasodium EDTA at 10%; [G5] Etidronate at 18%; [G6] Methylene blue at 5%; [G7] diode LASER; [G8] PDT (methylene blue + diode LASER). The time for action of the irrigating solutions, dye and irradiation, was 5 minutes for all groups. After the treatment, the canals were cleaned and filled with 5mL of pyrogen-free water, where it was agitated and aspirated by a pyrogen-free needle/syringe. The aspirated content of each specimen was kept under refrigeration at -80°C until posterior analysis of Limulus Amebocyte Lysate. The obtained data did not pass the Shapiro Wilks normality test, so they were submitted to the Kruskal-Wallis test followed by the Dunn's test. **RESULTS:** The obtained data presented relevant reduction only in the groups G3 (p = 0.0452) and G5 (p = 0.0097), in comparison to the control group G2. The medium percentage (Minimum/Maximum) of remaining endotoxin was: [G1] = 1.005 (0.3100/5.810); [G2] = 2.010 (0.8700/10.33); [G3] = 0.3800 (0.060/2.150); [G4] = 1.195 (0.110/3,420); [G5] = 0.9000 (0.0/1.540); [G6] = 1.320 (0.1800/9.410); [G7] = 1.210 (0.4700/9.400); [G8] = 3.560 (0.4800/5.460). **CONCLUSION:** The PDT did not present efficacy in neutralizing the LPS and, among the evaluated solutions, trisodium EDTA

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at 17% and etidronate at 18% presented efficacy in inactivating the bacterial endotoxin.

**Keywords:** Endotoxin. EDTA. Etidronic acid. Photochemotherapy.

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# **1 INTRODUCTION**

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## 1 INTRODUCTION

The necropulpectomy aims to obtain maximum reduction of the microorganisms present in the root canal system, allowing for the repair of the periapical tissues from the host immunopathological response. However, even after the removal of microorganisms, the presence of endotoxin is possible. The endotoxin is capable of inducing or maintaining periapical lesions if it remains in the root canal system, predisposing to an unsuccessful treatment (1).

Those endotoxins, called lipopolysaccharides (LPS), are present in the outer membrane of Gram-negative bacteria, which are released during the multiplication or lysis of these microorganisms. LPS is composed of: a polysaccharides chain that works as a specific antigen of bacterium, called O-Antigen; a group of oligosaccharides, called Core; and a lipid structure, known as Lipid A, responsible for toxic effect of the LPS (2).

When the LPS is released in the organism, it links to a receptor complex, such as CD14, TLR4, and MD2 and activates the release of a great number of chemical mediators and proinflammatory cytokines of immunocompetent cells, mainly macrophages (3). Thus, the LPS, in indirect way, causes a set of tissue alterations, inducing severe inflammatory reactions, bone resorptions, and highly painful symptomatology (1, 4, 5).

It is difficult to eliminate or inactivate the LPS of the root canal system since it irreversibly links to mineralized tissues, both bone and cementum (6). Furthermore, a study by de Oliveira *et al.*, in 2005 (7), showed that endotoxin presents high capacity to diffuse through the dentinal tubules, being able to reach the external root surface in just 24 hours.

During the endodontic treatment, irrigating solutions are used with good antimicrobial capacity, however, most of those solutions are not able to inactivate the by-product of Gram-negative bacteria (8, 9). In 2003, Tanomaru *et al.* (10) evaluated sodium hypochlorite solutions in different concentrations (1%, 2.5%, and 5%) and 2% chlorhexidine, but the inactivation of the LPS was not achievable. In 2001, Buck *et al.*

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(11) obtained similar results that verified that sodium hypochlorite solutions at 2.6%, chlorhexidine at 0.12%, and EDTA at 15% were not able to degrade the endotoxin. However, when the authors used a high pH mixture containing the same sodium hypochlorite, chlorhexidine, and ethanol concentrations, it was able to inactivate the LPS after 30 minutes by Lipid A hydrolysis.

Although the trisodium EDTA has not presented high capacity in inactivating the LPS, there is a study in where chelating solutions have the potential for linking to the calcium present in the part of Lipid A (12). New and high pH chelating solutions, such as tetrasodium EDTA and Etidronate, have presented satisfactory clinical results. Recent studies showed that those solutions remove the smear layer with lesser aggression to the dentin (13). However, there are presently no studies in the literature regarding the influence of high pH of those chelating solutions on the inactivation of the LPS.

Until now, *in vitro* (14, 15, 16) and *in vivo* studies (17, 18) showed that calcium hydroxide-based medication is still the most efficient method to neutralize the LPS. Because of its high pH, the calcium hydroxide-based medication can inactivate the endotoxin by lipid A hydrolysis, resulting in its degradation and formation of free fatty acids (11).

Different studies search for new resources capable of neutralizing or reducing the LPS effects. LASER is one of those resources that has been presenting promising results, such as Nd:YAG that can reduce the activated macrophage pro-inflammatory response caused by the LPS (19). Another treatment that also uses LASER is photodynamic therapy (PDT). In that case, it uses the low-power LASER, and the treatment is based on a combination of three obligatory factors: source of visible light, photosensitizing agent (dye), and molecular oxygen. The PDT presents high antimicrobial capacity (20, 21), mainly when it is combined with a conventional endodontic treatment (22).

Within the categories of phenothiazine dyes are toluidine blue and methylene blue, considered the most photosensitizing agents used in dental PDT (23). Their use can be explained because phenothiazine dyes are able to penetrate the bacterial wall, since its cation charge easily links to the negative charge of the lipopolysaccharides

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present in Gram-negative bacteria (24). Furthermore, in 2011, Giannelli *et al.* (25) showed that both methylene blue and red light (LASER) and red dye and blue light (LED), in the PDT were able to markedly reduce the macrophage activity stimulated by the LPS.

The present study evaluated, *in vitro*, the capacity of the photodynamic therapy (diode LASER + Methylene blue) and of chelating solutions, such as trisodium EDTA at 17%, tetrasodium EDTA at 10%, and etidronate at 18%, in inactivating the LPS present in the root canal. The null hypothesis was that the chelating solutions and the photodynamic therapy are able to inactivate the endotoxin in similar ways.



**2 ARTICLES**

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## **2 ARTICLES**

**2.1 ARTICLE 1** - Does photodynamic therapy interfere in the lipopolysaccharide neutralization?

**2.2 ARTICLE 2** - The capacity of different chelating solutions to inactivate the bacterial endotoxin.



## 2.1 ARTICLE 1 - Does photodynamic therapy interfere in the lipopolysaccharide neutralization?

### ABSTRACT

**Background:** The photodynamic therapy (PDT), initially proposed to combat tumor cells, had its clinical application increased in the area of dentistry due to its effective disinfection. This study assessed the influence of the PDT in the neutralization of lipopolysaccharides (LPS). **Methods:** Human single root teeth had their crowns sectioned and the root canals prepared using a mechanical technique until 40.04 diameter/taper. Sterilization procedure was performed in gamma radiation to eliminate microorganisms and remaining LPS. Next, 10 $\mu$ L of fresh *E. coli* LPS was inoculated into the root canals for 3 days. On the fourth day, the treatments were executed according to the following groups (n=10): [1] pyrogen-free water, [2] methylene blue 0.005%, [3] diode LASER, [4] methylene blue 0.005% + diode LASER, where the action time for each experimental solution and light irradiation was 5 minutes. After, the pyrogen-free water was placed and agitated into the root canal, then it was collected until completing 1mL for each specimen and stored in -80°C refrigeration for 1 month. The collected solutions were analyzed with the Limulus Amebocyte Lysate test to quantify the endotoxin. The obtained data were submitted to the normality test, and Kruskal-Wallis analysis followed by the Dunn's test. **Results:** The data analysis did not detect a statistical difference ( $p = 0.3341$ ) among the experimental groups. The median (minimum/maximum) percentage of reminescent endotoxin were: [Group 1] = 2.010 (0.8700/10.33); [Group 2] = 1.320 (0.1800/9.410); [Group 3] = 1.210 (0.4700/9.400); [Group 4] = 3.560 (0.4800/5.460). **Conclusion:** Based on these data, the PDT, and its components separately, was not effective to inactivate the Gram-negative endotoxin present in the root canal.

**Key-words:** lipopolysaccharide, phototherapy, methylene-blue.

### INTRODUCTION

In the effort to obtain success in endodontic treatment, the reduction of microorganisms and their remaining endotoxins in the root canal system is necessary [5]. Even after reducing most microorganisms, the presence of remaining endotoxins

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hinders the repair of periapical inflammatory lesions [30]. Lipopolysaccharide (LPS) is an endotoxin present in the cell wall of Gram-negative bacteria and it is released during bacterial multiplication or death [3]. It is composed of the Antigen-O, a chain of polysaccharides that functions as a specific antigen of a bacterium, the Core, a group of oligosaccharides, and the Lipid-A, a lipid structure responsible for the toxic effect of LPS [19].

When released into the organic tissues, the LPS binds to a receptor complex, such as CD14, TLR4, MD2, and activates the release of a large number of chemical mediators and pro-inflammatory cytokines of immunocompetent cells [32]. Thus, indirectly, the LPS causes a series of tissue changes, inducing severe inflammatory reactions, bone resorption, as well as the occurrence of highly painful symptoms [17, 39, 40]. Furthermore, there is great difficulty to eliminate or inactivate the LPS present in the root canal system since it binds irreversibly to mineralized tissues and both bone and cement [31]. In addition, a study by Oliveira et al. in 2005 [25], demonstrated that endotoxins have a high capacity to diffuse through the dentinal tubules, reaching the external surface in just 24 hours. In the sealed root canal, the endotoxin is able to penetrate through the obturation material and reach the periapical tissue in 23 days [36], faster than bacteria which requires 62 days [2].

Although endodontic treatment can reduce the number of bacteria in the root canal, the antimicrobial solutions used during the biomechanical preparation are unable to inactivate the byproduct of Gram-negative bacteria [1, 24, 35]. In 2003, Tanomaru et al. [35] analyzed the irrigation solutions most used in endodontic treatment and showed that 2% chlorhexidine and 1%, 2.5% and 5% sodium hypochlorite are not successful in inactivating the LPS. Furthermore, in a clinical study, Gomes et al (2009) showed that the irrigant solutions 2.5% NaOCl and 2% CHX have no detoxifying effect on endotoxins [10].

In the search for a better method to reduce the bacteria and their byproducts in the root canal, alternative procedures are often tested. Photodynamic therapy (PDT) has shown efficacy in the elimination of microorganisms, including bacteria, viruses, and yeasts due to the formation of singlet-oxygen, a cytotoxic species produced by the interaction of a photosensitizing compound with a light of an appropriate wavelength [26, 27]. The photosensitizing agents most used in dental PDT are toluidine blue and

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methylene blue, which are phenothiazine-based dyes [13]. Their use can be justified by the fact that these dyes manage to cross the bacterial wall since their cationic charge is easily linked to the negative charge of lipopolysaccharide present in Gram-negative bacteria [37]. Furthermore, in 2011 Giannelli et al. [9] demonstrated that PDT using methylene blue and red light (630nm), both LED and LASER, were able to markedly reduce the activity of macrophages stimulated by LPS.

## OBJECTIVE

The present study assessed, *in vitro*, the capacity of photodynamic therapy (diode LASER 660nm + 0.005% Methylene blue) in inactivating the LPS present in the root canal. The null hypothesis is that the photodynamic therapy will inactivate the endotoxin.

## MATERIAL AND METHODS

The present study was submitted and approved by the Research Ethics Committee. Forty-eight human single root canal teeth (incisors, canines and premolars) were obtained by means of Dental Clinic donation. During the first 24 hours, the teeth were cleaned and placed in 10% formalin, and then stored in saline until time of use.

### **Samples Preparation**

By means of radiographs, only single straight root canal human teeth were used in this study. The crowns were sectioned with a low-speed diamond disk, obtaining 14mm of the root. The root canal was explored using 10 and 15 K-files and prepared in all its extension with nickel-titanium Reciproc 25.07 and 40.04 files (VDW GmbH, Munique, German). The 2.5% sodium hypochlorite solution (Rioquímica, São José do Rio Preto, SP, Brasil) was used throughout the instrumentation procedure and 17% ethylenediaminetetraacetic acid (Biodinâmica – Ibiporã – Paraná – Brasil) as a final irrigation. To eliminate as much debris as possible, all specimens were submitted in ultrasonic agitation for 15 minutes in each solution: 2.5% sodium hypochlorite, 17% EDTA and saline solution.

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After the cleaning, a light-curing composite resin (Natural Look - DFL Industria e Comércio S.A. Taquaral, RJ) was used to seal the apical foramen, and all external root surface was waterproofed by two layers of epoxy adhesive (Araldite Brascola, São Paulo, Brazil), according to the Maekawa et al. 2011 [18].

The 12 specimens per plate were randomly distributed into 24-well cell culture plates and chemically activated acrylic resin was used to maintain the specimens in a vertical position (JET - Clássico – São Paulo – Brazil). Finally, the plates containing the specimens and all materials to be used in the research were submitted to gamma radiation sterilization (IPEN - São Paulo, Brazil).

### **Endotoxin Inoculation**

The present study used the endotoxin of *Escherichia coli* (n° 055:B5, Sigma, St Louis, USA) in the concentration of 200µg/mL, which was inoculated in the amount of 10µL in the root canal of the specimens. The plates were placed in a bacteriological oven at 37°C, 100% relative humidity for 24 hours [23]. The inoculation procedures were repeated daily to complete a total of three inoculations.

### **Experimental Groups**

Twenty-four hours after the last endotoxin inoculation, the root canals were dried with sterilized paper points and then treated according to the following experimental groups (n = 10):

Group 1: Pyrogen-free water (Eurofarma, Itapevi, SP, Brazil);

Group 2: Methylene Blue Chimiolux 0.005% (DMC Equipamentos LTDA. São Carlos, Brazil);

Group 3: Diode LASER 660nm (MMOptics LTDA, São Carlos, SP, Brazil);

Group 4: Photodynamic Therapy (Methylene Blue Chimiolux 0.005% + Diode LASER 660nm).

During the experiment, the root canals were filled with the evaluated solutions for 5 minutes, using pyrogen-free insulin-type syringes/needles, except the Groups 3 of the LASER irradiation. In the Group 4, the methylene blue was used for 5 minutes as pre irradiation time before the LASER. In Groups 3 and 4, the irradiation was

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performed for 5 minutes with an optic fiber attached to the LASER device, using up and down and spiral movements, according demonstrated in the Figure 1. At the end of the experiment, all samples have the root canals dried with sterilized paper points.

### **Collection of the remaining endotoxin**

The root canals were filled with pyrogen-free water, and using a new pyrogen-free syringe/needle, the solution was agitated, aspired, and stocked inside microtubes. That procedure was repeated until completing 1mL of the collected solution for each specimen. In the first 24 hours, the collected material was maintained under -20° C refrigeration and then under -80° C refrigeration until further analysis.

### **Limulus Amebocyte Lysate (LAL) Analysis**

In a 96-well cell culture plate, the following solution in duplicate were placed: pyrogen-free water (blank), endotoxin standards (LPS in 5%, 0.5%, 0.05% e 0.005% concentrations) and collected solutions from the root canal of the specimens. Subsequently, the plate was incubated in a QCL kinetic reader (Cambrex – São Paulo, SP, Brazil), attached to a microcomputer containing a Wink QCL software. After 10 minutes of incubation, 100 µL of the LAL chromogenic kinetic reagent were added in each well of the plate using an 8-channel multi pipette and pyrogen-free tips.

The plate reader software monitored the time necessary to increase the absorbance of each well by means of a linear log/log correlation of the reaction time and corresponding LPS concentration. Thus, the reports of the standard curve parameters and values of the number of LPS present in the specimens were obtained.

### **STATISTICAL ANALYSIS**

The collected data did not present homogeneity of variance in normality testing ( $\alpha=0.05$ ). Kruskal-Wallis analysis, followed by the Dunn's multiple comparison test was used to detect the difference between intra and inter-groups. All hypotheses were tested at a 95% confidence level.

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## RESULTS

The use of the methylene blue for 5 minutes showed the lowest average percentage of remniscent endotoxin. However, in the data groups comparison, a statistical difference was not observed ( $p = 0.3341$ ) between all the experimental treatments and the positive control. Median (minimum/maximum) percentages of the remniscent endotoxin were: [Group 1] = 2.010 (0.8700/10.33); [Group 2] = 1.320 (0.1800/9.410); [Group 3] = 1.210 (0.4700/9.400); [Group 4] = 3.560 (0.4800/5.460), according demonstrated in the Figure 2.

## DISCUSSION

The present study used the endotoxin derived from *Escherichia coli* bacteria, which is considered the standard endotoxin and the most used in studies with LPS *in vitro* [3,20,25,34]. In addition, so that the experiment was not affected by the presence of pre-existing endotoxins, all the specimens and materials used were sterilized in 60 Cobalt-Gamma-rays [6].

In the presence of molecular oxygen, the photosensitizing agent is stimulated when it is irradiated by light of an appropriate wavelength, producing a highly reactive oxygen-derived cytotoxic species [11]. As a photosensitizing agent, there are the phenothiazinic dyes, which were able to bind and cross the bacterial wall, affecting the structure of the Lipid A [37]. Lipid A is the structure responsible for the toxic effect of LPS [19] and it carries a total negative load [29]. In the present study, the photosensitizing agent chosen for the experiment was the Methylene blue, a dye that presents the peak of reactivity when stimulated by light within the red spectrum. However, meanwhile the Toluidine Blue-O demonstrated a dramatic effect on the biological activities of LPS [16], the Methylene blue was not able to inactivate significantly ( $p < 0.05$ ) the LPS, in comparison to the control group.

To compose the triad needed to perform the PDT (dye + oxygen + light), it is necessary to use a light within the visible light spectrum. The light source used in the present study was the red light 660nm derived from the low-energy diode LASER, and due to its coherence property, it is possible to guide the LASER light into the root canal

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by an optical fiber [21]. However, unlike high-energy or LASER ablatives, the diode LASER light is not able to promote favorable disinfection when used alone [8]. The present study obtained low values of inactivation of the endotoxin in the group treated only by the diode LASER light, not showing a significant difference to the control group ( $p < 0.05$ ). In 2011, Giannelli et al. [9] obtained a similar result when comparing different LASER lights to disinfect titanium implants contaminated with LPS and found that the isolated use of the diode LASER was inefficient to promote inactivation of the endotoxin.

The PDT has already shown to be a powerful antimicrobial procedure in endodontic area [7, 22] and presented the capacity to inactivate bacterial endotoxins, as demonstrated in Shrestha, Cordova, Kishen (2015) [33], Kömerik, Wilson, Poole (2000) [16] and Giannelli et al. (2011) [9] that have high inactivated values using Toluidine Blue-O + diode LASER. However, the present study did not obtain a favorable LPS inactivation using the PDT treatment, as shown in Group 4, where the specimens treated with methylene blue + diode LASER light did not have a significant difference compared to the control group ( $p < 0.05$ ). That data divergence with the literature outcome might be related to the photosensitizing agent used, where studies that showed a high endotoxin inactivation used the Toluidine Blue-O, and not the Methylene blue. Although, in 2017, a randomized clinical trial from Rabello *et al.* concluded that the PDT, performed as a supplement to the endodontic treatment, was able to optimize the disinfection of bacteria in the root canal, but it was not effective against the endotoxin [28].

Until now, *in vitro* [3, 24, 30] and *in vivo* [20, 34] studies have demonstrated that the intracanal medication based on calcium hydroxide is still the most effective treatment to neutralize the bacterial LPS. Due to the high pH, the calcium hydroxide can inactivate the LPS through the hydrolyzation of Lipid A, and that degradation results in the formation of fatty acids [4]. Therefore, efforts are done to find more alternatives besides calcium hydroxide to neutralize definitively the LPS inside root canal systems.

The Limulus Amebocyte Lysate (LAL) was the method used in the present study to quantify the endotoxin present in all specimens. It is a chromogenic test that uses a modified Limulus amoebocyte lysate and a synthetic color-producing substrate to

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detect and quantify chromogenically the endotoxin of Gram-negative bacteria [12]. That method has been used in several types of researches to quantify the endotoxin in different environments and has demonstrated a good performance in detecting the endotoxin concentration [14, 15, 18, 24, 25].

In the present study, the photodynamic therapy using diode LASER light 660nm + 0.005% Methylene blue was not effective to inactivate the Gram-negative endotoxin present in the root canal. Further studies with possible variations in application time are required to assess new methods to inactivate the LPS during the endodontic treatment.

#### ACKNOWLEDGEMENTES

The authors deny any conflicts of interest.

#### FIGURES

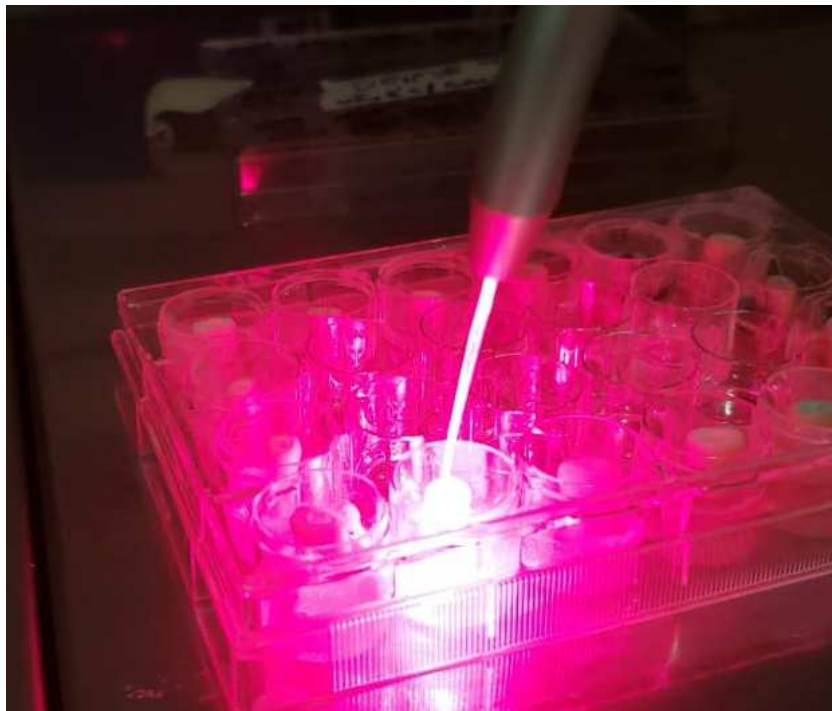


Figure 1: Irradiation of the root canal using an optic fiber attached to the LASER device.

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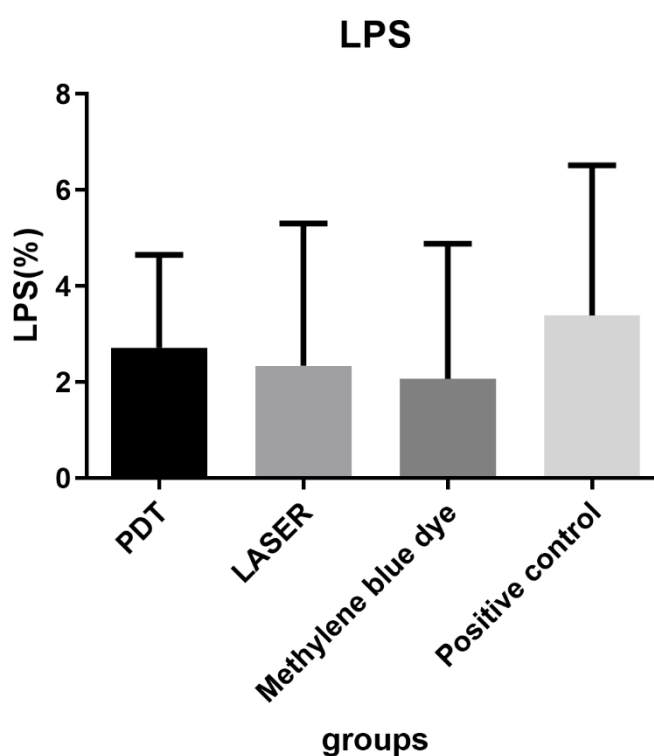


Figure 2: Graphic containing the median (minimum/maximum) percentage of remniscent endotoxin after the experimental treatments.

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## 2.2 ARTICLE 2 - The capacity of different chelating solutions to inactivate bacterial endotoxin

### ABSTRACT:

**Introduction:** The objective of necropulpectomy is to reduce microorganisms and remaining bacterial endotoxin. **Objective:** The present research assessed the influence of chelating solutions to decrease the lipopolysaccharide (LPS) activity. **Methods:** Human single-rooted teeth had their crowns sectioned and the root canals prepared. All samples and material were taken to sterilization in Cobalt-60 irradiation. 10 $\mu$ L of fresh LPS were inoculated into the root canals for 3 days. On the fourth day, the experimental treatments were executed according to the groups (n=10): [1] pyrogen-free water, [2] 2.5% sodium hypochlorite, [3] 17% trisodium EDTA, [4] 10% tetrasodium EDTA and [5] 18% etidronate. The action time for each solution was 5 minutes in the root canal. The pyrogen-free water was placed into the root canal, agitated, and collected, then stored in -80°C refrigeration. The samples were analyzed with the Limulus Amebocyte Lysate test to quantify the endotoxin. The obtained data were submitted to the Kruskal-Wallis analysis followed by the Dunn's test. **Results:** There were statistical difference among the groups [3] (p = 0.0452) and [5] (p = 0.0097) in relation to the control group [1]. The median (Minimum/Maximum) percentage of remaining endotoxin were: [Group 1] = 2.010 (0.8700/10.33); [Group 2] = 1.005 (0.3100/5.810); [Group 3] = 0.3800 (0.060/2.150); [Group 4] = 1.195 (0,110/3,420); [Group 5] = 0.9000 (0.0/1.540). **Conclusion:** Based on these data, the 17% trisodium EDTA and 18% etidronate when used for 5 minutes were able to inactivate the LPS present in the root canal.

**Keywords:** Endodontics, endotoxin (LPS), EDTA, sodium hypochlorite.

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## INTRODUCTION

The treatment of infected non-vital teeth aims to reduce the number of microorganisms and their endotoxins present in the root canal system (1). In case of remaining bacterial endotoxin after the endodontic treatment, it may induce painful symptoms and maintain chronic periapical lesions, leading the treatment to failure (2, 3).

That endotoxin is a component present in the external cell wall of Gram-negative microorganisms and it is released during bacterial duplication or death. It is known as Lipopolysaccharide (LPS), composed of a chain of polysaccharides that works as a specific antigen of the bacterium, called Antigen-O; a group of oligosaccharides called Core; and a lipid structure known as Lipid A responsible for the toxic effect of the LPS (4). When released into the organism, the LPS binds to a receptor complex and activates the release of a large number of chemical mediators and pro-inflammatory cytokines (5).

The elimination of LPS is difficult because it adheres irreversibly to the mineralized tissue such as bone and cementum (6). Furthermore, the endotoxin presents a high capacity to diffuse through the dentinal tubules and reaches the external surface in just 24 hours (7), where it will cause a series of tissue changes, such as inflammatory reaction, bone resorption, and highly painful symptoms (3, 8, 9).

Due to the complex anatomy of the root canal system, the endodontic treatment should combine efficient chemical and mechanical procedures to eliminate microorganisms and their endotoxins as much as possible (1). The association of irrigating solutions promotes a high disinfection in endodontic treatment, but most of these solutions fail to inactivate the LPS (10, 11). In 2003, Tanomaru *et. al* (12) demonstrated that different concentrations of the most used solution, 1%, 2.5%, and 5% sodium hypochlorite was unsuccessful on endotoxin. Another study of Buck *et. al* (2001), showed similar results using 2.6% sodium hypochlorite, 0.12%

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chlorhexidine and 15% EDTA; however, when the authors used a high pH solution (pH = 11.5), created by mixing sodium hypochlorite, chlorhexidine, and ethanol, the endotoxin was inactivated after 30 minutes through hydrolysis of the Lipid A (13).

Even though Trisodium EDTA cannot inactivate directly the LPS, it can be inactivated by the chelation process (14), because the Lipid A, an important structure of LPS and responsible for its toxic effect (2), is linked to calcium.. Furthermore, new chelating solutions presenting high pH have demonstrated favorable clinical results, such as the smear layer removal with low dentin aggression (15). Thus, it would be interesting to analyze the influence of these new high pH chelating solutions on the inactivation of LPS.

## OBJECTIVE

The objective of the present study was to assess the capacity of different chelating solutions (17% trisodium EDTA, 10% tetrasodium EDTA, and 18% etidronate) to inactivate the LPS present in the root canal.

The null hypothesis is that these chelating solutions will inactivate the endotoxin.

## MATERIAL AND METHODS

The present study was submitted and approved by the Research Ethics Committee.

Fifty human single root canal teeth (incisors, canines and premolars) were donated by an Odontology Clinic. The teeth were cleaned and placed in 10% formalin for 24 hours, then they were stored in saline until the time of use.

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### **Samples Preparation**

Using radiographic images, only a straight and single root canal human teeth were used in the present study. The crowns were sectioned with a lower-speed diamond disk, obtaining 14mm of the roots. The root canal was explored using #10 and 15 K-files and prepared in all its extension with nickel-titanium Reciproc 25.07 and 40.04 files (VDW GmbH, Munich, Germany). The 2.5% sodium hypochlorite solution (Rioquímica, São José do Rio Preto, SP, Brazil) was used throughout the instrumentation time and 17% ethylenediaminetetraacetic acid (Biodinâmica – Ibiporã – Paraná – Brasil) as final irrigation. To eliminate as much debris as possible, all the samples were submerged in ultrasonic agitation for 15 minutes in each solution: 2.5% sodium hypochlorite, 17% ethylenediaminetetraacetic acid and saline (Cristofoli Equipamentos de Biossegurança LTDA, Campo Mourão, PR, Brazil).

After the cleaning, a light-curing composite resin (Natural Look - DFL Industria e Comércio S.A. Taquaral, RJ) was used to seal the apical third, and all external root surfaces were waterproofed by 2 layers of epoxy adhesive (Araldite Brascola, São Paulo, Brazil) (16).

All 10 samples per plate were randomly distributed in 24-well cell culture plates and chemically activated acrylic resin was used to pin the samples in a vertical position (JET - Clássico – São Paulo – Brazil). In the end, the plates containing the samples and all material to be used in the research were submitted to Cobalt-60 gamma radiation sterilization (IPEN - São Paulo, Brazil).

### **Endotoxin Inoculation**

The present study used the endotoxin of *Escherichia coli* (n° 055:B5, Sigma, St Louis, USA) in the concentration of 200µg/mL, where 10µL was inoculated into the root canal of the samples. The plates were placed in a bacteriological oven at 37°C, 100% relative humidity, for

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24 hours (11). The inoculation procedures were repeated two more times, a day apart, to complete a total of three inoculations.

### **Experimental Groups**

Twenty-four hours after the last endotoxin inoculation, the root canals were dried with sterilized paper points and then treated according to the following experimental groups (n = 10):

Group 1: Pyrogen-free water (pH  $\cong$  6) (Eurofarma, Itapevi, SP, Brazil);

Group 2: 2.5% Sodium Hypochlorite (pH  $\cong$  11.8) (Rioquímica, São José do Rio Preto, SP, Brazil);

Group 3: 17% Trisodium EDTA (pH  $\cong$  7.3) (Biodinâmica – Ibiporã – Paraná – Brazil);

Group 4: 10% Tetrasodium EDTA (pH  $\cong$  12.2) (Zschjimmer & Schwarz do Brasil LTDA, São Leopoldo – RS, Brazil);

Group 5: 18% Etidronate (pH  $\cong$  10.8) (Zschimmer & Schwarz do Brasil LTDA, São Leopoldo – RS, Brazil).

The evaluated solutions were placed into the root canal until it was filled, using a pyrogen-free insulin-type syringes/needles, according demonstrated in the Figure 1. After 5 minutes, all samples had the root canals washed with pyrogen-free water and dried with sterilized paper points.

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Figure 1: The evaluated solutions were placed into the root canal using a pyrogen-free insulin-type syringes/needles.

### **Collection of the remaining endotoxin**

The root canals were filled with pyrogen-free water and then, using a new pyrogen-free syringe/needle, the solution was agitated, aspirated and stocked inside microtubes. That procedure was repeated until completing 1mL of the collected solution for each sample. In the first 24 hours, the collected material was maintained under -20°C refrigeration and then under -80° C refrigeration until further analysis.

### **Limulus Amebocyte Lysate (LAL) Analysis**

Into a 96-well cell culture plate, the following solutions were placed in duplicate: pyrogen-free water (blank), standard endotoxin (LPS in 5%, 0.5%, 0.05% and 0.005% concentrations) and collected solutions from the root canal samples. Subsequently, the plate was incubated in a QCL kinetic reader (Cambrex – São Paulo, SP, Brazil) attached to a microcomputer containing a Wink QCL software. After 10 minutes of incubation, 100µL of

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chromogenic kinetic reagent of LAL were added in each well of the plates using an 8-channel multi pipette and pyrogen-free tips.

Next, the plate reader software monitored the time necessary to increase the absorbance of each well using a linear log/log correlation of the reaction time and corresponding LPS concentration. Thus, the reports of the standard curve parameters and values of the number of LPS present in the samples were obtained.

#### STATISTICAL ANALYSIS

The collected data did not present a homogeneity of variance in the normality test ( $\alpha=0.05$ ). Kruskal-Wallis analysis, followed by the Dunn's multiple comparison test, were used to detect the difference between groups. All hypotheses were tested at a 95% confidence level.

#### RESULTS

The data analysis detected a statistical decrease of LPS among the experimental groups [3] ( $p = 0.0452$ ) and [5] ( $p = 0.0097$ ) in comparison to the control Group [1]. The median (minimum/maximum) percentage of remaining endotoxin were: [Group 1] = 2.010 (0.8700/10.33); [Group 2] = 1.005 (0.3100/5.810); [Group 3] = 0.3800 (0.060/2.150); [Group 4] = 1.195 (0.110/3.420); [Group 5] = 0.9000 (0.0/1.540), according demonstrated in the Figure 2.

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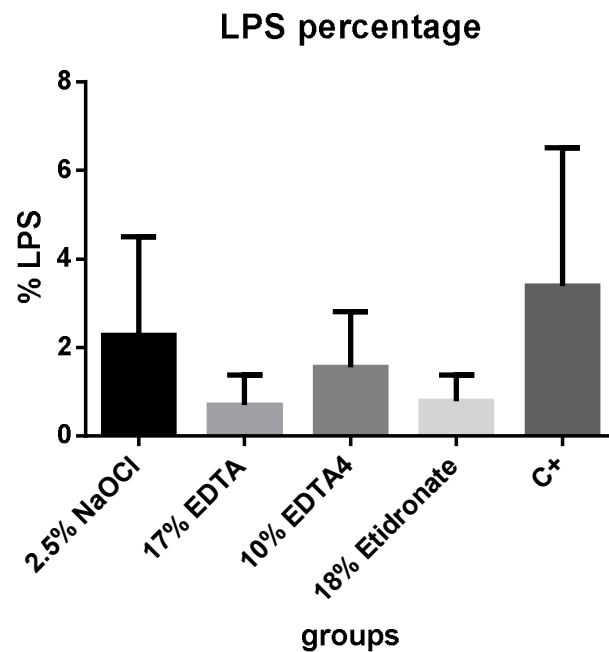


Figure 2: Graphic containing the median (minimum/maximum) percentage of reminiscent endotoxin after the experimental treatments.

## DISCUSSION

The present study was performed using the endotoxin derived from *Escherichia coli* bacterium, the standard and the most used endotoxin in studies with LPS *in vitro* (17, 18, 7, 1). Previous to the experiment, all the samples and materials to be used in the test were sterilized in Cobalt Gamma-rays (19) to eliminate the pre-existing endotoxins.

The Limulus Amebocyte Lysate (LAL) method was used to quantify the endotoxin present in all the evaluated specimens. It is a chromogenic test that uses a modified Limulus amebocyte lysate and a synthetic color-producing substrate to detect and chromogenically quantify the endotoxin of Gram-negative bacteria (20). The chromogenic method has demonstrated a good performance in detecting and quantifying the endotoxin concentration in different environments (21, 22, 16, 11).

Some studies (23, 24) have used absorbent paper points to collect the endotoxin from the samples; however, this method has limitations for it not being able to remove the endotoxin

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present in the dentinal tubules and root canal irregularities. In the present experiment, endotoxin collection was performed by completely filling the samples with pyrogen-free water using a new needle, where a great quantity of substrate was agitated and aspirated for analysis. Furthermore, the needle was passed against the dentinal walls to also collect contaminated dentin scraps.

Even now, according to *in vitro* (25, 17, 26) and *in vivo* (1, 18) studies, the calcium hydroxide-based intracanal medication is the most effective treatment to neutralize the bacterial LPS. The high pH of the calcium hydroxide promotes the hydrolyzation of Lipid A, resulting in the inactivation of the LPS (13). Thus, the present study aimed to evaluate the influence of high pH irrigating solutions on the inactivation of the LPS.

Sodium hypochlorite is the most used solution in endodontic treatment because it dissolves organic tissues and presents a high antimicrobial action (27). The present study used the solution in a 2.5% concentration, but it did not show effectiveness in reducing the endotoxin present in the root canal. This fact has also been observed in *in vitro* (28, 13, 29) and *in vivo* (12, 30, 31) researches where, even in high concentrations, the sodium hypochlorite has low or none efficacy in neutralizing the LPS.

Trisodium EDTA is another solution widely used during endodontic treatment. It is a chelating solution able to remove the contaminated smear layer and debris formed during the chemical-mechanical procedures, allowing a better cleaning and sealing of the dentinal tubules by obturator materials (32). That solution was able to reduce the LPS percentage present in the root canal when compared to those samples treated with pyrogen water. The decrease in the amount of LPS may be due to the ability of the EDTA to bind to the endotoxin structure (14), which, presumably, helps to increase it the disaggregation (33). A greater inactivation was observed when the chelating solution was combined with 30 seconds of ultrasonic activation,

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promoting a significant endotoxin reduction from infected root canals (34).

Q-mix, a novel slightly alkaline solution that combines the antimicrobial and substantivity properties of the chlorhexidine with the smear layer removing properties of the EDTA (35). When tested in samples contaminated with LPS, the Q-mix showed a low level of remaining endotoxin after 3 minutes of action. According to the authors (14), the LPS inactivation is the result of the conjunct action of all the components of the Q-mix solution, where the EDTA, besides the capacity to bind to the Lipid A, can expose the internal infected dentin; and the cetrimide, a cationic detergent, promotes a non-polar interaction with the Lipid A. In the end, all these factors are responsible for Q-mix reducing the LPS levels (14).

At the moment, there is no publication about the capacity of the etidronate and tetrasodium EDTA to reduce the bacterial endotoxin. The etidronate is a new chelating solution that, such as the EDTA, presents a great ability to remove the smear layer, but it promotes less aggression to the dentin (15). In the present study, only the 18% etidronate and the 17% EDTA solutions presented a significant endotoxin reduction in comparison to the control group using pyrogen-free water. Similar results are probably related to the high pH of both chelating solutions, and the alkaline solution should only influence the hydrolysis of the Lipid A (13) and, consequently, promote the LPS inactivation. Regarding the 10% tetrasodium EDTA, it was thought that it would present a favorable result because of its high pH (12.2), but even when showing values greater than 2.5% sodium hypochlorite group, the 10% tetrasodium EDTA had no significant difference in relation with the control group.

## CONCLUSION

In the present study, the 17% Trisodium EDTA and 18% Etidronate chelating solutions were effective to inactivate the Gram-negative endotoxin present in the root canal. Further

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studies are required to assess the different time of these evaluated solutions to inactivate the LPS during the endodontic treatment.

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## **3 DISCUSSION**

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### 3 DISCUSSION

The endotoxin used in the present study was derived from *Escherichia coli* bacterium, which is considered the most used standard endotoxin in *in vitro* studies (15, 16, 17, 7). Furthermore, all the samples and materials were sterilized in Cobalt-60 gamma radiation so that the pre-existing endotoxins in the material to be used would not affect the experiment (26).

In PDT, when the photosensitizer is stimulated with molecular oxygen by a visible light spectrum, it produces highly reactive cytotoxic species derived from oxygen (27). The role of photosensitizer can be performed by phenothiazine dyes, capable of linking and penetrating the bacterial wall, affecting the Lipid A structure (24), which is the structure responsible for the toxic effect of the LPS (2) and presents a negative charge (28). In the present study, the photosensitizing agent chosen was methylene blue, a cationic dye that presents peak reactivity when it is stimulated by light in the red spectrum. However, unlike the toluidine blue O, which showed in the literature a drastic effect in biological activities of the LPS (29), methylene blue, in the present study, was not able to significantly inactivate ( $p = 0.3341$ ) the LPS compared to the control group.

To obtain the necessary triad for the PDT (dye + oxygen + light), it is necessary to use light in the visible light spectrum. The light source used in the present study was 660nm red light derived from a low-power Diode LASER. Due to its coherence property, it was possible to guide its light into the root canal using an optical fiber (30). However, unlike the ablative or high-power LASER, diode LASER light is not able to promote a favorable disinfection when it is used alone (19). The present study obtained low values of the inactivation of endotoxin in the group treated only with Diode LASER, not presenting a relevant difference compared to the control group ( $p = 0.3341$ ). In 2011, Giannelli *et al.* (25) obtained similar results when they compared different LASER bulbs to disinfect contaminated titanium implants with LPS, where they verified that the isolated use of Diode LASER was ineffective in inactivating the endotoxin.

The PDT showed a potent antimicrobial procedure in the endodontic field (31, 32), and also has already showed its effectiveness in inactivation of bacterial endotoxin

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in the literature (29, 25). However, the present study did not obtain a favorable inactivation of the LPS using PDT. Unlike the cited in Giannelli *et al.* (2011) research (25), the specimens treated with methylene blue + Diode LASER, in the present study, did not show relevant reduction of endotoxin regarding the control group ( $p = 0.3341$ ). That difference of data may be related to the photosensitizing agent used since the favorable results in the literature were obtained using toluidine blue O (25). However, a randomized clinical trial of Rabello *et al.* in 2017 verified that the PDT performed as a supplement to the endodontic treatment was able to eliminate bacteria, but it was not effective to inactivate the endotoxin (33).

In endodontics, sodium hypochlorite is the irrigating solution most used during treatment due to its high capacity of dissolving organic material and high antimicrobial action (34). The present study used the solution at a concentration of 2.5%, however, the obtained results did not show effectiveness in the reduction or inactivation of endotoxin present in the root canal. This was also observed in *in vitro* (35, 11) and *in vivo* researches (36, 10, 37, 38), in which even in high concentrations, sodium hypochlorite showed low or none efficacy in neutralizing the LPS.

Until now, *in vitro* (14, 15, 16) and *in vivo* studies (17, 18) showed that the most effective treatment to neutralize bacterial LPS is still the calcium hydroxide-based intracanal medication. Due to high pH of calcium hydroxide, it can inactivate the LPS through Lipid A hydrolysis, resulting in fatty acids (11). Thus, the present study aimed to verify the influence of high pH irrigating solutions in the inactivation of the LPS.

Another widely used irrigating solution in endodontic treatment is Trisodium EDTA at 17%, a chelating solution that promotes the removal of the smear layer and detritus formed during the mechanical procedure, providing a better sealing of the dentinal tubules during the obturation (39). In the present study, the EDTA solution was able to reduce the percentage of LPS present in the root canal when it is compared to samples treated only with pyrogen-free water. The reduction in the amount of LPS may be related to the capacity of EDTA in linking calcium through the chelation process (12), which it presumably helps to increase the degradation of endotoxin (40). A greater inactivation was observed when the chelating solution received 30 seconds of ultrasonic activation, promoting a relevant reduction of LPS present in infected root canals (41).

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Qmix, a mixture of disodium EDTA and chlorhexidine digluconate, is a slightly alkaline solution that combines the detritus cleaning action with disinfection. When it is tested in contaminated samples with LPS, Qmix presented a low percentage of remaining endotoxin after 3 minutes of action. According to the authors (12), the inactivation of the LPS results from the joint action of all components of the QMix solution, mainly EDTA, which does not only have the capacity of linking to Lipid A by the chelation process, but also promotes the exposition of the infected and internal dentine.

In the literature, studies in relation to the capacity of etidronate at 18% and tetrasodium EDTA at 10% in inactivating the bacterial endotoxin was not found. Etidronate is a new chelating solution that, as EDTA, presents great capacity of smear layer removal combined with a lesser aggression on the dentinal wall (13). In the present study, only the etidronate at 18% and trisodium EDTA at 17% solutions were effective in significantly reducing the LPS in comparison to the control group treated only with pyrogen-free water. Similar results between those solutions were probably related to the high pH of both chelating solutions, since the alkaline solution influences the Lipid A hydrolysis (11) that consequently promotes the inactivation of the LPS. It was presumed that tetrasodium EDTA at 10% would present a favorable result due to its high pH (12.2), however, even presenting superior values of neutralization to sodium hypochlorite at 2.5%, tetrasodium EDTA at 10% did not obtain relevant differences in relation to the control group.

In the present study, Limulus Amebocyte Lysate (LAL) reaction was the method used to quantify the endotoxin present in all specimens. It is a chromogenic test that uses a modified Limulus amoebocyte lysate and a synthetic color-producing substrate to detect and chromogenically quantify the Gram-negative bacteria endotoxin (42). That method has been used in several types of researches to quantify the endotoxin in different environments and has demonstrated a good performance in detecting the endotoxin concentration (43, 44, 7, 45). Therefore, further studies are necessary to verify different times in LASER irradiation and different application protocols for using the chelating solutions.

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## **4 CONCLUSION**

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## **4 CONCLUSION**

Considering the experimental conditions and the methodologies used, the present study concluded that:

- Photodynamic therapy (PDT), using 660nm Diode LASER and methylene blue at 0.005%, was not effective to neutralize the endotoxin present in the root canal, even when the light source and blue dye were used alone;
- Within all the evaluated solutions, only Trisodium EDTA at 17% and Etidronate at 18% were efficient to reduce the amount of endotoxin present in the root canal



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**ANNEX**

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## ANNEX

### Confirming submission to Photodiagnosis and Photodynamic Therapy

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**PARECER CONSUBSTANCIADO DO CEP**

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** Efeito da PDT, e de novas soluções quelantes na inativação do LPS bacteriano

**Pesquisador:** DENISE FERRACIOLI ODA

**Área Temática:**

**Versão:** 2

**CAAE:** 28171519.0.0000.5417

**Instituição Proponente:** Faculdade de Odontologia de Bauru

**Patrocinador Principal:** Financiamento Próprio

**DADOS DO PARECER**

**Número do Parecer:** 3.970.612

**Apresentação do Projeto:**

Será realizado um experimento laboratorial in vitro para analisar a capacidade de determinados procedimentos odontológicos em inativar endotoxinas. Este experimento apresenta 2 fatores de variação: Tratamento, dividido em 8 níveis (Hipoclorito de sódio / Água Apirogênica / EDTA Trissódico / EDTA Tetrassódico / Etidronato / Terapia Fotodinâmica / LASER 660nm / Azul de Metileno; e a Endotoxina em único nível (Lipopolissacarídeo). A variável de resposta quantitativa será a concentração de endotoxina obtida após a realização dos tratamentos avaliados. Esta será verificada por meio de análise do grau de absorvância de cada amostra.

**Objetivo da Pesquisa:**

Avaliar, in vitro, a capacidade da terapia fotodinâmica (LASER de diodo + Azul de metileno), e de novas soluções quelantes, como o EDTA tetrassódico e o Etidronato, em inativar o LPS presente no interior do canal radicular.

**Avaliação dos Riscos e Benefícios:**

Os riscos informados pelos autores referem-se aos dentes que serão utilizados nos experimentos, pois estes serão extraídos por indicação clínica e previamente ao conhecimento dos pacientes da pesquisa supracitada. Esses dentes serão doados por Cirurgião-dentista que realizar as extrações por finalidade terapêutica, o que será comprovado através de um termo de cessão de dentes. Desta maneira, os pacientes não serão expostos a um risco maior que o estabelecido no tratamento empregado. Porém, para não haver o risco de as amostras serem utilizadas sem

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autorização do doador, as mesmas serão descartadas como material biológico de acordo com as normas da ANVISA após encerrados todos os experimentos.

Quanto aos benefícios, os autores referem que: "a descoberta um novo tratamento capaz de inativar a endotoxina bacteriana presente no interior do canal radicular e, conseqüentemente, seus efeitos deletérios".

**Comentários e Considerações sobre a Pesquisa:**

Trata-se de uma pesquisa relevante e que certamente trará resultados úteis em seu contexto de inserção.

**Considerações sobre os Termos de apresentação obrigatória:**

Os Termos de apresentação obrigatória estão adequados, exceto o cronograma do projeto apresentado por meio de arquivo pdf e na resposta ao CEP, uma vez que o mesmo não especifica o ano em que as atividades serão realizadas (apesar de estar dividido em etapas, não prevê data de início e fim do projeto). Contudo, na Plataforma Brasil, os autores atualizaram adequadamente o cronograma.

Destaca-se que os autores informaram estar cientes da necessidade de, após a aprovação do projeto, o Termo de Intenção de cessão de deve ser substituído pelo Termo de Cessão de Dentes, mesmo que ainda no relatório final.

**Recomendações:**

Destaca-se a necessidade de uma emenda para atualização do cronograma apresentado na carta de resposta ao CEP, ficando o mesmo de acordo com as datas atualizadas a Plataforma Brasil.

Destaca-se novamente que os autores informaram estar cientes da necessidade de, após a aprovação do projeto, o Termo de Intenção de cessão de deve ser substituído pelo Termo de Cessão de Dentes, mesmo que ainda no relatório final.

**Conclusões ou Pendências e Lista de Inadequações:**

Projeto aprovado; ratifico a necessidade de atenção aos itens citados nas RECOMENDAÇÕES.

**Considerações Finais a critério do CEP:**

Esse projeto foi considerado APROVADO na reunião ordinária do CEP de 01/04/2020, por e-mail, devido à pandemia da COVID-19 e por orientações da CONEP, com base nas normas éticas da Resolução CNS 466/12. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores, cronograma e quaisquer

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outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se pertinentes.

**Este parecer foi elaborado baseado nos documentos abaixo relacionados:**

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1217086.pdf	07/03/2020 00:22:28		Aceito
Outros	OficioDeRespostaAoCEP.pdf	07/03/2020 00:18:33	DENISE FERRACIOLI ODA	Aceito
Projeto Detalhado / Brochura Investigador	ProjetoDePesquisa.pdf	07/03/2020 00:12:14	DENISE FERRACIOLI ODA	Aceito
Cronograma	cronograma.pdf	07/03/2020 00:01:28	DENISE FERRACIOLI ODA	Aceito
Outros	checklist.pdf	20/01/2020 18:19:15	DENISE FERRACIOLI ODA	Aceito
Outros	termo_intencao_cessao_dentes.jpeg	11/12/2019 19:45:50	DENISE FERRACIOLI ODA	Aceito
Declaração de Instituição e Infraestrutura	t_aquiescencia.pdf	11/12/2019 19:37:54	DENISE FERRACIOLI ODA	Aceito
Declaração de Pesquisadores	declaracao_pesquisador.pdf	11/12/2019 19:36:28	DENISE FERRACIOLI ODA	Aceito
Orçamento	orcamento.docx	11/11/2019 10:53:01	DENISE FERRACIOLI ODA	Aceito
Folha de Rosto	folhaderosto.pdf	20/03/2019 10:46:05	DENISE FERRACIOLI ODA	Aceito

**Situação do Parecer:**

Aprovado

**Necessita Apreciação da CONEP:**

Não

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BAURU, 14 de Abril de 2020

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**Assinado por:**  
**Juliana Fraga Soares Bombonatti**  
**(Coordenador(a))**

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