Accuracy of Turbidimetric Limulus Amebocyte Lysate Assay for the Recovery of Endotoxin Interacted with Commonly Used Antimicrobial Agents of Endodontic Therapy

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Abstract

Introduction: This study was conducted to investigate whether the interaction between the turbidimetric limulus amebocyte lysate (LAL) substrate for endotoxin measurement and the substances/antimicrobial agents used in endodontic therapy can lead to the inhibition/enhancement of endotoxin recovery. **Methods:** Ten microliters of a suspension of *Escherichia coli* endotoxin (O55:B55) was inoculated and kept in contact for 1 hour with different substances categorized as follows: group 1: auxiliary chemical substances: 5.25% and 2.5% sodium hypochlorite solutions, 2% chlorhexidine (CHX) (gel and solution), 1% Natrosol gel (Drogal Chemicals and Pharmaceuticals Ltd, Piracicaba, SP, Brazil), 17% EDTA, 10% citric acid, 3% hydrogen peroxide, 5% sodium thiosulfate, and 0.5% Tween 80 associated with 0.07% soy lecithin (Drogal Chemicals and Pharmaceuticals Ltd) and group 2: intracanal medications: neomycin/polymyxin B/hydrocortisone (Otosporin; Glaxo Wellcome, Rio de Janeiro, RJ, Brazil); calcium hydroxide ($Ca[OH]_2$); $Ca(OH)_2$ + 2% CHX gel; $Ca(OH)_2$ + 2% CHX gel + zinc oxide eugenol; $Ca(OH)_2$ + camphorated paramonochlorophenol (Calen; S.S. White, Rio de Janeiro, RJ, Brazil); triple antibiotic paste; mineral trioxide aggregate (MTA); and iodoform. Positive and negative controls consisted of root canal hemorrhagic exudate and pyrogen-free sterile water, respectively. All samples were diluted up to a 10:4 dilution. Each dilution was individually examined by the turbidimetric LAL assay. Collected data were analyzed through performance characteristics of the LAL assay such as linearity, coefficient of variation percentage, and product positive control (PPC) values. **Results:** Correlation coefficient ($\geq$0.980) and coefficient of variation percentage (<10%) of the standard curve in triplicate showed the tests' linearity. Spike recovery of auxiliary chemical substances achieved PPC values ranging from 50%–197%, showing no interferences with LAL substrate. Conversely, 3% hydrogen peroxide achieved product inhibition in which endotoxin values were underestimated even after the 10:4 dilutions. Regarding intracanal medicaments, neomycin/polymyxin B/hydrocortisone also inhibited endotoxin detection in all dilutions investigated (PPC values <50%). In contrast, $Ca(OH)_2$ + 2% CHX gel + ZOE as well as triple antibiotic paste led to the enhancement of endotoxin detection in which endotoxin values could not be validated by the turbidimetric kinetic LAL assay (PPC value >200%). **Conclusions:** The performance characteristics of the kinetic turbidimetric assay for endotoxin measurement, such as precision and reproducibility, are modulated by the interaction of the LAL substrate with the substances/antimicrobial agents used in endodontic therapy. *(J Endod 2015;41:1653–1659)*

Key Words

Endotoxin, intracanal medicaments, irrigants, limulus amebocyte lysate methods, root canal

Endotoxin or bacterial lipopolysaccharide (LPS) is a general virulence factor present in the outer membrane of gram-negative bacteria predominantly involved in root canal infection (1, 2). Bacterial cells only secrete small amounts of LPS during multiplication; however, substantial amounts may be released during the destruction of the cells by antimicrobial substances or host immune cells (3). The presence of LPS in infected root canals has been consistently reported in clinical studies, revealing its dose-dependent association with the presence of clinical and radiographic features of endodontic disease (1–6).

To date, endotoxin detection can be performed by several methods (7–9). The rabbit pyrogen test was the first assay developed to test solutions intended for injection into humans. This test indicates the presence of endotoxins, which can cause a fever in rabbits and is sensitive to 0.5 endotoxin units (EUs)/mL (7, 10, 11). It is generally accepted that 1 EU equals approximately 0.1–0.2 ng EU/mL (12). Later, in the 1970s, an *in vitro* quantitative assay was developed using a lysate, a basic substrate of the limulus amebocyte test (LAL) test, produced by lysis of amebocytes from the horseshoe crab (*Limulus polyphemus*). The lysate contains a mixture of proteins that clot in the presence of very low levels of endotoxins (7, 10). Ever since, LAL has been recognized as the standard assay for the detection of endotoxins according to the US Food and Drug Administration guidelines for medical devices and parenteral drugs (12).

Besides the investigation of LPS in infected root canals (8–13), endodontic researchers have also attempted to evaluate the effect of root canal procedures on its

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elimination (8, 14–16) by using LAL with a variety of improved methods, such as the chromogenic end point (QCL-1000 test [BioWhittaker Inc, Walkersville, MD], detection limit: 0.1–1 EU/mL) (3, 13–16) and kinetic chromogenic (Kinetic-QCL test [BioWhittaker Inc], detection limit: 0.005–50 EU/mL) assays (17–19) (both determining the levels of endotoxin by yellow color intensity) and the kinetic turbidimetric assay (20–22) (turbidimetric test, detection limit: 0.01–100), which is based on a turbid reaction (coagulogen-based LAL assay). Martinho et al (23) showed that quantitative kinetic turbidimetric and kinetic chromogenic LAL methods are best suited for the analysis of endotoxins in root canal infection, both being more precise and allowing better reproducibility compared with the end point chromogenic assay.

Regardless of the method used, LAL-based assays contain a series of coagulation enzymes in which the pH, temperature, and ionic strength have a critical influence over the precise biochemical reactions to ensure the proper development of the serine protease catalytic coagulation cascade (24). Considering that a wide variety of antimicrobial agents have been tested for root canal detoxification, either in 1-visit or multiple-visit treatment (13, 15, 16, 18–20, 22), the interaction between LAL substrate and components of the test sample can interfere with any step of the coagulation cascade to an extent that the lysate is rendered insensitive or overreacts to endotoxin. Negative or underestimated results related to samples that inhibit the LAL test do not necessarily indicate the absence of or low level of endotoxins. The same is relevant for enhanced activity of the limulus coagulation cascade because of the cross-interaction mediated by the additional presence of interferences. Consequently, misleading results are expressed by the performance characteristics of the LAL assay.

Once validation criteria are established (a key element in endotoxin testing), it would be of interest to investigate the ability of the LAL substrate in detecting endotoxins when interacted with auxiliary chemical substances and intracanal medicaments of endodontic therapy. Reports about whether such associations are able to impair the accuracy of LAL substrate in detecting endotoxins have not been published so far. Therefore, this study was conducted to investigate whether the interaction between commonly used substances/antimicrobial agents of endodontic therapy and turbidimetric LAL substrate lead to the inhibition/enhancement of endotoxin recovery.

Materials and Methods

Exposure of Endotoxin to Selected Substances

The study was approved by the local institutional ethics committee (protocol no. 186/09). *Escherichia coli* O55:B5 endotoxin (Lonza, Walkersville, MD) was used for the experiments. Under sterile laminar flow, 50 μL of a standard solution containing endotoxin (1 EU/mL) was inoculated into different endodontic antimicrobial agents selected from those commonly used clinically as follows:

- *Group 1:* Auxiliary chemical substances: 5.25% sodium hypochlorite, 2% chlorhexidine (CHX) gel and solution, 1% Natrosol gel, 17% EDTA, 10% citric acid, 3% hydrogen peroxide, 5% sodium thiosulfate (sodium hypochlorite neutralizer), and 0.5% Tween 80 associated with 0.07% soy lecithin (CHX neutralizer); all substances were provided by Drogal Chemicals and Pharmaceuticals Ltd, Piracicaba, SP, Brazil
- *Group 2:* Intracanal medicaments: neomycin/polymyxin B/hydrocortisone (Otosporin; Glaxo Wellcome, Rio de Janeiro, RJ, Brazil); calcium hydroxide proanalysis ($Ca[OH]_2$; Biodinâmica Chemicals and Pharmaceuticals Ltd, Ibiporã, PR, Brazil); $Ca(OH)_2$ combined with 2% CHX gel (1: 1; v:v); $Ca(OH)_2$ combined with camphorated paramonochlorophenol (Calen; S.S. White, Rio de Janeiro, RJ, Brazil); $Ca(OH)_2$ combined with 2% CHX gel and zinc oxide eugenol (1:1:0.01 v:v:v) (Biodinâmica Chemicals and Pharmaceuticals Ltd); triple antibiotic paste; mineral trioxide aggregate (ProRoot MTA; Dentsply, Tulsa, OK); and iodoform (Biodinâmica Chemicals and Pharmaceuticals Ltd)

Auxiliary chemical substances were prepared 24 hours before the beginning of the experiment. Before inoculation of the endotoxin, 1 mL of each auxiliary chemical substance and the ready-to-use formulations of intracanal medications (Otosporin and Calen) were transferred to glass test tubes.

For powder substances, such as $Ca(OH)_2$, mineral trioxide aggregate, and iodoform, 1 g of each one was individually mixed with 1 mL endotoxin-free water (Lonza, Walkersville, MD). $Ca(OH)_2$ associated with 2% CHX gel was mixed in a 1:1 proportion. For the mixture of $Ca(OH)_2$, 2% CHX gel, and zinc oxide eugenol, a 1:1:0.01 proportion was used according to de Jesus Soares et al (25).

Triple antibiotic paste was prepared by crushing antibiotic ciprofloxacin (Ciplox, Drogal Chemicals and Pharmaceuticals Ltd), metronidazole (Metrogyl, Drogal Chemicals and Pharmaceuticals Ltd), and minocycline (Minoz, Drogal Chemicals and Pharmaceuticals Ltd) tablets separately using a mortar and pestle. The crushed powder was passed through a fine sieve to remove heavy filler particles and obtain a fine powder. The ciprofloxacin, metronidazole, and minocycline powders were weighed separately and mixed in a 1:1:1 proportion, respectively, to obtain a triple antibiotic mixture. A total of 300 mg of the antibiotic mixture was mixed with 1 mL endotoxin-free water.

The negative control was performed by the inoculation of endotoxins into 1 mL endotoxin-free water. The positive control consisted of root canal hemorrhagic exudate collected from root canals of teeth with irreversible pulpitis as described elsewhere (3, 15, 16, 22). The pH of all samples was measured right after preparation by using a pH meter (Procyon, digital pH meter model AS 720, electrode A 11489; Procy Instrumental Científica, São Paulo, SP, Brazil). All test tubes were vortexed for 5 minutes to ensure complete mixture and diluted with endotoxin-free water by 10-fold serial dilution up to a 10:4 dilution. All the glassware was rendered endotoxin free via heat treatment at 200°C for 4 hours (26). Endotoxin-free pipettes (Fisher Scientific, Pittsburgh, PA) were used for all procedures. All samples were left for 1 hour under constant agitation in an orbital shaker platform (Fanem, São Paulo, SP, Brazil). After the incubation period, each substance (as well as its respective dilutions) was assessed for further LAL assays.

Endotoxin Recovery Assay

LAL was used as a quantitative test for endotoxin level measurement and was performed according to the manufacturer's protocol (turbidimetric test [PYROGENT-5000, Lonza]).

Sample dilutions were performed according to the maximum valid dilution value in which at least 3 batches of each product should be tested for inhibition and enhancement (12). Moreover, all validation tests should be accomplished on an undiluted substance or an appropriate dilution.

As a parameter for the calculation of the endotoxin levels present in the samples, a standard curve was plotted by using a known endotoxin concentration supplied by the kit (100 EU/mL), with its dilutions reaching the final concentrations (0.01, 0.1, 1, and 10 EU/mL). A 96-well microplate (Corning Costar, Cambridge, MA) was placed on a heating block at 37°C and maintained at this temperature throughout the assay. First, 100 μL of the blank followed by the same volume of the standard endotoxin solutions (0.01, 0.1, 1, and 10 EU/mL) and 100 μL of the samples were added in duplicate to