Quantification of Endotoxins and Cultivable Bacteria in Root Canal Infection before and after Chemomechanical Preparation with 2.5% Sodium Hypochlorite

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Abstract

This clinical study was conducted to quantify endotoxins and cultivable bacteria in teeth with pulp necrosis and apical periodontitis before and after chemomechanical preparation with 2.5% sodium hypochlorite (NaOCl) and to investigate the possible correlation of endotoxin and cultivable bacteria with the presence of clinical symptomatology. Twenty-four root canals were selected. Samples were collected before (s1) and after chemomechanical preparation (s2). Culture techniques were used to determine the colony-forming unit. A limulus amebocyte lysate (LAL) assay was used to quantify endotoxins (lipopolysaccharide, LPS). LPS and bacteria were detected in 100% of the initial samples (s1), with a median concentration of 139 endotoxin units/mL and 2.64×10^5 colony-forming units/mL, respectively. Higher levels of LPS were found in teeth with clinical symptomatology ($p < .05$). At s2, mean endotoxin reduction of 59.99% and mean bacterial load reduction of 99.78% were found. Our findings indicated that chemomechanical preparation with 2.5% NaOCl was moderately effective against bacteria but less effective against endotoxins in root canal infection. Furthermore, a statistically significant association was found between higher levels and clinical symptomatology. *(J Endod 2008;34:268–272)*

Key Words

Chemomechanical preparation, cultivable bacteria, endotoxins, root canal infection, 2.5% sodium hypochlorite

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Bacteria and their by-products play a primary etiologic role in the development and perpetuation of apical periodontitis (1). The bacteria involved in primary endodontic infection are predominantly gram-negative anaerobic species (2) that present lipopolysaccharide (LPS) on the outer layers of their cells walls (2), which functions as an endotoxin in the host organism (3).

LPS (a virulence factor), generally referred to as endotoxin, released during disintegration of bacteria after multiplication and death (4), has been detected in teeth with pulp necrosis (5–8). Strong evidence correlates its presence in root canals with inflammatory reactions and bone resorption of the periradicular tissues (9–12). A high content of endotoxins in root canals has been associated with endodontic signs and symptoms such as spontaneous pain, pain on palpation, and tenderness to percussion (6, 7, 13). Furthermore, its egression through the apical foramen into periapex can even perpetuate an apical periodontitis (9, 11, 14).

Therefore, the main goal of an endodontic treatment and main property of the irrigant solution is based not only on bacterial load reduction and antimicrobial properties (15), respectively, but also on reducing endotoxins and on inactivating the toxic effects of LPS, which are important for the healing process of periapical tissues (9, 11, 14).

Sodium hypochlorite (NaOCl), widely used in endodontic infections at different concentrations (16), demonstrates a high capacity to reduce and eliminate bacteria from infected root canals (17, 18). However, in vitro (19–21) and in vivo (11, 22), studies with induced periapical lesions have shown a low or absent efficacy for NaOCl in neutralizing endotoxins. Such studies were performed with enterobacterial LPS, which displays toxicity that is not comparable to that of endodontic bacteria (23).

Therefore, the present clinical study was conducted to quantify endotoxins and cultivable bacteria in root canal infections with pulp necrosis and apical periodontitis before and after chemomechanical preparation with 2.5% sodium hypochlorite and to investigate the possible correlation of endotoxin and cultivable bacteria with the presence of clinical symptomatology.

Material and Methods

Patient Selection

Twenty-four patients at the Dental School of Piracicaba, São Paulo, Brazil, for root canal treatment were selected for this study. The age of patients ranged from 16–57 years. The selected teeth were single-root, presenting necrotic pulp tissues with radiographic evidence of apical periodontitis. None of the patients reported spontaneous pain. Eleven cases were asymptomatic, and 13 cases presented either tenderness to percussion and/or pain on palpation. A detailed medical and dental history was obtained from each patient. Patients who had received antibiotic treatment during the last 3 months or who had a general disease were excluded from the study. The Human Volunteers Research and Ethics Committee of the Dental School of Piracicaba approved a protocol describing the specimen collection for this investigation, and all patients signed their informed consent to participate in the study.

Sampling Procedure

The methods followed for the microbiologic procedures performed in this study have been previously described (7, 8, 24). The teeth were isolated from the oral cavity

with a rubber dam, and the disinfection of their external surfaces and the surrounding structure field was carried out by using 30% hydrogen peroxide followed by 2.5% NaOCl. The solutions were inactivated with 5% sodium thiosulfate to avoid interference with bacteriologic sampling. The sterility of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it on blood agar plates, which was incubated aerobically and anaerobically. NaOCl was prepared by Proderma (Farmácia de Manipulação Ltda, Piracicaba, São Paulo, Brazil). The manufacturer diluted NaOCl in sterile water without preservatives. The solution was prepared 24 hours before the beginning of the experiment, always in small portions. All subsequent procedures were performed aseptically.

A 2-stage access preparation was performed. The access cavity was made without the use of water spray but under manual irrigation with sterile apyrogenic saline and by using sterile apyrogenic high-speed diamond bur. This first stage was performed to promote a major removal of the contaminants (microorganisms and endotoxins). In the second stage before entering the pulp chamber, the access cavity was disinfected following the protocol described above. Its sterility was checked by taking swab samples of the cavity surface and streaking on to blood agar plates, with subsequent incubation at 37°C under both aerobic and anaerobic conditions. A new sterile apyrogenic bur was used, accomplished by irrigation with sterile apyrogenic saline, to access the canal. A sterile pyrogen-free paper point (size 35; Dentsply-Maillefer, Ballaigues, Switzerland) was then introduced into the full length of the canal, which was determined radiographically, and retained in position during 60 seconds for sampling. The procedure was repeated with 5 paper points. Afterwards, one paper point was placed in a pyrogen-free glass for the chromogenic limulus amebocyte lysate (LAL), and the other paper points were pooled in a sterile tube containing 1 mL of VMGA III transport medium for microbial cultivation. The endotoxin samples were frozen at −20°C, and microbial cultivation was performed after all sample collections. All samples were collected by using the same technique.

Clinical Procedures

After the first microbiologic sampling, the pulp chamber was thoroughly cleaned with 2.5% NaOCl. A K-file (Dentsply-Maillefer), size 10 or 15, was placed in the total length of the root canal, as calculated by the preoperative radiograph. The coronal two thirds of each root canal were prepared initially by using rotatory files (GT Rotatory Files size .10/20 and .08/20; Dentsply-Maillefer) at 350 rpm, reaching 4 mm before the total length. Gates-Glidden drills, sizes 5, 4, 3, and 2 (Dyna-FFDM, Bourges, France) were used until 2 mm before the total length, prepared with GT files. The working length (1 mm from the radiographic apex) was checked with a radiograph after inserting an anatomic file in the canal to the estimated working length, confirmed by the apex locator (Forum Technologies, Rishon Le-Zion, Israel). The apical stop was established by using K-files (Dentsply-Maillefer). The apical stop ended after the use of 3 files larger than the initial one. Step-back flaring of the canal was performed with larger files at intervals manipulated in a filling action. The file used to prepare the apical stop was used to recapitulate. Stepping back ended after the use of 3 files larger than the file that prepared the apical stop (18).

The use of each instrument was followed by an irrigation of the canal with 5 mL of 2.5% NaOCl solution. The working time for the chemomechanical procedure was established at 20 minutes for all cases. Before the second sampling (S2), NaOCl was inactivated with 5 mL of sterile 0.5% sodium thiosulfate during a 1-minute period.

Culture Procedure

The transport media containing the root canal samples were shaken thoroughly in a mixer inside an anaerobic chamber for 60 seconds (Vortex, Marconi, Piracicaba, São Paulo, Brazil). Serial 10-fold dilutions were made up to 10^{-4} in tubes containing fastidious anaerobe broth (LAB M, Bury, UK). Fifty microliters of the serial dilutions was plated, by using sterile plastic spreaders, into 5% defibrinated sheep blood fastidious anaerobe agar (FAA; Lab M) to culture nonselectively obligate anaerobes and facultative anaerobes. The plates were incubated at 37°C in an anaerobic atmosphere for up to 14 days. After this period, the colony-forming units (CFUs) were visually quantified for each plate.

Determination of Endotoxin Concentration

The quantitative chromogenic LAL assay—QLC-100 LAL test kit (BioWhitaker, Inc, Walkersville, MD) was used to measure endotoxin concentrations in the root canals before and after chemomechanical procedures.

Standard Curve

As a parameter for calculation of the amount of endotoxins in root canal samples, a standard curve was plotted with endotoxins supplied in the kit (*Escherichia coli* 0111:B4) with a known concentration (25 endotoxin units [EU]/mL) and following the manufacturer's instructions. After the LAL test procedure (described below), the absorbencies of endotoxin standard solutions with a series of endotoxin concentrations (ie, 0.1, 0.25, 0.5, and 1.0 EU/mL) were measured individually with an enzyme-linked immunosorbent assay plate-reader (Ultramark; Bio-Rad Laboratories, Inc, Hercules, CA) at 405 nm. The standard curve fulfilled the criteria of linearity ($r \geq 0.980$), as reported by the guideline on validation of LAL tests(25).

Spike Concentration

To avoid the inhibition or enhancement of LAL, the addition of a known concentration of *E. coli* endotoxins (spiking procedure) is recommended. For all tests, the spike recovery was 0.4 EU/mL. This activity was chosen because of its lower position on a logarithmic standard curve (its value was set as zero).

Test Procedure

Initially, serial dilutions of the samples were made to 10^{-4}. LAL reagent water (blank) was used as a negative control. All reactions were accomplished in duplicate to validate the test. A 96-well microplate (Corning Costar, Cambridge, MA) was used in a heating block at 37°C and maintained at this temperature throughout the assay. Initially, 50 μL of the blank was added followed by the standard endotoxin solutions, and the samples were consecutively added to the wells. This was followed by the addition of 50 μL LAL to each well, and the microplate was then briefly shaken. Ten minutes later, 100 μL of substrate solution (prewarmed to 37°C) was added to each well, always maintaining the same sequence. The plate was mixed and incubated at 37°C for 6 minutes. Afterwards, 100 μL of a stop reagent (acetic acid, 25% v/v) was added to each well, and the absorbance (405 nm) was read by using a spectrophotometer (Ultramark; Bio-Rad Laboratories).

Calculation of Endotoxin Concentrations

The mean absorbance value of the blank was subtracted from the mean absorbance value of the standards and the value of samples to calculate the mean absorbance of the samples. Because this absorbance value was directly proportional to the concentration of endotoxins present, the endotoxin concentration was determined from the standard curve.