Efficacy of Endodontic Treatment for Endotoxin Reduction in Primarily Infected Root Canals and Evaluation of Cytotoxic Effects

Luciane Dias de Oliveira, PbD,* Cláudio Antonio Talge Carvalho, PbD,† Alessandra Sverberi Carvalho, MSc,† Jéssica de Souza Alves,* Marcia Carneiro Valera, PbD,† and Antonio Olavo Cardoso Jorge, PbD*  

Abstract

Introduction: Endotoxins are one of the etiologic agents involved in the pathogenesis of apical periodontitis. The objectives of this clinical study were to investigate the effects of endodontic treatment by using different irrigants on endotoxins in root canals with pulp necrosis and apical periodontitis and to evaluate the cytotoxic effects. Methods: Thirty-six root canals were selected. Samples were collected before (S1) and after instrumentation (S2). The root canals were divided into 3 groups (n = 12) according to the irrigant combination used: CLX + LW, 2% chlorhexidine gel + calcium hydroxide (0.14%, limewater); CLX + PmB, chlorhexidine + polymyxin B; CLX (control), chlorhexidine + saline. The third sampling (S3) was performed after ethylenediaminetetraacetic acid and 54 after intracanal medication (CLX + calcium hydroxide for 14 days). Endotoxins were quantified by the chromogenic Limulus amebocyte lysate assay, and cytotoxic effects were evaluated by the production of cytokines (interleukin-1β, tumor necrosis factor α) in macrophages (RAW 264.7) stimulated with the root canal content. Results: Endotoxins were detected in all root canals before instrumentation (S1). Group CLX + LW presented the greatest endotoxin reduction after instrumentation (99.18%), which was similar to group CLX + PmB (96.42%, P > .05) and different from group CLX (90.78%, P < .05). The intracanal medication promoted important endotoxin neutralization, with a reduction of 99.2% to 100%. The root canal content induced a higher production of tumor necrosis factor α and interleukin-1β in S1 samples compared with samples obtained after treatment. Conclusions: The combination of CLX and limewater as irrigant was the most effective in reducing endotoxins in root canals, and intracanal medication was important to neutralize the cytotoxic effects. (J Endod 2012;38:1053–1057)  

Key Words: Calcium hydroxide, cytokines, endotoxin, macrophages, polymyxin B, root canal.

Most pathologic alterations involving the pulp and periapical tissues present a microbial etiology, with bacteria and their products playing a significant role in the induction and maintenance of these lesions. Studies demonstrated a relationship between polymicrobial infection of root canals, especially infections caused by anaerobic gram-negative bacteria, and clinical signs and symptoms such as spontaneous pain, pain on palpation, edema, and purulent exudate (1–6).

Endotoxins consist of lipopolysaccharide (LPS) complexes that are part of the outer membrane of the cell wall of gram-negative bacteria. Endotoxins are the main virulence factor of these bacteria (7) and exert various biological effects that result in the amplification of inflammatory and immune responses (8). Studies suggest that endotoxins are involved in the pathogenesis of pulpal and periapical inflammation (6, 9–15). A positive correlation between endotoxin concentration in the root canal and the presence of endodontic signs and symptoms has been shown (16). Endotoxins have been detected in 100% of first samplings of root canals with necrotic pulp, with levels significantly higher in symptomatic teeth (17–21). Therefore, treatment of infected root canals should not only destroy bacteria but should also inactivate their endotoxins and other toxic products.

Sodium hypochlorite and chlorhexidine, which are used as auxiliary chemical solutions during biomechanical preparation, have shown an excellent antimicrobial activity, but little or no effect on LPS has been reported (12, 15, 17–19, 22). In an in vitro study (15), 2.5% and 5.25% sodium hypochlorite and 2% chlorhexidine were unable to inactivate LPS in root canals. However, the application of 0.14% calcium hydroxide (limewater) and polymyxin B as irrigation agents significantly reduced the level of endotoxins in root canals. It would therefore be interesting to evaluate in vitro the combined application of irrigants with antimicrobial properties such as chlorhexidine gel and substances able to inactivate endotoxins such as calcium hydroxide and polymyxin B in an attempt to enhance the benefits of endodontic therapy. In addition, it would be important to investigate the in vivo effect of the administration of intracanal medications (calcium hydroxide + 2% chlorhexidine gel) for 14 days on LPS. We hypothesized that these medications abolish the cytotoxic effects induced by endotoxins and other bacterial factors such as the production of different cytokines (interleukin [IL]-1β and tumor necrosis factor [TNF]-α) involved in the process of periapical bone resorption.
The objectives of the present clinical study were to investigate the effects of biomechanical preparation by using different combinations of irrigation agents and intracanal medication on endotoxins in root canals with pulp necrosis and apical periodontitis and to evaluate the cytotoxic effects of the root canal content in macrophages.

Materials and Methods

Patient Selection

Thirty-six patients seen at the Endodontics Clinic of the São José dos Campos Dental School (UNESP), São José dos Campos, São Paulo, Brazil, participated in this study. The age of the patients ranged from 19–55 years. Thirty-six single-rooted teeth (superior incisors, superior and inferior canines, and inferior premolars) with pulp necrosis and radiographically visible apical periodontitis were selected. Patients who had received antibiotic therapy during the last 3 months and teeth with periodontal pockets larger than 4 mm were excluded. The project was approved by the Ethics Committee of the São José dos Campos Dental School, UNESP (protocol 096/2007-PH/CEP), and all patients signed a free informed consent form.

The presence of caries, spontaneous pain, pain on percussion or palpation, a history of pain, periodontal health (probing), and presence of a fistula, edema, and abscess were evaluated. In addition, the size of a fistula, edema, and abscess were evaluated. Patients who had received antibiotic therapy during the last 3 months and teeth with periodontal pockets larger than 4 mm were excluded. The project was approved by the Ethics Committee of the São José dos Campos Dental School, UNESP (protocol 096/2007-PH/CEP), and all patients signed a free informed consent form.

Experimental Groups

Files, instruments, and all materials used in this study were treated with Co60 gamma radiation (20 kGy for 6 hours) for sterilization and elimination of preexisting endotoxins (23) (EMBRARAD; Empresa Brasileira de Radiação, Cota, SP, Brazil).

The teeth were isolated with a rubber dam, and the operative field was disinfected with 30% hydrogen peroxide for 30 seconds, followed by 2.5% sodium hypochlorite for an additional 30 seconds. The sodium hypochlorite solution was neutralized with 5% sodium thiosulfate (16, 17, 19–21, 24).

Access to the pulp cavity was made with sterile apyrogenic high-speed burs (KG Sorensen, Cotia, SP, Brazil) under irrigation with sterile apyrogenic saline solution. For collection of the first root canal sample, which was collected immediately after opening of the crown and before application of any antimicrobial solution (S1), the canals were filled with sterile apyrogenic saline for 1 minute. Care was taken to avoid overflow of the solution. The content was then aspirated with an insulin syringe and needle. This procedure was repeated until a final volume of 100 µL was obtained.

Next, the cervical and middle thirds of the root canals were prepared by the crown-down technique by using Endo-Eze oscillating files (Ultradent Products, South Jordan, UT), according to the manufacturer’s instructions. The files of the Endo-Eze system were adapted to the Endo-Eze contra-angle in a handpiece (Kavo do Brasil, Ltda, Sangaúl Joinville, SC, Brazil). K-files size 15 or 20 (Dentsply-Maillefer, Ballaigues, Switzerland) were always used between each file of the oscillating system.

The lumen of the canal was identified by using a K-file size 10 (Dentsply-Maillefer). Next, cervical interferences were eliminated with the 13/.060 instrument of the Endo-Eze system by using the same principles of the crown-down pressureless technique. Instrumentation was continued by using an oscillating 13/.045 file, K-file (N1 or N2) until reaching a depth that was 3 mm shorter than the full length of the root canal calculated from preoperative radiographs.

During preparation of the cervical and middle thirds, the root canal was filled with 2% chlorhexidine gel as adjunct, followed by irrigation and aspiration of 5 mL sterile apyrogenic saline after use of the oscillating instrument. This procedure was repeated at each file change. The working length (1 mm from the radiographic apex) was calculated on the basis of a radiograph after introducing a file into the root canal.

Apical preparation (0.5–1 mm beyond the radiographic apex) was performed with 4 K-files. The teeth were then divided into 3 groups (n = 12) according to the combination of adjunct substances used during apical root canal preparation (Table 1): CLX + LW, 2% chlorhexidine gel + apyrogenic saline, followed by limewater [0.14% Ca(OH)2]; CLX + PmB, 2% chlorhexidine gel + apyrogenic saline, followed by polymyxin B; CLX, 2% chlorhexidine gel + apyrogenic saline.

In group CLX + LW, 2% chlorhexidine gel was applied during the first 2 files used for apical preparation, followed by irrigation with 5 mL apyrogenic saline. Next, 5 mL limewater [0.14% Ca(OH)2] was used as irrigation agent for the last 2 files to neutralize possible endotoxins after the death of gram-negative bacteria. After the last file, the canals were filled with limewater, which was mixed for 3 minutes (#25 K-file) before final irrigation with apyrogenic saline.

In group CLX + PmB, 2% chlorhexidine gel was applied during the first 2 files used for apical preparation, followed by irrigation with 5 mL apyrogenic saline. Next, polymyxin B + saline were used as irrigation agent for the last 2 files to neutralize possible endotoxins after the death of gram-negative bacteria. After application of the last file, the canals were filled with polymyxin B, which was mixed for 3 minutes (#25 K-file) before final irrigation with apyrogenic saline.

In group CLX (control), 2% chlorhexidine gel was applied during the 4 files used for apical preparation, followed by irrigation with 5 mL apyrogenic saline at each file change.

At the end of biomechanical preparation, all root canals were irrigated with 5 mL sterile apyrogenic saline, and a second sample was collected (S2). All root canals were flooded with 17% ethylenediaminetetraacetic acid (EDTA), which was mixed with a file for 3 minutes and then removed with 5 mL sterile apyrogenic saline. A third root canal sample was then obtained (S3).

The canals were dried with absorbent (sterile and apyrogenic) paper points and filled with intracanal medication consisting of a paste of 2% chlorhexidine gel and calcium hydroxide pro-analysis (equal volumes) (Table 1). The medication was introduced into the root canals with a lentulo spiral run at low speed, and a provisional restoration of glass ionomer cement was placed. Fourteen days after use of the intracanal medication, the surgical field was isolated and disinfected.

### Table 1. Groups Treated with Different Combinations of Adjunct Substances during Apical Root Canal Preparation

<table>
<thead>
<tr>
<th>Groups (n = 12)</th>
<th>Adjunct substances</th>
<th>Intracanal medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLX + LW</td>
<td>2% chlorhexidine gel + apyrogenic saline, followed by limewater [0.14% Ca(OH)2]</td>
<td>Paste of 2% chlorhexidine gel and Ca(OH)2 pro-analysis</td>
</tr>
<tr>
<td>CLX + PmB</td>
<td>2% chlorhexidine gel + apyrogenic saline, followed by polymyxin B</td>
<td>Paste of 2% chlorhexidine gel and Ca(OH)2 pro-analysis</td>
</tr>
<tr>
<td>CLX</td>
<td>2% chlorhexidine gel + apyrogenic saline</td>
<td>Paste of 2% chlorhexidine gel and Ca(OH)2 pro-analysis</td>
</tr>
</tbody>
</table>
and the provisional restoration was removed. Next, the root canals were irrigated with 10 mL apyrogenic saline. The solution was aspirated, and the root canal content was again collected (S4).

For completion of endodontic treatment, all root canals were filled with gutta-percha points and AH+ cement (Dentsply) by using the lateral condensation technique. The teeth were restored, and the cases will be followed during a period of 2 years for evaluation of treatment success.

Collection of Root Canal Content

Four root canal samplings were performed: S1, immediately after coronal opening; S2, after biomechanical preparation; S3, after EDTA application; and S4, after removal of the intracanal medication. All samples were submitted to the quantification of endotoxins and the evaluation of cytotoxic effects in macrophage cultures (RAW 264.7).

Quantification of Endotoxins (LPS) by the Kinetic Chromogenic Limulus Amebocyte Lysate Assay

The kinetic chromogenic Limulus amebocyte lysate (LAL) (Lonza, Walkersville, MD) assay was used for the quantification of endotoxins. Escherichia coli endotoxin was used as standard. A positive control (root canal sample contaminated with a known amount of endotoxin) was included for each sample to determine the presence or absence of interfering agents. For the test, 100 μL of apyrogenic water (reaction blank), 5 standard endotoxin solutions (0.005–50 EU/mL), the root canal samples, and the positive controls were added to a 96-well apyrogenic plate. The tests were carried out in quadruplicate. The plate was incubated at 37°C ± 1°C for 10 minutes in the Kinetic-QCL reader, which was coupled to a microcomputer with the WinKQCL software. Next, 100 μL of the chromogenic reagent was added to each well. After the beginning of the kinetic test, the software continuously monitors absorbance at 405 nm in each microplate well and automatically calculates the log/log linear correlation between the reaction time of each standard and the corresponding endotoxin concentration.

The results were analyzed by Friedman analysis of variance and Dunn test for the evaluation of percent reduction of endotoxins within the same group. Comparisons between experimental groups were done by the Kruskal-Wallis and Dunn tests. The level of significance was set at 5% for all tests.

Evaluation of the Cytotoxic Effects of Root Canal Content by the Production of Cytokines (IL-1β and TNF-α) in Macrophage Cultures

A mouse macrophage line (RAW 264.7) obtained from the cell bank of Associação Técnico Científica Paul Ehrlich (APABCAM, Rio de Janeiro, Brazil) was maintained in Dulbecco modified Eagle medium (DMEM) (LGCBio, São Paulo, Brazil) enriched with 10% fetal bovine serum (FBS) (Invitrogen Brasil Ltda, São Paulo, SP, Brazil). The culture medium was changed every 2 days, and the cells were maintained at 37°C in a 5% CO2 atmosphere.

For the tests, a cell suspension was prepared by mechanical removal of the cells with a cell scraper (Gorning Costar, Cambridge, MA). Next, the culture medium containing the cells was transferred to tubes and centrifuged at 9000 rpm for 5 minutes at 25°C. The supernatant was discarded, and the pellet was resuspended in DMEM + 10% FBS. Cell viability was evaluated by trypan blue exclusion, and viable cells were counted in a Neubauer chamber. Each well of a 24-well polystyrene plate (Gorning Costar) was inoculated with 1 × 105 viable cells, and DMEM + 10% FBS was added until a final volume of 500 μL. These cells were activated with the samples collected from the root canals and incubated at 37°C in a 5% CO2 atmosphere. After 24 hours, the supernatants were collected, and cytokines (IL-1β and TNF-α) were quantified by an enzyme-linked immunosorbent assay. The DuoSet anti–IL-1β or anti–TNF-α kit (R&D Systems, Minneapolis, MN) was used according to manufacturer’s instructions. After determination of optical densities, the levels of IL-1β (pg/mL) and TNF-α (pg/mL) in the macrophage culture supernatants were calculated by using the GraphPad Prism 5.0 (La Jolla, CA) program. The results were analyzed statistically by analysis of variance and the Tukey test, adopting a level of significance of 5%.

Results

Quantification of Endotoxins (LAL Assay)

Endotoxins were detected in 100% of S1 samples (baseline) (Table 2). A significant reduction of endotoxin levels was observed at S2 in all groups (CLX + LW, 99.18%; CLX + PmB, 96.42%; CLX, 90.78%) (Table 3). This reduction was significantly higher in group CLX + LW (instrumentation with chlorhexidine gel + limeswater) than in group CLX (chlorhexidine gel + saline solution) (P < .05) and was similar compared with group CLX + PmB (chlorhexidine gel + polymyxin B) (P > .05). No significant difference in the reduction of endotoxin levels was observed for CLX + PmB when compared with groups CLX + LW and CLX (P > .05) (Table 3). Group CLX presented the lowest reduction in endotoxin levels.

A slight increase of endotoxin levels was observed after the application of EDTA (S3) in groups CLX + LW and CLX + PmB and a small reduction in group CLX (Table 3). However, the difference was not statistically significant (P > .05). A significant reduction of endotoxin levels was observed after the intracanal medication (chlorhexidine + calcium hydroxide paste) (S4) in all groups (CLX + LW, 100%; CLX + PmB, 100%; CLX, 99.2%), with no significant difference between groups (P > .05) (Table 3).

Evaluation of Cytotoxic Effects

Production of IL-1β. Mean IL-1β production was higher at S1 compared with the other samplings, with no significant difference between the experimental groups (P > .05) (Table 4). The root canal content collected after biomechanical preparation (S2) promoted low production of IL-1β in all groups, with no significant difference between groups.
groups ($P > .05$). No statistical differences in IL-1β production were observed after EDTA application (S3). Root canal content collected after the intracanal medication (S4) promoted little or no IL-1β production, with no significant difference between groups ($P > .05$) (Table 5). A lower mean production of TNF-α was observed after biomechanical preparation (S2) compared with S1. However, TNF-α levels continued to be high in all groups, and there was no significant difference between the experimental groups ($P > .05$). TNF-α production after EDTA application (S3) was similar to S2, with high production in all groups and no significant difference between groups ($P > .05$). A reduction of TNF-α production was observed after the intracanal medication (S4) in many cases when compared with the previous samplings, but production continued to be relatively high in various samples. There was no significant difference between the 3 groups ($P > .05$) (Table 5).

**Discussion**

Quantification of endotoxins revealed their presence in all root canals with necrotic pulps, in agreement with the literature (16–20, 25). The endotoxin concentration observed in the first sampling (S1) ranged from 29.2–42,400 EU/mL. These results agree in part with those reported by other authors (17, 18), who obtained endotoxin concentrations ranging from 10–200 EU/mL (mean, 151.61 EU/mL) and from 17–696 EU/mL (mean, 228 EU/mL), respectively. Similar levels were observed in most of the patients studied here. However, some samples presented much higher levels, in agreement with Jacinto et al (16), who found endotoxin concentrations ranging from 2,390–22,100 EU/mL in necrotic root canals. These differences between results might be attributed to the sensitivity of the test that detects minimum variations in endotoxin levels, the method of sampling of root canal content, time of sampling, and anatomical diversity of root canals, among others.

With respect to clinical data, only 8 of the 36 patients reported painful symptoms. Six of these patients had high endotoxin levels in the first sampling (S1), in agreement with studies showing a correlation between clinical signs and symptoms and higher endotoxin levels (16, 18, 21, 25). However, endotoxin levels were not elevated in the other 2 patients reporting pain. Regarding the size of the lesions, 2 patients with extensive lesions (>10 mm) accompanied by an exudate presented the highest levels of endotoxins in the first sampling (42,400 and 29,200 EU/mL). However, no elevated endotoxin levels were observed in 3 other patients with lesions measuring ≥10 mm. In the remaining patients, the small variation in the extent of apical periodontitis and endotoxin concentration did not permit any correlation of the data.

A significant reduction in endotoxin levels compared with the first sampling (S1) was observed after biomechanical preparation (S2), irrespective of the irrigation agent used. This reduction was more than 90% in all cases. These results agree with other authors (17–19) who suggested that chemomechanical preparation reduces the levels of endotoxins in root canals. However, these authors reported mean reductions of 44.4%–47.12% or 57.98%–59.99% when 2% chlorhexidine or 2.5% NaOCl was used, respectively. In the present study, the mean reduction was 90.78% when 2% chlorhexidine was used as adjunct. These divergent results might be explained by the different techniques used for root canal preparation, including the size of the instruments used for apical preparation and the volume of irrigants. In the present study, 4 files of the Endo-Eze system were used for preparation of the cervical and middle thirds and 4 K-files for preparation of the apical third. Thus, part of the reduction in endotoxin levels seems to be due to the process of instrumentation itself, irrigation and aspiration of root canal content, irrespective of the irrigant used.

In the present study, a significantly higher reduction in endotoxin levels was observed when limewater (0.14% calcium hydroxide) was used in combination with 2% chlorhexidine gel during biomechanical preparation of the root canals. The use of limewater for the last 2 files during apical preparation promoted a significantly higher neutralization of endotoxins (mean of 99.18%) than the use of 2% chlorhexidine gel alone throughout preparation (90.78%). In group CLX + LW, no endotoxins were detected in 3 root canals after instrumentation. These results indicate that the use of limewater during the final steps of root canal instrumentation is feasible and has benefits for endodontic therapy, neutralizing most endotoxins after the death of gram-negative bacteria. Further studies combining limewater with different irrigants during biomechanical preparation are needed to evaluate whether this combination increases the success of endodontic treatment. All cases of this study will be followed up for a minimum period of 2 years to evaluate clinical success or failure. In group CLX + PmB, the use of polymyxin B for the last 2 files during apical preparation also resulted in an important reduction of endotoxins in root canals (mean of 96.42%), demonstrating that final application of polymyxin B is able to neutralize a large quantity of endotoxins. However, the difference was not significant when compared with the CLX group.

No endotoxins (levels <0.005 EU/mL) were detected in 20 root canals after the intracanal medication (chlorhexidine gel + calcium hydroxide) for 14 days, corresponding to a reduction of 99.20% and 100%. These results disagree with those of Vianna et al (17), who found that in vivo administration of intracanal medication (calcium hydroxide paste, chlorhexidine gel, or chlorhexidine gel + calcium hydroxide paste) for 7 days only promoted a mean endotoxin reduction in root canals of 14%. According to these authors, the use of intracanal medications for 7 days does not bring any improvement. The longer

**TABLE 4.** Levels of IL-1β (mean ± standard deviation, pg/mL) in Macrophage Culture Supernatants after Activation with the Root Canal Content

<table>
<thead>
<tr>
<th>Sampling</th>
<th>CLX + LW</th>
<th>CLX + PmB</th>
<th>CLX</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>50.34 ± 71.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.02 ± 130.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.28 ± 92.94&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>S2</td>
<td>4.26 ± 5.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.41 ± 6.93&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.07 ± 7.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>S3</td>
<td>3.14 ± 3.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.81 ± 7.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.17 ± 4.84&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>S4</td>
<td>2.36 ± 3.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.91 ± 6.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.33 ± 3.87&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscript letters<sup>a,b</sup> indicate statistically significant differences ($P < .05$).

**TABLE 5.** Levels of TNF-α (mean ± standard deviation, pg/mL) in Macrophage Culture Supernatants after Activation with the Root Canal Content

<table>
<thead>
<tr>
<th>Sampling</th>
<th>CLX + LW</th>
<th>CLX + PmB</th>
<th>CLX</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>1132.26 ± 782.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1115.37 ± 538.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1046.02 ± 580.79&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>S2</td>
<td>628.57 ± 426.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>518.64 ± 420.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>791.75 ± 403.23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>S3</td>
<td>674.26 ± 310.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>532.58 ± 279.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>694.67 ± 237.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>S4</td>
<td>345.40 ± 261.53&lt;sup&gt;c&lt;/sup&gt;</td>
<td>291.61 ± 240.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>323.94 ± 273.50&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscript letters<sup>a,b,c</sup> indicate statistically significant differences ($P < .05$).
time of application (14 days) in the present study might have promoted a greater action of the intracanal medication deep within the dentinal tubules. The present in vitro results confirm previous in vitro studies in which application of the intracanal medication (chlorhexidine gel + calcium hydroxide) for 14 days neutralized most of the endotoxins that remained in the root canals after biomechanical preparation (22, 26). The biological properties of LPS require the presence of ester-linked hydroxy fatty acids, and calcium hydroxide cleaves these bonds (27). Studies demonstrated the ability of calcium hydroxide to neutralize endotoxins in root canals when used as intracanal medication (10–12, 22, 26, 28). In this respect, the present results showed that calcium hydroxide did not lose its LPS-neutralizing capacity in the presence of chlorhexidine gel, and that this combination had positive effects on the elimination of endotoxins from root canals, supporting its importance as an intracanal medication.

Analysis of the cytotoxic effects of root canal content showed that samples collected before treatment (S1) promoted a higher mean production of TNF-α and IL-1β than content collected after treatment. Martinho et al (24) showed a correlation between the number of gram-negative bacteria and the levels of IL-1β and TNF-α (P < .05). Elevated levels of endotoxin were followed by the secretion of TNF-α, and higher levels of IL-1β and endotoxin were related to a larger size of the radio-opaque area (24). These authors concluded that the antigenicity of the endodontic content is not only related to the concentration of endotoxin found in the root canal but also to the number of different species of gram-negative bacteria involved in the infection. In the present study, production of IL-1β was lower than that of TNF-α in all cases, with almost undetectable levels in many samples after treatment (S2, S3, and S4). TNF-α production was relatively high in all cases after biomechanical preparation and EDTA application (S2 and S3), demonstrating the cytotoxic effect of these samples. Mean TNF-α production was lower in samples after the intracanal medication. However, elevated levels were observed in some cases, demonstrating that the production of TNF-α was more sensitive to the presence of several bacterial factors such as endotoxin (LPS), lipoteichoic acid, and peptidoglycan than IL-1β in the cells studied. The results showed no significant difference in the production of IL-1β or TNF-α between the substances used for biomechanical preparation (CLX + LW, CLX + PmB, and CLX), and the intracanal medication was found to promote a lower production of these cytokines.

In conclusion, the present study demonstrated that the combination of 2% chlorhexidine gel and calcium hydroxide 0.14% (limewater) as irrigant was the most effective in reducing endotoxins in root canals, and the use of intracanal medication (2% chlorhexidine gel + calcium hydroxide) for 14 days was important to neutralize the cytotoxic effects.

Acknowledgments

The authors deny any conflicts of interest related to this study.

References