This clinical study investigated the effects of endodontic treatment by using different irrigants (limewater + NaOCl and polymyxin B + NaOCl) and intracanal medication on endotoxins in teeth with primary endodontic infection and radiographically visible apical periodontitis. Thirty-three teeth with necrotic pulp and periapical lesions from different patients were selected for this study. Samples were collected after the coronal opening (S1) and after instrumentation (S2). Root canals were divided in 3 groups (n = 11) according to the irrigant combination used: NaOCl + LW: 2.5% NaOCl + calcium hydroxide solution (0.14%, limewater); NaOCl + PmB: 2.5% NaOCl + 10.000 UI/mL polymyxin B; 2.5% NaOCl (control). The third sampling (S3) was performed after ethylenediaminetetraacetic acid and the fourth (S4) after samples got 14 days with intracanal medication with 2% chlorhexidine gel + calcium hydroxide. Endotoxins (lipopolysaccharide) were quantified by chromogenic Limulus amebocyte lysate (LAL). Endotoxins were detected in all root canals after the coronal opening (S1). NaOCl + PmB group presented the greatest endotoxin reduction after instrumentation (76.17%), similar to NaOCl + LW group (67.64%, p<0.05) and different from NaOCl group (42.17%, p<0.05). After intracanal medication period (S4), there was significant increase of endotoxins neutralization. It was concluded that NaOCl + PmB promoted the greatest reduction of endotoxin levels, followed by NaOCl + LW. Intracanal medications had no significant complementary role in the reduction of endotoxins at the end of the treatment.

Key Words: Endotoxins, limewater, polymyxin B, sodium hypochlorite.
on dentin for up to 12 weeks and biocompatibility to the periapical tissues (15).

Polymyxin B, often used in patients with endotoxic shock, is a cationic polypeptide antibiotic that acts primarily on the cell wall of Gram-negative bacteria, causing fast changes in the permeability of the cytoplasmic membrane and causing cell death (16). In addition to the antimicrobial activity, this substance is able to neutralize endotoxins (5, 17) and may be used in endodontics because of its effectiveness in the neutralization of LPS, present in root canals (13).

Therefore, this clinical study evaluated the endotoxin levels in root canals with necrotic pulp and radiographically visible periapical lesion, before endodontic treatment, after biomechanical preparation with different combinations of irrigants (NaOCl + limewater, NaOCl + polymyxin B) and after intracanal medication (calcium hydroxide + chlorhexidine gel).

Material and Methods

Patient Selection

After Ethics Committee’s approval, 33 patients attending the Dental School of São José dos Campos (UNESP), São José dos Campos (SP), Brazil, for primary endodontic treatment of radiographically visible apical periodontitis were included in this study. A detailed dental history was obtained from each patient. Those who had received antibiotic treatment during the last three months or who had any general disease were excluded. The Human Research Ethics Committee of the Dental School of São José dos Campos (UNESP) approved the protocol (023/2008-PH/CEP) describing the samples collection for this investigation and the patients were all volunteer who signed an informed consent form. The protocol (023/2008-PH/CEP) was approved by the Dental School of São José dos Campos (UNESP), São José dos Campos, SP, Brazil). This procedure was repeated until a final volume of 100 μL was obtained.

After the first collection, cervical and middle thirds of the root canals were prepared by the crown-down technique, using Endo-Eze oscillating files (Ultradent Products, South Jordan, UT, USA), according to the manufacturer’s instructions.

The lumen of the canal was identified by using a K-file size 10 (Dentsply/Maillefer Instruments SA, Ballaigues, Switzerland). Next, cervical interferences were eliminated with Endo-Eze instrument 13/0.60 by using the same principles of the crown-down pressureless technique.

Instrumentation was continued by using oscillating file 13/0.45, K-file (#15), oscillating file 13/0.35, K-file (#15), and oscillating file 10/0.25, until achieving a depth 3 mm shorter than the full length of the root canal calculated from preoperative radiographs.

During the preparation of cervical and middle root canal thirds, 5 mL of 2.5% NaOCl solution were used as irrigant and renewed at each change of instrument. The working length (1 mm shorter than the radiographic apex length) was calculated, taking into consideration the tooth radiograph after introduction of a file into the root canal. Apical preparation (0.5–1 mm shorter than the radiographic apex) was performed with 4 K-files. Then, teeth were then divided into 3 groups (n = 11), according to the combination of adjunct substances used during apical root canal preparation (Table 1): NaOCl + LW: 2.5% NaOCl + calcium hydroxide solution (0.14%, limewater); NaOCl + PmB: 2.5% NaOCl + 10.000 UI/mL polymyxin B, and 2.5% NaOCl (control).

In group NaOCl + LW, 5 mL of 2.5% NaOCl was used for the two first files for apical preparation. Next, 5 mL of limewater was used as irrigation agent for the two last files.

In group NaOCl + PmB, 5 mL of 2.5% NaOCl was used for the two first files for apical preparation. Next, polymyxin B (Ophalmos Fórmulas Oficiais Ltda, São Paulo, SP, Brazil) was applied during the two last files, followed by irrigation with 5 mL of apyrogenic saline at each file change.

In group NaOCl (control), for the 4 files for apical preparation, 5 mL of 2.5% NaOCl solution was used and renewed at each file change.
At the end of biomechanical preparation, all root canals were irrigated with 5 mL of apyrogenic saline and the second sample was collected (S2). Root canals were then filled with 17% EDTA (Asfer Indústria Química Ltda, São Caetano do Sul, SP, Brazil), which was mixed with a file for 3 minutes and then removed with 5 mL of apyrogenic saline before third sample collection (S3).

The root canals were dried with sterile and apyrogenic absorbent paper points of compatible size and filled with intracanal medication consisting of a paste of 2% chlorhexidine gel (Terapêutica Farmácia de Manipulação, São José dos Campos, SP, Brazil) associated with pro-analysis calcium hydroxide (prepared in equal proportions in volume). 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. The intracanal medication was maintained for 14 days.

After this period, the patients returned. The teeth were isolated and disinfected, according to the protocol described above, and the temporary restoration was removed. Root canals were irrigated with 10 mL of apyrogenic saline to remove intracanal medication, and then, the fourth sample collection (S4) was performed. In order to finish the endodontic treatment, root canals were obturated with gutta-percha points and AH plus cement (Dentsply/Maillefer SA, Ballaigues, Switzerland) by using the active lateral condensation technique.

**LAL assay (KQCL test) - Quantification of Endotoxins**

All samples collected (S1, S2, S3 and S4) during endodontic treatment (100 μL) were transferred to polypropylene Eppendorf tubes, containing 900 μL of apyrogenic and sterile water, which were used for quantification of endotoxins.

The kinetic chromogenic limulus amebocyte lysate (LAL) (Lonza, Walkersville, MD, USA) assay was performed in order to quantify 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 endotoxin units (EU)/mL], root canal samples, and positive controls [each root canal sample contaminated with a known concentration of endotoxin (10 EU/mL)] were added to a 96-well apyrogenic microplate. 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 WinKQCL software (Lonza). Next, 100 μL of the chromogenic reagent was added to each well. After the beginning of the kinetic test, the software continuously monitored the absorbance at 405 nm in each microplate well and automatically calculated the log/log linear correlation between reaction time of each standard and the corresponding endotoxin concentration (3,5,6).

**Statistical Analysis**

The results were analyzed statistically by Kruskal-Wallis and Dunn tests, adopting a level of significance of 5%.

**Results**

All the samples from first collection (S1) showed presence of endotoxins (33/33 - baseline). It was possible to verify a progressive reduction in endotoxin levels according to the collection (Table 2). The main objective of the endotoxin test was to assess the effectiveness of different irrigants (NaOCl, NaOCl + LW, NaOCl + PmB). The comparison between the reduction percentages observed for each patient after instrumentation (S1 to S2) allowed a better screening of the results obtained for the irrigants analyzed. Descriptive statistics of reduction from S1 to S2, S3 and S4 is presented in Table 3. Kruskal-Wallis test revealed a value for $P = 0.0228$ (P <.05), which confirms the presence of statistically significant differences between the groups. Application of Dunn’s test identified the difference between groups NaOCl and NaOCl + PmB (Table 3).

Application of EDTA was also evaluated but no difference between the groups was found, which was confirmed by Kruskal-Wallis test for $P = 0.1829$ (P >.05). In order to evaluate the endodontic treatment by analyzing the effectiveness of intracanal medications for 14 days, it was also considered the percentage of reduction between S1 and S4 for each patient in the study. Kruskal-Wallis test revealed a value of $P = 0.4475$ (P >.05) for the percentage of reduction between S1 and S4, which no statistically significant difference between the groups.

**Table 1. Groups treated with different combinations of adjunct substances during root canal preparation**

<table>
<thead>
<tr>
<th>Group (n=11)</th>
<th>Adjunct substances</th>
<th>Intracanal medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOCl</td>
<td>2.5% NaOCl solution</td>
<td></td>
</tr>
<tr>
<td>NaOCl + LW</td>
<td>2.5% NaOCl solution, followed by limewater [0.14%, Ca(OH)2]</td>
<td>2% chlorhexidine gel + calcium hydroxide</td>
</tr>
<tr>
<td>NaOCl + PmB</td>
<td>2.5% NaOCl solution, followed by polymyxin B</td>
<td></td>
</tr>
</tbody>
</table>
statistics of the values and reductions from S1 to S2, S3 and S4 is also presented in Table 3.

**Discussion**

In this study, the mean reduction from S1 to S2 (after biomechanical preparation) was 42.17% for the group NaOCl; 67.64% for the group NaOCl + LW; and 76.17% for the group NaOCl + PmB. The mean reduction found for the group NaOCl in this study was slightly lower than the values reported by Gomes et al. (19), who observed a reduction percentage of 57.98% of endotoxins after using the same irrigant, and by Martinho and Gomes (20), who found a percentage of reduction of 59.99%. It is possible that the difference in the reduction percentage found in other studies may occur due to the different technical instrumentation used. In those studies, rotary techniques were used, whereas we have used oscillatory system during the endodontic treatment.

These data reveal that 2.5% NaOCl used alone during biomechanical preparation is not very effective against endotoxins present in infected root canals, suggesting that 2.5% NaOCl is not successful for elimination of endotoxins, which remained in the root canal system. Aiming to enhance the effectiveness of the biomechanical preparation, we decided to associate NaOCl to calcium hydroxide solution and polymyxin B, whose action against endotoxins has been demonstrated elsewhere (5,9,21).

The results found for the association NaOCl + LW revealed a reduction percentage of 67.64% from S1 to S2 and 76.17% for the association of NaOCl + PmB, demonstrating the improved effectiveness of irrigation for both associations, even though only the group NaOCl + PmB was significantly different from NaOCl used alone. Oliveira et al. (5) used limewater and polymyxin B in association with chlorhexidine as irrigants, since the associations showed better results in comparison with chlorhexidine alone. Moreover, the combination of chlorhexidine with limewater was more effective in reducing endotoxins from root canals, indicating that these associations presented the best results.

The use of calcium hydroxide in a solution of apyrogenic water (calcium hydroxide solution) and polymyxin B, used as irrigants, was reported by Oliveira et al. in vitro (13) and in vivo (5). The authors evaluated the detoxification of endotoxin by using LAL test and production of polyclonal antibodies by B cells. The results revealed that calcium hydroxide and polymyxin B presented an excellent effect on the elimination of endotoxins. Even though a reduction in endotoxin levels was found with the use of associations compared to NaOCl used alone, a small quantity of endotoxin remained in the root canals after biomechanical preparation. The remaining endotoxin probably persisted because calcium hydroxide and polymyxin B, though effective against the LPS, did not penetrate sufficiently into the dentinal tubules to eliminate the remnants present in deepest regions. Safavi and Nichols (11) demonstrated that treatment of LPS with calcium hydroxide releases a high quantity of fatty acids by the hydrolysis of ester bonds in lipid chains of bacterial LPS. Thus, treatment with calcium hydroxide would detoxify LPS, inhibiting its pathological effects (2, 11).

The effectiveness of polymyxin B against endotoxins observed in this study is supported by previous works in the literature (9,13,17,21). Hong et al. (21) reported that the systemic administration of polymyxin B in rats reduced the size of periapical lesions by approximately 80%. According to the author, polymyxin B is a cationic peptide with powerful antioxidant activity. Its molecule bonds to lipid A, negatively charged, altering the three-dimensional conformation of the LPS molecule. In the present study, the polymyxin B associated with 2.5% NaOCl presented the highest percentage of reduction of endotoxin levels (76.17%) from S1 to S2, with statistically significant difference from NaOCl used alone. On the basis of the results currently available for polymyxin B group, it seems fair to suggest that the combined use of NaOCl with this drug may be a viable option for use in root canals in order to obtain greater endotoxin neutralization.

Data gathered in this study demonstrated that

### Table 2. Levels of endotoxins (mean ± standard deviation, EU/mL) in the root canal samples obtained from different experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOCl + LW</td>
<td>1551.26 ± 2726.08</td>
<td>1669.30 ± 168.11</td>
<td>169.75 ± 209.78</td>
<td>85.19 ± 146.64</td>
</tr>
<tr>
<td>NaOCl + PmB</td>
<td>1162.69 ± 1319.77</td>
<td>48.23 ± 82.17</td>
<td>37.80 ± 39.34</td>
<td>77.05 ± 174.13</td>
</tr>
<tr>
<td>NaOCl (control)</td>
<td>5147.74 ± 14907.97</td>
<td>1172.52 ± 2051.23</td>
<td>189.12 ± 318.96</td>
<td>112.79 ± 179.82</td>
</tr>
</tbody>
</table>

### Table 3. Descriptive statistics of the percentages of reduction of endotoxin levels regarding first, second, third and fourth samples found in different experimental groups (NaOCl, NaOCl + LW, NaOCl + PmB)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1-S2</td>
</tr>
<tr>
<td>NaOCl + LW</td>
<td>67.64±32.30 AB</td>
</tr>
<tr>
<td>NaOCl + PmB</td>
<td>76.17±33.24 A</td>
</tr>
<tr>
<td>NaOCl (control)</td>
<td>42.17±36.97 B</td>
</tr>
</tbody>
</table>

Different letters indicate statistically significant differences (p<0.05).
the use of intracanal medication (calcium hydroxide paste associated with 2% chlorhexidine gel) did not increase the neutralization of endotoxins. This medication was selected based on studies that demonstrate its effectiveness against microorganisms and endotoxins (5, 14). This way, in the end of the endodontic treatment (irrigation + intracanal medication), the mean percentages of endotoxin reduction were 75.23% for the group using only NaOCl solution during instrumentation + medication; 79.97% for NaOCl + LW and medication and 80.59% for NaOCl + PmB and medication. It may be noticed that, comparing the percentages obtained after the second collection (S2), the mean reduction obtained after using intracanal medication was greater but not statistically different. It is believed that the use of intracanal medication has adequate antimicrobial action because it remains in contact with dentin and diffuses through the tissues (22). These results show that there is still no technique considered ideal for elimination of infection from the root canal system.

In conclusion, biomechanical preparation with the combined use of 2.5% NaOCl + polymyxin B as irrigant presented the greatest reduction of endotoxin levels, followed by 2.5% NaOCl + limewater. Intracanal medication had no significant complementary role in the reduction of endotoxins at the end of the treatment.

**References**


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