Evaluation of pH, available chlorine content, and antibacterial activity of endodontic irrigants and their combinations against Enterococcus faecalis

Juliane M. Guerreiro-Tanomaru, DDS, PhD, a Renata D. Morgental, DDS, MSc, a Danilo L. Flumignan, DDS, MSc, b Fabricia Gasparini, DDS, MSc, b José E. Oliveira, DDS, PhD, b and Máximo Tanomaru-Filho, DDS, PhD, a Araraquara, SP, Brazil

UNIV ESTADUAL PAULISTA

Objectives. The objectives of this study were to evaluate pH, available chlorine content, and antibacterial activity of endodontic irrigants and their combinations.

Study design. The pH and chlorine content of sodium hypochlorite (NaOCl) were analyzed pure and in combination with 10% citric acid (CA) and apple vinegar (AV). The antibacterial effect of the following solutions was measured by direct contact test against Enterococcus faecalis: 2.5% NaOCl, 2.5% NaOCl + 10% CA (7:3), 2.5% NaOCl + AV (5:5), 10% CA, and AV. Sterile saline was used as control. The colony-forming units were determined by serial decimal dilutions.

Results. The combination of 2.5% NaOCl with CA or AV lowered the pH and the chlorine content. NaOCl, alone or in combination was able to eliminate E. faecalis in 30 seconds, and CA, after 10 minutes. AV promoted reduction (32.2%) after 10 minutes.


Endodontic infection plays an essential role in the etiology of pulpal and periapical disease.1,2 Thus, one of the main objectives of endodontic therapy of teeth with periapical lesions is the control of infection.3 The persistence of microorganisms in the root canal system after endodontic therapy is determinant to treatment failure.4,5

Instrumentation and irrigation of the root canals are extremely important, but these procedures alone are unable to eliminate endodontic infection.6,7 This is attributable to microbial propagation into the root canal system and apical cementum,8,9 which are challenging areas to reach during the root canal preparation.

Sodium hypochlorite is the most widely used irrigating solution in endodontics for its antimicrobial activity, tissue-dissolving property, detergent action, and the ability to neutralize toxic products.10,11 Some factors may affect the efficacy of NaOCl, such as solution concentration,12 temperature,13 and pH.14,15 The 2.5% sodium hypochlorite solution has been used because of its antimicrobial and solvent activity, with better biocompatibility than more concentrated solutions.10-13

At lower pH, the chlorine in the NaOCl solution is predominantly available as hypochlorous acid (HOCI), more active than the hypochlorite anion (OCl-) prevalent at more alkaline pH.16 HOCI is able to better penetrate the bacterial cell membrane, because of its lack of electrical charge and molecular structure similar to water. Once within the cell, HOCI presents bacteriostatic effect, reacting with the DNA, RNA, and other nucleotides. Additionally, this substance acts as a bacteriostatic agent, reacting with amino acids and producing chloramines.17 Therefore, the antimicrobial effect of NaOCl can potentially be enhanced by lowering the pH of the solution.14,15

Citric acid was first suggested as an irrigant by Wayman et al.18 This substance is generally recommended as a final irrigant because of its ability to remove the smear layer19,20 and it can be associated with NaOCl solutions to lower their pH. Irrigation with NaOCl followed by citric acid may reduce the microbiota in root canals.21 Apple vinegar, containing mainly acetic acid and malic acid, also has the ability to remove the smear layer22 and is active against endodontic microorganisms,23 thus being another alternative for use in combination with NaOCl.

The goal of this study was to evaluate the pH and available chlorine content in 2.5% NaOCl solutions,
Table 1. Mean and standard deviation of pH and available chlorine content in 2.5% NaOCl solution, pure or in combination with 10% citric acid and apple vinegar

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ratios</th>
<th>pH</th>
<th>Chlorine, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5% NaOCl</td>
<td>—</td>
<td>11.75 ± 0</td>
<td>2.52 ± 0.02</td>
</tr>
<tr>
<td>10% Citric acid</td>
<td>—</td>
<td>1.51 ± 0.01</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Apple vinegar</td>
<td>—</td>
<td>2.76 ± 0.01</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>2.5% NaOCl + 10% citric acid</td>
<td>8:2</td>
<td>6.415 ± 0.035</td>
<td>2.085 ± 0.005</td>
</tr>
<tr>
<td>2.5% NaOCl + 10% citric acid</td>
<td>7:3</td>
<td>4.82 ± 0.04</td>
<td>1.815 ± 0.005</td>
</tr>
<tr>
<td>2.5% NaOCl + 10% citric acid</td>
<td>6:4</td>
<td>3.79 ± 0.08</td>
<td>1.555 ± 0.005</td>
</tr>
<tr>
<td>2.5% NaOCl + 10% citric acid</td>
<td>5:5</td>
<td>3.08 ± 0.02</td>
<td>1.30 ± 0.01</td>
</tr>
<tr>
<td>2.5% NaOCl + 10% citric acid</td>
<td>4:6</td>
<td>2.685 ± 0.005</td>
<td>1.045 ± 0.005</td>
</tr>
<tr>
<td>2.5% NaOCl + 10% citric acid</td>
<td>3:7</td>
<td>2.40 ± 0.01</td>
<td>0.785 ± 0.005</td>
</tr>
<tr>
<td>2.5% NaOCl + 10% citric acid</td>
<td>2:8</td>
<td>2.125 ± 0.045</td>
<td>0.53 ± 0</td>
</tr>
<tr>
<td>2.5% NaOCl + apple vinegar</td>
<td>8:2</td>
<td>7.365 ± 0.045</td>
<td>2.07 ± 0</td>
</tr>
<tr>
<td>2.5% NaOCl + apple vinegar</td>
<td>7:3</td>
<td>6.565 ± 0.055</td>
<td>1.805 ± 0.005</td>
</tr>
<tr>
<td>2.5% NaOCl + apple vinegar</td>
<td>6:4</td>
<td>5.43 ± 0.02</td>
<td>1.545 ± 0.005</td>
</tr>
<tr>
<td>2.5% NaOCl + apple vinegar</td>
<td>5:5</td>
<td>4.69 ± 0.01</td>
<td>1.285 ± 0.005</td>
</tr>
<tr>
<td>2.5% NaOCl + apple vinegar</td>
<td>4:6</td>
<td>4.345 ± 0.035</td>
<td>1.035 ± 0.005</td>
</tr>
<tr>
<td>2.5% NaOCl + apple vinegar</td>
<td>3:7</td>
<td>4.03 ± 0.02</td>
<td>0.775 ± 0.005</td>
</tr>
<tr>
<td>2.5% NaOCl + apple vinegar</td>
<td>2:8</td>
<td>3.76 ± 0.04</td>
<td>0.52 ± 0</td>
</tr>
</tbody>
</table>

pure and in combination with 10% citric acid or apple vinegar in different ratios, and to investigate the in vitro antibacterial action of these substances and their combinations.

MATERIAL AND METHODS
pH level and available chlorine assessment

The pH and the chlorine content of 2.5% NaOCl solution (Araquêmica, Araraquara, SP, Brazil) were measured pure and in combination with 10% citric acid (Dinâmica, Diadema, SP, Brazil) or apple vinegar (Castelo, Jundiaí, SP, Brazil) in different ratios (by volume): 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, and 2:8.

The pH was assessed using a DMFH-2 pH meter (Digimed, São Paulo, SP, Brazil), calibrated at 25°C. The chlorine content was evaluated by iodometry, using an AT500-N2 automatic potentiometric titrator AT500-N2 (KEM—Kyoto Electronics Manufacturing, Tokyo, Japan). All analyses were conducted in triplicate.

Antibacterial activity against Enterococcus faecalis

The microbiological assays were carried out in a laminar flow chamber (VecoFlow Ltda, Campinas, SP, Brazil). The following solutions and combinations were investigated: 2.5% NaOCl, 2.5% NaOCl + 10% citric acid at 7:3 ratio, 2.5% NaOCl + apple vinegar at 5:5 ratio, 10% citric acid, and apple vinegar. Because sodium hypochlorite solutions show better activity at pH of approximately 5,24 when the chlorine is available mostly as hypochlorous acid,16 the solutions with this pH (Table I) were selected for these tests. Sterile saline was used as negative control and it allowed the determination of the initial number of colony-forming units (CFU).

The antibacterial activity was evaluated by direct contact test, using a standard strain of E. faecalis (ATCC 29,212). The bacteria were plated onto tryptic soy agar (Difco, Detroit, MI), 12 hours before each test. Purity of the inoculum was confirmed by Gram stain.

The inoculum was suspended in sterile saline and the optical density was adjusted using a spectrophotometer (model 600 Plus, Femto, São Paulo, SP, Brazil). The bacterial suspension was adjusted to a concentration of 3 × 10^7 CFU/mL and used within 60 minutes.

Automated pipettes were used to carry 1.45 mL of each solution or combination into 2-mL Eppendorf test tubes. Following that, a 50-µL aliquot of E. faecalis suspension was added to the tube, and the mixture was agitated for 30 seconds (Vortex AP 56, Phoenix, Araraquara, SP, Brazil). Contact times were 30 seconds, and 1, 3, and 10 minutes.

After the contact period, serial decimal dilutions up to 10^-5 were made. Then, 100-µL aliquots of the mixture were transferred to another test tube containing 0.9 mL of neutralizing agent. For the pure NaOCl solution, the neutralizer was 1% sodium thiosulfate, whereas for the other groups, 1% sodium thiosulfate + 1% Tween 80 solution was used. The contents from the first dilution were homogenized and 100 µL was transferred to a third test tube containing 0.9 mL of the corresponding neutralizing solution. The fourth, fifth, and sixth tubes contained 0.9 mL sterile saline.

Finally, 20-µL aliquots from each dilution were seeded in triplicate on the surface of agar plates, then
incubated at 37°C for 48 hours under aerobic conditions. Readings were carried out on the dilution samples with 5 to 50 colonies in each area of bacterial growth. The mean values were used to calculate the number of CFU/mL at each period of contact between the irrigant and the bacterial suspension. These experiments were repeated 3 times. Mean values obtained were expressed as mean percentages of viable colonies after contact with the irrigants at each experimental period.

RESULTS

pH and chlorine content

The combination of 2.5% NaOCl with 10% citric acid lowered both the pH and the available chlorine content. The higher the amount of acid in the solution, the lower the values found for pH and chlorine availability. A similar effect was observed for the combination of 2.5% NaOCl with apple vinegar (Table I).

Antibacterial activity

Plain 2.5% NaOCl solution, as well as in combination with 10% citric acid or apple vinegar fully eliminated *E. faecalis* in 30 seconds of contact. Plain 10% citric acid eliminated *E. faecalis* in 10 minutes. Apple vinegar promoted only a reduction in the number of viable cells by 32.20% at the end of the experiment, as shown in Table II.

DISCUSSION

Investigations on the influence of pH values on the antibacterial activity of NaOCl have been described. 14,15,25 *E. faecalis* was used because of its high prevalence in endodontic treatment failures, 26 which is likely related to its ability to invade dentin tubules and bind to collagen. 27

The use of chloridric acid and acetic acid to adjust the pH of NaOCl solutions has been previously assessed. 14,15 In this study, citric acid and apple vinegar were used with the same purpose. Citric acid has been used in endodontics as a final irrigant, 19,21 and apple vinegar has shown activity against endodontic microbiota. 23

In a preliminary study, a remarkable reduction on the available chlorine content in combinations of NaOCl with acidic solutions was observed after 10 minutes. For this reason, the substances were mixed only immediately before the physical-chemical and microbiological analyses took place. Combination of 2.5% NaOCl with 10% citric acid gradually lowered the pH and the available chlorine content as the ratios progressed from 8:2 to 2:8. Zehnder et al. 28 studied the combination of 1% NaOCl with 10% citric acid in different ratios and observed absence of available chlorine after the first minute. A possible explanation for this fact would be evaporation of chlorine in the form of gas during interaction of the substances, as reported by Baumgartner and Ibay. 29 Similar results were observed by mixing NaOCl with apple vinegar.

According to Hugo and Russel, 24 NaOCl solutions show stronger antimicrobial activity when the pH is close to 5, which corresponded to the ratio of 7:3 for the combination of NaOCl with citric acid and 5:5 for NaOCl with apple vinegar. These were the selected ratios for the direct contact test. However, in these solutions, the chlorine contents were 1.815 and 1.285, respectively. It is important to point out that the antibacterial effect of NaOCl is directly related to the amount of HOCl available, besides the total chlorine present in the solution.

NaOCl solution in high concentration presents more aggressive action to the periapical tissues. 11 In this study, the combinations of irrigating solutions were tested with the aim of improving the antibacterial activity of 2.5% NaOCl by lowering the pH.

Camps et al. 14 and Mercade et al. 15 demonstrated, in extracted human teeth, that NaOCl solutions with neutral pH are more active against *E. faecalis* than conventional solutions with pH 12. In the present study, NaOCl was mixed with acidic solutions, resulting in a pH of approximately 5, showing antibacterial activity comparable with that of the pure solution with alkaline pH. All the solutions analyzed were able to eliminate *E. faecalis* in the minimum period (30 seconds). In vitro, 2.5% NaOCl was capable of eliminating *E. faecalis* and adjustment of the pH proved unnecessary. In addition, plain citric acid solution and apple vinegar were less efficient.

The direct contact test allows comparisons between the substances without the influence of extrinsic factors that could decrease or increase their antimicrobial effect. So, it should be the first step in the evaluation of antimicrobial agents. In this methodology, the first and the second serial dilution tubes contained a neutralizing
solution to prevent trace amounts of irrigant from being transferred into the culture medium.\textsuperscript{30}

In clinical situations, the results of this study suggest that when NaOCl solution is combined with acidic solution, these substances should be placed directly into the root canal instead of being mixed in a syringe, to prevent the potential loss of chlorine that would occur by previously mixing the solutions. Thus, it would be interesting to study the efficacy of an initial irrigation of the canal with the acidic solution, followed by irrigation with NaOCl.

Considering the methodology used and the results obtained, the present study allows the conclusion that the combination of 2.5% NaOCl with 10% citric acid or apple vinegar lowered the pH and the chlorine content, but did not affect the antibacterial activity, compared with pure NaOCl solution. Plain citric acid and apple vinegar showed weak activity against \textit{E. faecalis}. Additional studies are necessary to clarify the physical and chemical interactions between NaOCl and different acids.

REFERENCES


