THE EFFECTS OF ANTIBACTERIAL SOLUTIONS ON MICROORGANISMS ISOLATED FROM INFECTED ROOT CANALS IN VIVO

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ABSTRACT
Objective: This study assessed the in vivo antimicrobial activity of NaOCl, Chx, Ca(OH)2, and H2O2 against five different microorganisms: Enterococcus faecalis, Porphyromonas endodontalis, Streptococcus salivarius, Lactobacillus sp., and S. aureus. Study design: Eighty infected teeth in seventy patients with pulpal or periapical pathology were studied. After preparing a standard access cavity, sterilized paper points were used to take samples from the root canals. These were incubated in test tubes at 37°C for 72 hours. Of the resulting bacteria, the five most frequent strains were examined. The teeth were divided into four equal groups. After preparing the root canal using the step-back technique until a number 35 file, each group was subjected to one of the following irrigation solutions: 2% chlorhexidine gluconate (Chx), 5% NaOCl, Ca(OH)2 in distilled-water, or 3% H2O2. After irrigation, a temporary filling was placed in each cavity. The same irrigation procedure was repeated three and six days later. Then, the microbiologic sampling was also repeated. The chi-squared test was used for statistical comparisons. Results: Of the irrigants tested in this in vivo study, chlorhexidine was the most effective against all the microorganisms isolated from infected root canals, followed by NaOCl, Ca(OH)2, and H2O2. However, differences among the three groups were not statistically significant, except against Enterococcus faecalis (p>0.05). Conclusions: Our results did highly confirm the fact from previous studies that chx with 2% percent is the most effective antibacterial agent after three consecutive irrigations. Furthermore, in vitro and in vivo studies are necessary to assess whether the Chx imparted substantive dissolve organic material.

Introduction
The success of endodontic treatment is directly influenced by the elimination of microorganisms from infected root canals. Irrigant solutions are essential during root canal preparation because they help clean the root canal, lubricate the files, flush out debris, kill bacteria, and dissolve tissue, without damaging the periapical tissues. The selection of the ideal irrigant depends on its antibacterial action and lack of toxicity to periapical tissues.

The mechanical action of the instruments alone is not capable of cleaning a root canal satisfactorily owing to the complexity of the internal dental anatomy (apical deltas, lateral canals, accessory canals, etc.), and because direct contact between the instruments and all walls of the root canal system is impossible. Therefore, the physical and chemical actions of irrigating solutions are obvious alternatives.
For many years, the most frequently used irrigants have been sodium hypochlorite (NaOCl) and hydrogen peroxide (H₂O₂), alone or in combination. Their benefits, which include good tissue dissolution and disinfecting capability, have been demonstrated in several studies (14, 6, 12). Unfortunately, NaOCl has toxic effects on vital tissues, resulting in hemolysis, skin ulceration, and necrosis (13, 17). The concentrations of the irrigants also remain controversial; many authors recommend 5.25% NaOCl, while others prefer 3% or even 0.5% (1).

For two decades, chlorhexidine gluconate (Chx) has been widely used in periodontics owing to its antibacterial activity. Its use in endodontics has been proposed as both an irrigant and as an intracanal medication. Chlorhexidine inhibits the Gram-positive and -negative microorganisms commonly found in endodontic infections. Its efficacy is based on the interaction between the positive charge of the molecule and the negatively charged phosphate groups on the bacterial cell wall, which allows the chlorhexidine molecule to penetrate bacteria with toxic effect (14, 7, 24, 1).

Another irrigating solution that is used extensively in endodontics is H₂O₂. This oxidizing solution acts via the reaction of superoxide ions to produce hydroxyl radicals, which are the strongest oxidants known. This radical can attack membrane lipids, DNA, and other essential cell components. Its antimicrobial action is the result of the oxidation of sulfydryl groups and double bonds in proteins, lipids, and surface membranes. In the presence of myeloperoxidase, chloride in the bacteria may be oxidized to hypochlorite (3, 20).

As a root-canal irrigant, the antimicrobial effectiveness of Ca(OH)₂ is based on its ability to release OH⁻ ions. It is strongly alkaline, with a pH of ca. 12.5. In aqueous solution, Ca(OH)₂ dissociates into Ca²⁺ and OH⁻. Various biological properties have been attributed to this substance, such as antimicrobial activity, tissue-dissolving ability, inhibition of tooth resorption, and induction of repair by hard tissue formation (16, 21, 22, 23).

This study assessed the in vivo antimicrobial activity of NaOCl, Chx, Ca(OH)₂, and H₂O₂ against five different microorganisms: Enterococcus faecalis, Porphyromonas endodontalis, Streptococcus salivarius, Lactobacillus sp., and S. aureus.

Materials and Methods
This in vivo study examined 70 patients who attended Dicle University for endodontic treatment. Their ages ranged from 18 to 55, and they had a total of 80 teeth with pulpal or periapical pathology. A detailed medical and dental history was obtained from each patient. None of the patients had received antibiotic treatment in the previous 3 months, and none had any systemic disease.

Samples were collected using strict asepsis. A rubber dam was used to isolate the tooth. After radiographic examination in a holding device, complete isolation antisepsis of the operating field was obtained with 10% povidone iodine solution (Batticon; Adeka, Samsun, Turkey).

A two-stage access cavity preparation was performed by employing sterile burs and manual irrigation with sterile saline solution instead of water spray. All coronal restorations, posts and carious lesions were completely removed. The tooth and surrounding field were then cleaned with 30% H₂O₂ and decontaminated with a 2.5% NaOCl solution for 30 s each. The solution was inactivated with sterile 5% sodium thiosulfate. The root canal was accessed using sterile burs with water spray. Aseptic techniques were used for instrumentation, during access to and removal of the contents of the pulp space, and sample collection. After initial entry to the pulp space, the root canal was enlarged with minimal instrumentation,
TABLE 1

Prevalence of microbial species in 80 root canals

<table>
<thead>
<tr>
<th>Microbial species</th>
<th>Total Number of Microbial Genera</th>
<th>Genera Found in Primary Infected Canals</th>
<th>Genera Found in Secondary Infected Canals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococcus faecalis</td>
<td>60</td>
<td>35</td>
<td>25</td>
</tr>
<tr>
<td>Streptococcus salivarius</td>
<td>18</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>15</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Porphyromonas endodontalis</td>
<td>28</td>
<td>19</td>
<td>9</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>16</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Fusobacterium spp</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

where possible, and without the use of any irrigant. A sterile paper point was then introduced into the full length of the canal and retained in position for 60 s for microbial sampling. If the root canal was dry, a small amount of sterile saline solution was introduced into the canal to ensure viable sample acquisition. Chemically active irrigants were never used before sampling. Under aseptic conditions, the operative field was again disinfected, and the first microbiological sample was collected by introducing a sterile paperpoint with a diameter compatible with that of the canal into each root canal.

The paperpoints were immediately placed in test tubes containing reduced transport fluid and sent to the Microbiology Laboratory, Dicle University Medical School, for microbiological processing. For microbiologic processing, sterile metal wings and glass beads were placed in the tube with the paperpoint under aseptic conditions. Microbial specimens were cultured in 10 ml of thioglycollate broth (Difco Laboratories, Detroit, MI, USA) with 5 μg of hemin/ml and 0.5 μg of menadione/ml or on enriched blood agar plates composed of trypticase soy agar (Difco) enriched with 5% sheep blood, 5 μg of hemin/ml, 0.5 μg of menadione/ml, 5 mg of yeast extract/ml, and 0.4 mg of cytine/ml. The cultures were incubated at 37°C for 5 to 7 days in an Oxoid Anaerobic jar (Anaero-General-OXOID®; Basingstoke, Hampshire, UK).

Biomechanical preparation was subsequently performed with K type files (Dentsply-Maillefer, Switzerland) and Gates-Glidden (Mani, Japan) burs using the classical neutralization and step-back technique with programmed backing. Each instrument change was accompanied by irrigation with 2 ml of Chx, NaOCl, Ca(OH)₂, or H₂O₂ for the respective group. After drying the root canals with sterile paperpoints, a final root sample was obtained in the manner described above. Consequently, a sterile cotton pellet was placed at the canal entrance and the root canals were left empty and temporarily sealed with zinc oxide-eugenol cement (Cavex, Haarlem, the Netherlands) for 72 h.

Twenty root canals each were irrigated with the following irrigating solutions:
1. Group 1: 2 ml of 2% Chx (Drogsan, Ankara, Turkey).
2. Group 2: 2 ml of 5% NaOCl (Wizard, Ankara, Turkey).
3. Group 3: 2 ml of 3% H₂O₂ (Merkez, Istanbul, Turkey).
4. Group 4: 2 ml of Ca(OH)₂ (Visium, Germany) in distilled water.

The same canal irrigation procedure and microbiological sampling were repeated three and six days later. To explore the antibacterial efficiencies of the four irrigation solutions statistically, the chi-squared test
Results and Discussion

Figures 1-4 present the effectiveness of the antimicrobial action of the irrigating solutions (2% Chx, 5% NaOCl, Ca(OH)_2 solution, and 3% H_2O_2) on the numbers of microorganisms at the three sampling times. Comparing the 1st and 2nd or 3rd samples, the chi-squared test showed a statistically significant reduction in all bacteria (p<0.01) with 2% Chx and 5% NaOCl (Figs. 1 and 2). Chx and NaOCl were efficacious against E. faecalis. The same irrigants were equally effective against the other microorganisms isolated from the root canals in this study (p>0.05).

For S. salivarius, Lactobacillus sp., and S. aureus (Figs. 1-4), NaOCl and Chx killed 100% of these organisms at the 2nd and 3rd samplings. The numbers of P. endodontalis were reduced, and the differences between the four irrigants were not significant (p>0.05).

For Streptococcus salivarius, there was a significant difference (p<0.01) between the second and third samplings for each of the solutions. There was no significant difference in the effectiveness of NaOCl, Chx, and Ca(OH)_2, in reducing the numbers of S. salivarius (p<0.05), while H_2O_2 was less effective.

Similarly, H_2O_2 was less effective at killing S. aureus (p<0.01), while there was no significant difference between 2% Chx, 5% NaOCl, and Ca(OH)_2 solutions, in reducing the numbers of Staphylococcus aureus (p>0.05).

For Lactobacillus sp., comparing the 1st, 2nd and 3rd samplings, there was no significant difference between NaOCl, Chx, and Ca(OH)_2 (p>0.01), while H_2O_2 did not reduce the numbers of Lactobacillus sp. (p>0.05).

Figures 1-4 show the presence of microorganisms at the first sampling. E. faecalis was most prevalent, followed by P. endodontalis, and S. aureus. After irrigating the root canals four times, there was a significant reduction in the numbers of microorganisms, supporting the observation that endodontic instrumentation and irrigation...
alter the microflora of the root canal.

We used thioglycollate medium in screw-capped vials to transport and culture specimens from root canals. This medium has a high capacity to reduce oxygen, and no toxic intermediates of oxygen accumulate in the medium when it is exposed to atmospheric oxygen. In addition, the agar in the medium prevents any oxygen to which the medium is exposed during sampling from diffusing deep into the medium. When the vial is closed with the screw cap after sampling, the enclosed oxygen is consumed by the medium and an anaerobic environment is maintained for the specimens (8, 4).

The strains evaluated in this study were Enterococcus faecalis, Porphyromonas endodontalis, Lactobacillus sp., Staphylococcus aureus, and Streptococcus salivarius, which are frequently isolated during routine endodontic treatment of an infected root or from teeth with periapical pathology. In particular, E. faecalis, which is a facultative anaerobic Gram-positive bacteria, has been implicated in persistent root canal infections and has been used in several previous studies on the efficacy of endodontic irrigants owing, in part, to its high resistance to a wide range of antimicrobial agents (9).

The majority of the infectious bacteria, together with their principal substrate, necrotic pulp debris, are probably removed during routine endodontic procedures, such as instrumentation and irrigation of the pulp space. However, the removal is not always total in clinical practice, because of the anatomic complexity of many root canals, and limited access to the microcanal system by therapeutic agents (2).

Comparing the antimicrobial actions of the irrigating solutions at the first, second and third samplings, it was clear that 2% Chx and 5% NaOCl had a significant effect on the facultative anaerobes. The same did not occur with H2O2 and Ca(OH)2. When the results for the three samplings were compared, the antimicrobial actions of NaOCl and Chx were significant, especially for E. faecalis. Ca(OH)2 did not significantly reduce either E. faecalis or

Fig. 2. Antibacterial effect of 5.25% Sodium Hypochlorite.
TABLE 2

The effectiveness of the antimicrobial action of the irrigating solutions

<table>
<thead>
<tr>
<th></th>
<th>E. faecalis</th>
<th>P. endodontalis</th>
<th>S. salivarius</th>
<th>Lactobacillus</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>2% Chlorhexidine gluconate</td>
<td>15</td>
<td>3</td>
<td>3</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>5.25% Sodium Hypochlorite</td>
<td>14</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Calcium Hydroxide</td>
<td>15</td>
<td>10</td>
<td>10</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>3% Hydrogen peroxide</td>
<td>16</td>
<td>12</td>
<td>12</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

I- 1st harvesting; II- 3 day harvesting; III- 6 day harvesting

![Fig. 3. Antibacterial effect of Calcium Hydroxide](image)

P. endodontalis, while it had slight antimicrobial effects on S. salivarius, Lactobacillus sp., and S. aureus (Fig. 3).

In this study, 2% Chx clearly showed antimicrobial activity against E. faecalis after 3 and 6 days. This agrees with other results (15, 5, 10), although such studies utilized chlorhexidine in solutions or gels, and at concentrations of 0.05, 0.12, 0.2, and 0.5%.

We found that Ca(OH)2 was an ineffective endodontic irrigant. Concern has been growing about the insufficient antimicrobial efficacy of Ca(OH)2, not only against E. faecalis, but also against other microbial genera commonly present in infected root canals, such as P. endodontalis. This is concurs with previous findings (22, 10, 19, 25).

The capability of Ca(OH)2 solution to dissolve infected pulpal tissue was insufficient. Although Ca(OH)2 did have some antibacterial action under the experimental conditions, it was unable to kill and eliminate sufficient E. faecalis at any period.

We clearly demonstrated that 3% H2O2 had limited success in eliminating bacteria from the root canals, which is in agreement with previous reports that E. faecalis and
other bacteria remain viable after using H$_2$O$_2$ as an irrigant in infected canals (18). H$_2$O$_2$ reduced the numbers of S. salivarius and Lactobacillus sp. significantly, and slightly reduced both P. endodontalis and streptococci between samplings (Fig. 4).

Although we found that Chx was an alternative irrigant, one must remain cautious because of its ability to dissolve organic materials. Comparing the biocompatibility of the irrigants used in this study, the use of concentrated NaOCl as a root canal irrigant might cause severe clinical problems when extruded into vital tissues.

We confirmed previous findings that 2% Chx is the most effective antibacterial agent after three consecutive irrigations. This is probably due to the diffusion ability and long-lasting release of Chx molecules, which hinder re-infection of the root canal. When evaluating root-irrigants, the ability of materials to diffuse, as well as their toxicity, should also be considered. Further in vitro and in vivo studies are necessary to assess whether Chx dissolves organic material.

REFERENCES