One-versus Two-visit Endodontic Treatment of Teeth with Apical Periodontitis: A Histobacteriologic Study

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Abstract

Introduction: This study analyzed the in vivo microbiological status of the root canal systems of mesial roots of mandibular molars with primary apical periodontitis after 1- or 2-visit endodontic treatment. Methods: Mesial root canals were instrumented by using either a combination of K3 and LightSpeed instruments (mesiobuccal canals) or the ProTaper system (mesiolingual canals), with 5% NaOCL irrigation. Patency files were used. Smear layer was removed, and a final rinse with 5 mL of 2% chlorhexidine was performed. In the 2-visit group (7 roots, 14 canals), canals were medicated with calcium hydroxide for 1 week and then obturated by using the continuous wave of compaction technique. In the 1-visit group (6 roots, 12 canals), canals were immediately obturated after chemomechanical procedures. Teeth were extracted 1 week after root canal instrumentation and processed for histobacteriologic analysis. Results: In the 1-visit group, no case was completely free of bacteria; residual bacteria occurred in the main root canal (5 of 6 cases), isthmus (5 of 6), apical ramifications (4 of 6), and dentinal tubules (5 of 6). In the 2-visit group, 2 cases were rendered bacteria-free; residual bacteria were found in the main canal only in 2 cases (none of them with persistent dentinal tubule infection), in the isthmus (4 of 7 cases), and in ramifications (2 of 7). The 2 instrumentation techniques performed similarly. When filling material was observed in ramifications, it was usually intermixed with necrotic tissue, debris, and bacteria. Conclusions: The 2-visit protocol by using an interappointment medication with calcium hydroxide resulted in improved microbiological status of the root canal system when compared with the 1-visit protocol. Residual bacteria were more frequent and abundant in ramifications, isthmuses, and dentinal tubules when root canals were treated without an interappointment medication. Apical ramifications and isthmuses were never completely filled. The use of an antibacterial interappointment agent is necessary to maximize bacterial reduction before filling. (J Endod 2012;38:1040–1052)

Key Words

Calcium hydroxide, endodontic infection, endodontic treatment, 1-visit endodontics, sodium hypochlorite

The microbiological goals of the endodontic treatment of teeth with apical periodontitis are to reduce the microbial bioburden to levels compatible with periradicular tissue healing and to prevent microbial recolonization of the treated canal. The former can be attained by antimicrobial measures involving chemomechanical procedures and intracanal medication, whereas the latter is a role for root canal obturation. One of the most controversial issues in endodontics is whether an interappointment medication is really needed to improve disinfection and then enhance treatment outcome (1).

Several clinical studies have evaluated the intracanal antimicrobial activity of chemomechanical preparation by using NaOCl as the irrigant in concentrations ranging from 0.5%–5%, but most of them demonstrated that 40%–60% of the canals still exhibit detectable cultivable bacteria (2–12). Because residual bacteria have been shown to adversely affect treatment outcome (4, 13), the use of an interappointment intracanal medication has been recommended to supplement the antibacterial effects of chemomechanical procedures and maximize bacterial reduction (3, 5, 7, 9, 10, 14). Calcium hydroxide is arguably the most commonly used intracanal medication, but its effectiveness in significantly increasing the number of culture-negative root canals after chemomechanical preparation has been demonstrated to be inconsistent (3, 5, 15).

There are many studies showing that root canals that cultured negative before filling have a greater potential of improved outcome (4, 16, 17). Thus, the choice for clinical protocols that predictably lead to negative cultures as demonstrated by the literature has been recommended (18). Nevertheless, culture has limitations, because it has low sensitivity, many endodontic bacterial species remain to be cultivated, and the method used for sampling only shows the microbiological conditions of the main canal (19). The histobacteriologic approach used in previous studies (20–22) has the great advantage of providing information about the spatial location of residual bacteria, including those present in areas distant from the main canal, such as tubules, isthmuses, and ramifications, which are difficult to treat and sample. Therefore, this method has great potential to provide information about clinical protocols that are better suited to control the infection in the root canal system.
The present histobacteriologic study was undertaken to analyze the \textit{in vivo} microbial status of the middle and apical segments of the root canal system of mesial roots of human mandibular molars with primary apical periodontitis after 1- or 2-visit endodontic treatment. Two different nickel-titanium rotary instrumentation techniques were used for both groups, and calcium hydroxide was used in the 2-visit treatment protocol.

\textbf{Materials and Methods}

\textbf{Clinical Procedures}

Teeth selected for this study were human mandibular molars with necrotic pulps and radiographic evidence of apical periodontitis that were extracted for reasons not related to this study (nonrestorability because of extensive caries/crown fractures or periodontal disease). The study protocol was approved by the institutional review board of Tlaxcala University, Mexico, and informed consent was obtained from the individuals participating in the study after the clinical procedures were thoroughly explained. Thirteen teeth were selected and randomly assigned to either of 2 experimental groups by using a coin toss. Only the 2 mesial canals were included in the experiment. All clinical procedures were carried out by 1 experienced endodontist (J.V.).

\textbf{2-visit Group}

This group included the mesial canals of 7 mandibular molars. After local anesthesia, the cusps were cut down, carious dentin was excavated, and the tooth was isolated with a rubber dam. Thorough disinfection of the tooth and the rubber dam was done by using 5\% NaOCl, and the endodontic access cavity was prepared with sterile high-speed carbide burs. Next, the access cavity and the distal canal were profusely irrigated with 5\% NaOCl, calcium hydroxide was placed at the entrance of the distal canal, and the distal aspect of the access cavity was separated from the mesial one by building a mechanical barrier with LC Block-out resin (Ultradent, South Jordan, UT).

After exploration with a scouting #10 K-file, the 2 separate mesial canals were coronally flared by using Gates-Glidden burs sizes 3 and 2, along with copious irrigation with 5\% NaOCl. The working length (WL) was determined with the aid of an electronic apex locator (Elements Diagnostic Unit; Sybron Endo, Orange County, CA) at the 0.0 reading of the device with a #10 K-file. Mesio-angled and/or disto-angled

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radiographs were taken to visualize and confirm the presence of distinct mesiobuccal and mesiolingual canals. All mesiobuccal canals were instrumented by using K3 rotary instruments (Sybron Endo) until a size 40/.06 reached the WL, after which, further enlargement of the apical portion of the canal was completed by using LightSpeed instruments from size 42.5 to size 60 (Sybron Dental Specialties, Orange, CA). All mesiolingual canals were instrumented by using the ProTaper system (Dentsply/Maillefer, Ballaigues, Switzerland) up to the F2 instrument (25/08). Copious irrigation with 5% NaOCl (5 mL after each instrument used) was done throughout the instrumentation procedure. A 27-gauge side-vented needle (Endo-Eze; Ultradent) was used to deliver the irrigant, initially to the deepest possible penetration of the needle and then to a distance of 2 mm from the WL. Special care was taken to instrument and irrigate the canals with NaOCl for a total period of about 45 minutes.

Subsequent to the use of every instrument, a #10 K-file was used to maintain patency of the apical foramen by taking it 1 mm beyond the WL. The root canals were rinsed with 5 mL of 17% ethylenediaminetetraacetic acid (EDTA) (Smear Clear; Sybron Endo), followed by 5 mL of sterile saline solution. Chemomechanical procedures were completed by performing a final rinse with 5 mL of 2% aqueous chlorhexidine solution (Consepsis; Ultradent). The canals were then dried with sterile paper points.

All canals from the 2-visit group were subsequently medicated with a freshly prepared paste of calcium hydroxide (Sultan, Fenix, NJ) in sterile saline for a period of 1 week. The pH of the calcium hydroxide paste was measured before application and was found to be above pH 12.5. The paste was spun into the canals with the aid of a lentulo spiral. Care was taken to properly fill the root canal with the calcium hydroxide paste without any radiographically visible air bubbles. The paste was condensed at the canal orifice level with the aid of a sterile cotton pellet. Another sterile cotton pellet moistened in absolute alcohol was used to clean the pulp chamber walls from calcium hydroxide residues. Access cavies were closed with intermediate restorative material (IRM) (Dentsply DeTrey GmbH, Konstanz, Germany).

After a period of 1 week, the patient was anesthetized, rubber dam was applied, the operative field was disinfected as above, and the root canals were reinstrumented and obturated. The procedures consisted of repeating instrumentation with the last instrument operated at the WL with 10 mL of 5% NaOCl as the irrigant. Thereafter, the canals were rinsed with 10 mL of EDTA, followed by 5 mL of sterile saline solution. A final disinfecting rinse was performed once again with 5 mL of 2% chlorhexidine (Consepsis). A patency file was placed 1 mm beyond the WL to check for patency before filling.

The root canals were obturated with gutta-percha and Pulp Canal Sealer (Sybron Endo) by using the continuous wave of compaction technique (25). For this, the Elements Obturation System (Sybron Endo) was set on the down-pack mode at 200°C, and the Buchanan Heat Plugger F06 (Sybron Endo) was used 3–5 mm short of the WL. Compaction of the gutta-percha mass was achieved by using the Buchanan Hand Plugger #1 (Sybron Endo). Backfill was done by injecting thermoplastized gutta-percha from the obturator cartridge of the Elements Obturation System set on the backfill mode at 100°C and by using a 23-gauge needle. After compaction of the injected gutta-percha, excess material was removed from the pulp chamber, and the access cavities were sealed with IRM.

Immediately thereafter the teeth were carefully extracted. A round sterile carbide bur size 1/4 was used to prepare a shallow groove on the buccal surface of the exposed root to allow recognition of the mesiobuccal canal and orientate the tooth processing. The teeth were immersed immediately in a chilled fixative solution consisting of 4% paraformaldehyde (Merck, Darmstadt, Germany) in phosphate buffer (776 mOsm, pH 7.2). The patients were given oral and written post-extraction instructions.

### Table 1. Microbiological Status and Location of Residual Microorganisms in the Middle and Apical Root Canal System of Teeth with Apical Periodontitis after Treatment in 1 or 2 Visits

<table>
<thead>
<tr>
<th>Specimen</th>
<th>MBC</th>
<th>MLC</th>
<th>IST</th>
<th>DT*</th>
<th>AR</th>
<th>Overall†</th>
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<tr>
<td>DLH</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>Extraradicular biofilm</td>
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<td>OP</td>
<td>–</td>
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<td></td>
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<td>RPC</td>
<td>+</td>
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<td>+</td>
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<td>Canals confluent</td>
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<tr>
<td>IMI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Canals confluent</td>
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<tr>
<td>EGH</td>
<td>+</td>
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<td>–</td>
<td>–</td>
<td>+</td>
<td>Canals confluent; extraradicular biofilm</td>
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<tr>
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<td></td>
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<td>Canals confluent</td>
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</tbody>
</table>

**AR:** apical ramifications; **DT:** dentinal tubules; **IST:** isthmus; **MBC:** mesiobuccal canal; **MLC:** mesiolingual canal.

*Residual dentinal tubule infection denotes bacterial presence in dentin surrounding the main root canal and the isthmus area.

†Presence (+) or absence (–) of microorganisms in 1 or more of the 5 anatomic locations examined.

### Table 2. Summary of Microbiological Status of Teeth Treated in 1 or 2 Visits and Detailed in Table 1

<table>
<thead>
<tr>
<th>Microbiological status</th>
<th>1 visit</th>
<th></th>
<th>2 visits</th>
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<tbody>
<tr>
<td>Total number of treated teeth</td>
<td>6</td>
<td>100</td>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>Teeth exhibiting residual microorganisms</td>
<td>6</td>
<td>100</td>
<td>5</td>
<td>71</td>
</tr>
<tr>
<td>Mesiobuccal canals with residual microorganisms</td>
<td>3</td>
<td>50</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Mesiolingual canals with residual microorganisms</td>
<td>4</td>
<td>67</td>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td>Teeth with residual microorganisms in isthmuses</td>
<td>5</td>
<td>83</td>
<td>4</td>
<td>57</td>
</tr>
<tr>
<td>Teeth with residual microorganisms in dentinal tubules</td>
<td>5</td>
<td>83</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Teeth with residual microorganisms in apical ramifications</td>
<td>4</td>
<td>67</td>
<td>2</td>
<td>29</td>
</tr>
</tbody>
</table>
This group included the mesial canals of 6 mandibular molars that were treated in 1 visit. These canals were treated exactly as described for those in the 2-visit group, except that they were instrumented and filled at the same appointment. No interappointment dressings with calcium hydroxide were used. The teeth were extracted in accordance with the procedures described above 7 days after the intracanal procedures without reentering the canal.

**Controls**

Four healthy mandibular third molars with vital pulps and extracted because of pericoronitis were processed histologically to serve as negative controls. Four untreated mandibular molars with necrotic pulps and radiographic evidence of apical periodontitis were extracted because of the reasons explained above and served as positive controls.

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**Figure 2.** One-visit group. (A) Severely broken-down mandibular right first molar in 30-year-old woman. Caries has involved the furcation, and apical periodontitis lesions are present on both roots. (B) Postobturation radiograph. (C) Radiograph of mesial root after extraction. (D) Specimen immersed in clearing agent before embedding in paraffin. The root canal filling is slightly overextended apically. (E) Cross-cut section taken from the middle third at the level of line 1 in (D). Both canals appear well-prepared, with dentin removed circumferentially and a round shape of similar diameter at this level. Note the wide isthmus harboring obturation material connecting the 2 canals and the lingual extension of the lingual canal (arrow) (Taylor modified Brown & Brenn stain; original magnification, ×16). (F) Detail of lingual canal. A large mass of necrotic debris colonized by bacteria occupies the central part of the canal lumen, embedded in the obturation material. High-power view from center of this mass shows amorphous material (likely food remnants) surrounded by heavy concentration of bacterial profiles (original magnification, ×50; inset, ×400). (G) Detail of isthmus in (E) (original magnification, ×50). (H) Magnification of area indicated by upper arrow in (G). Bacterial biofilm covers the irregularity in dentin wall of the isthmus (original magnification, ×400). (I) Magnification of area on opposite isthmus wall indicated by lower arrow in (G). In addition to biofilm present on dentin wall, bacteria are also colonizing dentinal tubules (original magnification, ×400).
Tissue Processing

After fixation for a minimum period of 1 week, the mesial roots were separated from the teeth with a diamond disk under water spray just beyond the root canal orifices, and radiographs were taken in a mesiodistal projection. Demineralization of the roots was carried out in an aqueous solution consisting of a mixture of 22.5% (vol/vol) formic acid and 10% (wt/vol) sodium citrate for 4 weeks, with the end point being determined radiographically. All specimens were washed in running tap water for 48 hours, dehydrated in ascending grades of ethanol, and cleared in xylene. Photographs of the mesial and distal views of the cleared roots were taken while immersed in xylene.

With a sharp razor blade the roots were arbitrarily cross-sectioned, under adequate magnification, approximately at the transition between the coronal and the middle thirds and between the middle and the apical thirds. The middle and apical segments were finally infiltrated and embedded separately in paraffin (melting point 56°C) according to standard procedures. With the microtome set at 4-5 μm, 200 consecutive cross-cut sections were taken respectively of the middle and apical thirds in an apical direction for all specimens. The apical segments were then removed from the paraffin blocks and re-embedded upside down to obtain cross-cut serial sections from the root tip in a coronal direction; 300–600 sections were cut for each root tip. To prevent detachment of the apical small tissue during the bath in acetone-containing solutions, all sections from the root tips were placed on special polarized slides (Superfrost Plus; Gerhard Menzel GmbH, Braunschweig, Germany). Thus, sections were divided according to the 3 regions analyzed: middle third, apical third, and root tip.

Slides were stained with the Taylor modified Brown and Brenn technique for bacteria (24). The accuracy of the bacterial staining method was tested by using the protocol described by Ricucci and Bergenholtz (25). Slides were examined under the light microscope by 2 evaluators (D.R., S.L.). Evaluations were performed separately, and whenever disagreement occurred, it was resolved by joint discussion. The following were specifically looked for in the histologic examination: presence of residual bacteria in the main canal, isthmuses, ramifications, and dentinal tubules (around the main canals or the isthmus area); presence of debris; type of tissue in apical ramifications as well as the presence of bacteria, debris, and filling material therein.

Figure 2. (continued). (J) Section taken from apical third approximately at level of line 2 in (D) where the 2 canals join. Note that the size of the preparation of the lingual canal, showing an extension, is considerably smaller than that of the buccal canal (original magnification, ×50). (K) Magnification of confluence area indicated by upper left arrow in (J). Amorphous material and necrotic debris heavily colonized by bacteria (original magnification, ×400). (L) Magnification of area indicated by lower arrow in (J). Abundance of bacteria colonizing debris packed onto dentin wall at confluence between the 2 canals. This histologic picture corresponds to the dark area in the apical third, indicated by arrow in (D) (original magnification, ×400). (M) Magnification of area of dentin wall indicated by upper right arrow in (J). Debris and bacteria fill the irregularity and appear packed by the obturation material (original magnification, ×400). (N) Cross-cut section from apical third, not far from that shown in (J). Buccal portion of root dentin (original magnification, ×50). (O) Magnification of more external dentin indicated by left arrow in (N). Few tubules show bacteria in reduced numbers (original magnification, ×400). (P) Magnification of inner dentinal area indicated by right arrow in (N). Bacterial colonization of dentinal tubules is heavier at this level (original magnification, ×400). (Q) Overview of cross-cut section passing through apical ramification shown in (D) (original magnification, ×16). (R) Detail of portion of ramification more proximal to the root canal. High-power view shows bacterial biofilm intermixed with the obturation material squeezed into this area (original magnification, ×100; inset, ×400).
Figure 3. One-visit group. (A) Tooth #19 with destructive carious process in 35-year-old man. Caries has destroyed the furcation. (B) Postobturation radiograph. Considerable amount of obturation material has been extruded through a ramifications. (C) Radiograph of mesial root. There is confluence of the 2 canals. (D) Distal view of cleared specimen. Obturation material did not remain attached to the root at extraction, but 2 apical ramifications “filled” by obturation material can be seen. (E) Cross-cut section from middle third taken at level of line 1 in (D). Overview shows a wide and short isthmus connecting the 2 canals (Taylor modified Brown & Brenn stain; original magnification, ×25). (F) Detail of isthmus. Necrotic debris heavily colonized by bacteria and surrounded by remnants of obturation material (original magnification, ×100; inset, ×400). (G) Detail from lingual canal wall showing colonization of dentinal tubules (original magnification, ×100; inset, ×400). (H–J) Sequence of sections encompassing the whole course of the most apical ramifications, cut in a coronal direction, and taken approximately 60 sections from each other (original magnification, ×16). (K and L) Progressive magnifications of content of ramifications in (J). Thick bacterial biofilm with high density of filamentous forms and surrounded by obturation material can be seen (original magnification, ×100 and ×400). (M) Section passing at the periodontal ligament end of the more coronal ramification in (D) (original magnification, ×16). (N) Detail of ramification in (M). Obturation material is enmeshed in thick bacterial biofilm (original magnification, ×100). (O) High-power view of area indicated by arrow in (N). Biofilm fills the irregularities of dentin wall (original magnification, ×400).
Results

All specimens were available for analyses after the histobacteriological processing. Although some occasional sections were lost during the cutting process, neighboring sections allowed the full picture to be seen. Some artifacts were observed in a few cases because of partial detachment of the sections from the slides during the staining procedures. This involved only sections taken in the middle and apical thirds and not sections taken from the root tip, which were processed on special slides. Another common artifact was the displacement of the obturation material remnants onto the cut dentin surface by the microtome blade during cutting or because of their tendency to float on the mounting medium. However, these artifacts have not significantly interfered with the overall histologic assessment.

Histologic sections confirmed the presence of mesiobuccal and mesiolingual canals in all 13 teeth included in this study. In 4 cases (3 from the 1-visit and 1 from the 2-visit group), the 2 mesial canals showed confluence toward the apices. Isthmus connecting the 2 mesial canals were observed in all mesial roots. Although at some levels these communications appeared sometimes incomplete (Fig. 1F), the high number of sections taken allowed the observation of complete communication between the 2 main canals (Fig. 1F). Ramifications (apical and/or lateral) were also present in all 13 roots, varying in number and size, and were particularly concentrated in the apical third. In 10 cases (3 from the 1-visit group and all 7 cases from the 2-visit group), the overfilling observed radiographically and in the cleared specimens was confirmed histologically; small to moderate amount of obturation material was present beyond the main apical foramen (Fig. 1G and H).

Sections that stained positive for microorganisms demonstrated various different bacterial morphotypes, including cocci, rods, and filamentous forms. Microorganisms were found in the middle and/or apical third of 11 of the 13 root canal treated specimens (85%) (all 6 roots of the 1-visit group and 5 of the 7 roots of the 2-visit group) (Tables 1 and 2). Microbial distribution was different from case to case and between groups (Tables 1 and 2).

Microbiological Status

In the 1-visit group, no tooth had its root canal system rendered bacteria-free. Only in 1 case were residual bacteria not observed in the main root canals at the 3 levels. However, in this specific case, residual bacteria were present in the isthmus and within dentinal tubules. In 2 cases, bacteria were not seen in the main canals at the middle and apical thirds, but they occurred in sections from the root tip. Bacteria were observed only in the lingual canal (ProTaper) in 2 specimens, only in the buccal canal (K3/LightSpeed) in 1 specimen, and in both canals in 2 cases (Table 1). Microorganisms were observed in the isthmuses from 5 of 6 specimens (Figs. 2E–I, 3E and F, and 4H and I) and in dentinal tubules from 5 of 6 cases (Figs. 2I and N–P, 3G, and 4F). This intratubular infection involved tubules of the dentin surrounding the main canals (Figs. 2N–P, 3G, and 4E and F) and the isthmus area (Fig. 2G–I). Bacterial persistence into tubules was observed at varying depths, sometimes reaching more than two thirds of the dentin thickness (Fig. 2N and O).

In the 2-visit group, 2 teeth had their root canal systems rendered free of bacteria. Residual bacteria were not observed in the main root canals of 5 of the 7 roots. Bacteria were, however, demonstrated in the isthmuses in 4 of the 7 cases (Fig. 5D) (Table 1). No bacteria were seen in the dentinal tubules surrounding the main canals from all 7 roots. Typical bacterial profiles within tubules were identified only in 1 specimen, which showed a few bacterial cells in the dentin surrounding the isthmus. In 2 specimens, some bodies were seen within tubules at varying depths. These bodies were faintly stained, reddish or bluish, but without the typical appearance profile of bacterial cells (Fig. 6C and D).

Debris

In the 1-visit group, debris was always present in varying amounts in the isthmuses and in most cases in the root canals at different levels. It was observed as masses of amorphous material including dentin shavings, sometimes apparently embedded in the obturation material (Fig. 2E and F) or packed onto the instrumented root canal walls (Fig. 2J–M) and sometimes layering the entire circumference of the canal. In some instances, necrotic masses were apparently free from bacteria, but in some cases they were heavily infected (Figs. 2E and F, J–M and 3E and F).

Debris was also present in the isthmuses of the 2-visit group, more frequently in the most apical part of the canals (Fig. 5G–I). In some cases, at the root tip the canal lumen in cross section appeared completely filled with debris, which was densely packed apical to the root canal filling (Fig. 7D and E).

Biofilm on the External Root Surface

In 3 cases (2 from the 1-visit group and the other from the 2-visit group), a bacterial biofilm was observed beyond the root canal space on the external apical root surface (Table 1; Figs. 4J and K and 7D and F). One of these teeth was extracted because of periodontal disease, but the apical biofilm was clearly independent of the periodontal biofilm. In 1 case a consistent portion of the buccal apical profile was covered by a thick bacterial biofilm with abundance of extracellular matrix (Fig. 7D and F). All cases of extraradicular biofilm also showed residual bacteria in some intraradicular areas (Fig. 4E–I).

Apical Ramifications

Apical ramifications were present in varying numbers in all 6 cases of the 1-visit group. In all these cases, debris was observed within the ramification. Of the 6 cases, residual bacteria were seen in 4 (Figs. 2Q and R and 3H–O) and vital tissue in 4; obturation material was observed within some ramifications from all cases. Combinations of these observations were observed in all cases.

Likewise, all 7 cases of the 2-visit group showed apical ramifications in varying numbers (Fig. 8). All of them had debris. Bacteria were present in 2 cases and vital tissue in 2 cases; obturation material was seen in some ramifications from 3 cases. Combinations of these observations occurred in all cases.

Controls

In the negative controls, the pulp tissue at the middle and apical root segments was normal, and no bacteria were detected either in the root canal or in the periradicular tissues. Positive controls showed bacterial colonization of the main canal, usually forming biofilm structures adhered to the main root canal walls. Some bacterial cells were also enmeshed in the necrotic tissue in the main canal. Bacterial biofilms were seen in ramifications and isthmuses, and dentinal tubular invasion was invariably observed, especially in dentin underneath biofilms (data not shown).

Discussion

The ideal microbiological goal of the endodontic treatment of teeth with apical periodontitis is total eradication of the infection, but this can rarely be reached by using the currently available techniques and substances (2, 26–28). This was confirmed by the present study, in which only 2 cases showed complete absence of bacteria after...
treatment (both cases were treated in 2 visits). Considering the high success rate of the endodontic treatment, it is reasonable to assume that what is clinically achievable and possibly sufficient for an optimal outcome is maximal reduction of the microbial bioburden to levels that are compatible with periradicular tissue healing (18). The present findings indicate that the root canal systems of teeth treated in 2 visits with 1-week calcium hydroxide interappointment medication had an improved microbiological status when compared with teeth treated in a single visit.

Bacteria arranged in biofilms were commonly seen on untouched root canal walls of treated teeth as well as in isthmuses and ramifications (Figs. 2E–H, J–M, Q, and R, 3E–O, 4G–K, and 5D). These findings, along with those from the positive controls, are in agreement with studies demonstrating that bacteria in primary infections are usually organized in biofilm communities (21, 29). There are many advantages of the biofilm lifestyle; one of them is the ability to protect the community members from the effects of antimicrobial therapeutic measures, especially when the community is not mechanically affected by instruments and the irrigant flow. Histobacteriologic analyses of teeth with post-treatment disease reveal biofilms associated with many cases, especially in those areas inaccessible to treatment (21, 22). This represents strong indirect evidence that bacterial biofilms remaining undisturbed in those difficult-to-reach areas can jeopardize the endodontic treatment outcome, and efforts should be directed toward improving disinfection of those anatomic complexities.

**Figure 4.** One-visit group. (A) Tooth #19 with complicated crown fracture in 73-year-old man. (B) Postobturation radiograph. (C) Radiograph of mesial root after extraction. (D) Cleared specimen. (E) Cross-cut section taken at middle third at level of line 1 in (D). Root canal walls of lingual canal appear well-prepared (Taylor modified Brown & Brenn stain; original magnification, ×50). (F) High magnification from lingual canal wall. Bacterial colonization of some dentinal tubules (original magnification, ×400). (G) Distal root canal wall of lingual canal. Bacteria entrapped between obturation material and dentin wall (original magnification, ×400). (H) Cross-cut section taken from apical third at level of line 2 in (D). Buccal canal connected to a wide isthmus (original magnification, ×25). (I) High-power view of area of isthmus indicated by arrow in (H). Debris heavily colonized by bacteria (original magnification, ×400). (J) Section taken at level of line 3 in (D), encompassing only the lingual canal. The foramen ends on the distal aspect of the apex (original magnification, ×50). (K) High-power view shows biofilm on opposite sides of apical constriction, extending to external root surface (original magnification, ×400).
The anatomic complexity of the apical root canal system is one of the most important factors limiting the attainment of proper disinfection (30). In infected root canals associated with apical periodontitis, bacteria can be located not only in the lumen of the main canal but also in recesses, dentinal tubules, isthmuses, lateral canals, and apical ramifications. In these areas, they are usually protected from the effects of chemomechanical procedures. This is because it is physically impossible for instruments to reach most of these areas, and the antibacterial irrigants are left to act in the canal for only a short time, which is usually insufficient for them to diffuse and reach effective concentrations to kill bacteria harbored in anatomic complexities (31) (Figs. 2I–R, 3E–O, and 4E–I). This has been previously demonstrated (26) and was evidently confirmed in the present study for the 1-visit group; residual bacteria were seen in the isthmuses (5 of 6 cases), apical ramifications (4 of 6 cases), and dentinal tubules (5 of 6 cases). The main reason to use an interappointment medication is to allow time for the medication to diffuse and reach bacteria in those areas inaccessible to instruments and irrigants. Our results indicate that this strategy is really effective in reducing the bacterial bioburden in the whole system, because residual bacteria were only seen in the isthmuses of 4 cases and in ramifications of 2 cases in the 2-visit group and usually in lower abundance than in the teeth treated in 1 visit. No residual dentinal tubule infection was found in the middle and apical thirds of the main root canal after 1 week of calcium hydroxide medication. The present findings confirm that it requires time for the therapeutic antibacterial procedures to affect bacteria located in anatomic complexities and then maximize bacterial reduction to levels that cannot be achieved in 1 visit.

Data from many studies using culture also confirm the advantage of using intracanal medication in reducing the bacterial load and the number of cases with positive culture because of residual bacteria (3, 5, 10). This is interesting because the method of sampling commonly used provides information of the bacteriologic conditions in the main canal, an area where instruments and irrigants are expected to act. Nonetheless, even in the main canal there are areas that remain uninstrumented (32, 33) (Fig. 2J and M). The additional benefit of using an intracanal medication for reduction of bacteria in the main canal was confirmed by this histobacteriologic study. Although in the 1-visit group residual bacteria were found in the root canals of 5 of the 6 teeth, in the 2-visit group only 2 teeth showed residual bacteria in the main canal. In addition to the effects of the medication per se, the reinstrumentation with the last apical instrument and the final rinse with NaOCl and chlorhexidine in the second visit might have contributed to the improved disinfection of the main canals.

Two instrumentation protocols were used in the present study. Our results demonstrated that both performed equally well in terms of cleaning and disinfecting the main canal. This is curious if one considers that...
the size of apical preparation differed in the 2 protocols. Some previous studies demonstrated that the larger the apical preparation, the larger the bacterial reduction (34–36). This is because larger preparations increase the chances for incorporating recesses and irregularities, which might harbor bacteria, in addition to removing more infected dentin. However, this was not apparently confirmed in the present study, suggesting that the level of enlargement achieved in the smallest preparations (ProTaper group, apical file dimensions 25/.08) along with copious irrigation with 5% NaOCl was sufficient to favor bacterial elimination from the mesial canals of mandibular molars.

It is also worth pointing out that apical patency was performed in all cases with a #10 K-file, but debris and bacteria were seen in various cases in the apical canal (Figs. 5H and I and 7D and E). Debris and/or bacteria were present in the main foramina in 8 of the 13 cases. More specifically in the 1-visit group, debris and bacteria were present in 3 cases and only debris in another case. In the 2-visit group, debris and bacteria were present in 1 case and only debris in 3 other cases. Although this might suggest that patency files are not as effective in helping disinfection and cleaning as suggested (37), any further discussion on this topic would not be appropriate because a group with no patency files for comparison was not included in the present investigation. Further studies are required to help elucidate the importance, if any, of using patency files to improve apical disinfection and cleaning.

Evidence of extraradicular infection was observed in 3 cases. In all those cases, infection was primarily organized as biofilms adhered to the external apical root surfaces (Figs. 4J and K and 7D and F), which were beyond the reaches of instruments, irrigants, and intracanal medication. Whether these remaining extraradicular biofilms can remain active after root canal treatment to the point of jeopardizing the treatment outcome cannot be answered by the present study. However, reports suggest that the presence of a biofilm on the external apical root surface might be implicated in the failure of apical periodontitis to heal (38, 39).

All mesial roots analyzed showed apical ramifications. Obturation material was observed in ramifications from all cases treated in 1 visit and in 3 cases of the 7 teeth treated in 2 visits. Although this might suggest that calcium hydroxide interferes with “filling” ramifications, the main question is whether such a “filling” means adequate sealing and disinfection. In fact, residual bacteria were more commonly seen in ramifications from the 1-visit group than in the 2-visit group.

Figure 6. Two-visit group. Tooth #30 in 39-year-old woman with 8-mm-deep periodontal pocket caused by fracture on the pulp chamber floor. (A) Cleared mesial root. Obturation material is present in the communications between the 2 canals. (B) Cross-cut section taken from middle third at level of line 1 in (A). Overview shows that the canals are well-prepared at this level, and there is an incomplete isthmus (Taylor modified Brown & Brenn stain; original magnification, ×16). (C) Buccal canal. Some tubules exhibit red-stained structures (original magnification, ×100). (D) High magnification demonstrates that these structures do not have the appearance of usual bacterial profiles (original magnification, ×400). (E) Cross-cut section taken at apical third at level of line 2 in (A). An extension of the buccal canal and an incomplete isthmus are present (original magnification, ×16). (F) Detail of buccal extension (original magnification, ×100). (G) High magnification shows that the tissue in the extension is partly vital. Absence of bacteria (original magnification, ×400). Area of the isthmus indicated by arrow in (E) is magnified in the inset. Obturation material and debris. Absence of bacteria (original magnification, ×400).
Debris was present in ramifications from all cases treated regardless of the group. In most cases in which the filling material was observed in ramifications, it was intermixed with necrotic tissue and debris. In some cases, bacteria were also present along with the filling material (Figs. 2Q and R and 3H–O). Voids were usually observed. Therefore, thorough filling, seal, and disinfection cannot be expected by squeezing the filling material into ramifications. These findings are in line with previous reports (20, 40).

The optimal outcome depends on the ability of treatment procedures to restore healthy conditions by creating favorable conditions to healing (41). The best root canal environmental conditions that are highly conducive to periradicular healing are represented by absence of microorganisms, a condition similar to that found in vital cases that have a very high potential for success. Therefore, clinicians are encouraged to pursue treatment protocols that predictably control the root canal infection and allow for establishment of an environment that is favorable to healing. Studies comparing the success rate of the endodontic treatment of teeth with apical periodontitis performed in 1 or more visits revealed that 2 or more visits with calcium hydroxide as the intracanal medication offer a success rate that is 10%–20% higher than 1-visit treatment (4, 17, 42–44). However, other studies showed virtually no percentage difference (45) or even 10% more success for 1-visit treatment (46, 47). Data available have been limited and did not allow precise conclusions about superiority of 1 method over the other (48). However, virtually all these studies were plagued by a small sample size. A very recent study with a larger number of cases demonstrated a significantly better outcome for teeth whose canals were treated in 2 visits with calcium hydroxide as interappointment medication as compared with 1-visit treatment (49). This is in line with the better disinfection observed in the present study for the 2-visit group and is consistent with the notion that root canal disinfection is integral to success.

One argument in favor of treating infected root canals in 1 visit is that residual bacteria surviving treatment are entombed by obturation and die because a source of nutrients is denied (46, 50). This argument might be valid to bacteria remaining on untouched canal walls or within dentinal tubules (18). However, bacteria remaining in the very apical part of the root canal (Fig. 2J–M), in apical ramifications (Figs. 2Q and R and 3H–O), and in lateral canals can maintain long-standing infections. These bacteria are in direct contact with the periradicular tissues, which are a sustainable source of nutrients. The simple fact that bacteria can be found in the main root canal of many cases with
post-treatment disease (51, 52) indicates that entombment is not reliable. Findings from this and previous studies (13, 22, 53, 54) suggest that even bacteria sequestered in the root canal by obturation might derive nutrients from debris and tissue remnants until a more sustainable source of nutrients is established.

A great advantage of the method used in this study to evaluate the antimicrobial performance of clinical protocols is that it provides information as to the anatomic localization of residual bacteria. This gives a clear idea of the deficiencies of the different approaches and allows for further development of strategies to enhance disinfection. When compared with the correlative light and transmission electron microscopy used in a previous study (26), the present approach has the advantage of permitting the analysis of the bacterial persistence within tubules. However, a significant disadvantage of the method is the cross-sectional nature of the analysis, which does not permit to evaluate the specimen before and after clinical procedures. Also, the technique is more effective to detect gram-positive bacteria, and the possibility exists that gram-negative bacteria might have passed unnoticed. Another possible limitation is that light microscopy does not provide information about bacterial viability, even though the bacterial arrangements suggest that bacteria were unaffected by procedures and then possibly remained viable. Also, 1 week after instrumentation we observed polymorphonuclear neutrophils in neighboring areas where residual bacteria were observed. This is also suggestive evidence of bacterial viability.

Another limitation of the present study was the small number of cases analyzed. This is because this type of study is very difficult to conduct, and cases are hard to find, select, and collect. As a consequence of the small sample size, statistical analysis was not really useful. Molars were selected because they are, by and large, the teeth most difficult to treat. However, this is different from the material analyzed in several previous microbiological studies, which usually used single-rooted teeth.

Research has been conducted to disclose procedures that are able to expedite bacterial elimination in the root canal system and then optimize single-visit disinfection (55). Approaches such as performing a final rinse with chlorhexidine have been suggested to supplement the antimicrobial effects of chemomechanical procedures. Although ex vivo (56) and in vivo studies (57) have shown that a final rinse with chlorhexidine improves disinfection, the present study revealed that these effects were not satisfactory enough to obviate the need for an interappointment medication with calcium hydroxide. Ultrasonic activation of NaOCl is another supplementary approach that has the potential to improve disinfection, but the literature has shown inconclusive results about its effectiveness (56, 58). Studies are underway that use the present methodology to evaluate the antimicrobial clinical efficacy of ultrasonic activation of NaOCl.

In conclusion, this study demonstrated that the 2-visit protocol with an interappointment medication with calcium hydroxide resulted in improved microbiological status of the root canal system when compared with a single-visit protocol. No histobacteriologic difference was observed between minimal apical preparation and apical enlargement. Residual bacteria were more commonly observed and in higher abundance in ramifications, isthmuses, and dentinal tubules of teeth treated without interappointment medication. Apical ramifications and isthmuses were never completely filled, and the filling material, when forced to these areas, was usually interspersed with debris, necrotic tissue, and bacteria. The present results reinforce the concept that current instruments, irrigants, and techniques cannot predictably disinfect the root canal system in a single visit and the use of an antibacterial interappointment agent is necessary to maximize bacterial reduction before filling.

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