Antibacterial Efficacy of Chlorhexidine Gluconate Intracanal Medication In Vivo

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Abstract
The antibacterial efficacy of intracanal medication with 2% chlorhexidine liquid (CHX) was assessed in teeth with apical periodontitis. Canals in 22 teeth were instrumented at the first session, medicated with CHX, and reaccessed after 7 to 15 days. Bacteriological samples were aspirated at the first and second sessions, before (1A, 2A) and after (1B, 2B) canal instrumentation. Viable bacterial counts were obtained by culture (CFU) and microscopy using vital dyes. Microscopic counts were higher than CFU counts. Consistently high CFU counts in 1A samples (mean, $2 \times 10^5 \mu L^{-1}$ canal volume) decreased significantly ($p < 0.0001$) in 1B samples, increased significantly ($p < 0.04$) in 2A samples, and decreased in 2B samples to the level of 1B samples, increased significantly ($p < 0.0001$) in 2A samples, and decreased in 2B samples to the level of 1B samples. Proportions of negative cultures followed the pattern of CFU counts. Intracanal medication with CHX did not reduce the bacterial concentration. Bacterial counts expressed per microliter canal volume added information beyond the counts per tooth as expressed in previous studies. (J Endod 2007;33:788–795)

Key Words
Apical periodontitis, chlorhexidine, epifluorescence microscopy, intracanal medication, microbiology, root canal infection

Bacterial invasion of the root canal systems of teeth most frequently results in infection and apical periodontitis, a periradicular inflammatory response developed by the host to prevent dissemination of the bacteria into the bone (1). Elimination of bacteria from the root canal system is paramount to the healing of apical periodontitis; therefore, it is the focus of endodontic treatment. Indeed, persistence of bacteria in the root canal system at the time of root canal filling has a negative impact on the outcome of endodontic treatment (2).

Bacterial elimination during endodontic treatment is attempted by chemomechanical preparation of the root canal, and bacterial culturing has been used as an outcome measure to assess the efficacy of different treatment regimens (3–14). Two measures of bacterial elimination have been used: (1) the proportion of canals from which no bacteria can be recovered (no growth or negative culture) and (2) the bacterial concentrations in the canals that yield positive growth. Mechanical preparation alone can yield negative cultures in only 10% to 50% of root canals (4, 9, 10), whereas the addition of sodium hypochlorite irrigation increases this proportion to a total of 28% to 92% (5, 6, 8, 11–14). Bacterial concentration can similarly be reduced, from the typical count of up to $10^7$ per mL of sample at the beginning of treatment (13, 14), to levels between $10^3$ and $10^5$ at the end of chemomechanical preparation (9–12, 14). Importantly, bacteria that survive in the root canal system after chemomechanical preparation have been shown to rapidly multiply when the canals are left empty (4, 5, 7), suggesting that the conditions within empty root canal systems favor bacterial proliferation. In order to prevent bacterial regrowth, and preferably to eliminate residual bacteria, antibacterial intracanal medication has been used between subsequent treatment sessions (7, 11, 12, 14). The most widely used intracanal medication has been calcium hydroxide. It effectively kills the majority of root canal bacteria (7) but is less effective against Enterococcus faecalis (15) and yeasts (Candida spp) (16).

In the past 2 decades, chlorhexidine has been investigated as an alternative intracanal medication both in vitro (17–21) and in vivo (22). Chlorhexidine is a broad-spectrum antibacterial agent that is effective against both E. faecalis (20) and Candida albicans (16). In addition to its immediate action on bacteria, chlorhexidine can be adsorbed onto and subsequently released from dental tissues, resulting in substantive antibacterial activity or “substantivity” (23). Such substantivity has been shown in vitro in root canals medicated with chlorhexidine by using different vehicles (i.e., liquid, gel, or controlled-release devices) (17–20). However, the clinical efficacy of chlorhexidine as an intracanal medication appears to have been assessed in only one study (22), with 78% negative cultures reported after 7 days. Clearly, further assessment of the in vivo efficacy of chlorhexidine intracanal medication is warranted.

The culturing methodology for endodontic microbiological analysis used consistently in the majority of studies (2–14) was established by Möller (3). This methodology relies on root canal sample acquisition with paper points, cultivation of the recovered bacteria on culture media, and enumeration by counting colony-forming units (CFU). Le Goff et al. (24) introduced an alternative method for sample acquisition that relies on aspiration of the root canal contents rather than absorption with paper points. Both sampling methods were compared in a recent in vitro study (25) where canals were inoculated in a standardized manner and then subjected to root canal instrumentation procedures. Higher bacterial counts were obtained with the aspiration technique than with paper points. It was suggested that bacteria entrapped in the paper points might not be released into the culture, resulting in underestimation of the bacterial presence in
canals (24). In addition, because only a small proportion of sampled bacteria are cultivable under laboratory conditions (26), better specificity and sensitivity in detecting sampled bacteria can be achieved by the use of epifluorescence microscopy techniques (26, 27). The bacterial counts reported in all previous studies (2–14) have been expressed per tooth or root canal, overlooking the possibility that canals with a large volume might harbor more bacteria than canals with a smaller volume. This might explain the wide variation in counts reported by previous authors.

The purpose of this study was two-fold: (1) to assess the efficacy of intracanal medication with 2% chlorhexidine gluconate liquid (CHX) in eliminating bacteria beyond that obtained by chemomechanical preparation and (2) to quantify the concentrations of viable bacteria per root canal unit volume, before and after chemomechanical preparation and intracanal medication, using three independent methods. It was hypothesized that (1) the use of CHX as an intracanal medication would increase the proportion of negative cultures beyond that achieved by chemomechanical preparation, and (2) bacterial counts obtained by epifluorescence microscopy would be higher than CFU counts.

Materials and Methods

Study Cohort

Before data collection, the research protocol was approved by the Health Sciences Research Ethics Board. Subjects were recruited from among patients in the Graduate Endodontics Clinic, Faculty of Dentistry, University of Toronto, Canada, in accordance with specific inclusion and exclusion criteria. Included were only premolar or anterior teeth presenting with radiographic evidence of apical periodontitis and with sinus tract, and 73% with percussion sensitivity.

Preclinical Laboratory Procedures

Reduced transport fluid (RTF) containing the reducing agent dithiothreitol was used as transfer medium due to its proven ability to maintain the vitality of the sampled bacteria (28). Aliquots (1.4 mL) of RTF were placed into microtubes prepared for root canal sampling. In addition, two empty microtubes were used in the first treatment session for measuring the volume of the sampled root canal before and after chemomechanical preparation (described later). Microtubes were weighed three times with a precision balance (Sartorius, Göttingen, Germany), and the average weights calculated to allow measurement of sample volumes.

Microbiological Samples

Two types of samples were acquired during two treatment sessions: (1) access cavity samples (AC), obtained before accessing the pulp chamber, and (2) treatment step samples, obtained from the root canals before (A) and after (B) chemomechanical preparation. Each root canal sample was placed in a microtube containing 1.4 mL RTF. Immediately after acquisition, samples were stored at 4°C to minimize changes in the bacterial populations and transferred to the microbiology laboratory for processing within 2 hours.

First Treatment Session

Möller’s methodology (3) was used with modifications. Each treated tooth was anesthetized, isolated with a rubber dam, and the tooth crown cleaned with pumice and rinsed with sterile saline. Opal-Dam (Ultradent Products, South Jordan, UT) was placed along the tooth-dam interface to prevent leakage of saliva to the operative field. The tooth crown and surrounding rubber dam were disinfected with 30% hydrogen peroxide (Wiler-PCA, London, ON, Canada), followed by 5% iodine tincture (Wiler-PCA) for 60 seconds each, using sterile cotton rolls and cotton pellets.

All canals were removed with sterile high-speed carbide burs under sterile saline irrigation, without penetration into the pulp chamber. The access cavity was again disinfected with 30% hydrogen peroxide and 5% iodine tincture for 30 seconds and 60 seconds, respectively. The disinfecting agents were then inactivated with filter-sterilized 5% sodium thiosulphate for 60 seconds (3), rinsed with sterile saline, and an access cavity sample (AC1) was obtained as follows: the access cavity was filled with RTF (without dithiothreitol, because of unknown toxicity in clinical use) and the contents aspirated with a sterile endodontic syringe and a 27-G needle (Monoject; Sherwood Medical, St Louis, MO). Subsequently, new sterile burs were used to penetrate the pulp chamber. An ISO size 10 or 15 K-type file (Flexofile; Dentsply/Maillefer, Balleinque, Switzerland) was negotiated apically to 1 mm short of the estimated working length by using sterile saline only. The first root canal sample (1A) was then obtained as follows: RTF was injected into the canal leaving approximately 200 µL in the syringe barrel to facilitate transfer of the collected sample to microtubes, and a size 10 or 15 file was pumped circumferentially to 1 mm short of the estimated working length. The canal contents were then aspirated with a sterile endodontic syringe and a 27-G needle inserted up to 2 mm short of the estimated length (25). The canal was dried with paper points (Dentsply/Maillefer) and subsequently filled with sterile water to enable measurement of its initial volume (described later).

The working length was established with an apex locator (Root ZX; J. Morita, Irvine, CA). Canals were enlarged in a crown-down sequence with Gates-Glidden drills #4, #3, and #2 (Dentsply/Maillefer) followed by ProFile .04 and .06 taper rotary instruments (Dentsply/Maillefer) and nickel titanium hand files (Dentsply/Maillefer) to at least an ISO size of 35. Irrigation with 1 mL of 2.5% sodium hypochlorite was applied between subsequent instruments, with a 27-G needle inserted up to 2 mm short of the working length. At completion of preparation, the canal was irrigated with 3 mL filter-sterilized 5% sodium thiosulphate to inactivate sodium hypochlorite (3) and then flushed with 3 mL of sterile saline and dried with sterile paper points. The second root canal sample (1B) was obtained in the same manner as sample 1A, and the final volume of the prepared canal was measured.

Before medication, the canal was dried and irrigated with 2 mL 2% chlorhexidine liquid (CHX). At the end of irrigation, the canal was left filled with CHX. An ISO size 20 K-type file was inserted to the working length to assist distribution of CHX to the apical portion of the canal. A sterile cotton pellet soaked with CHX was placed at the canal orifice. The tooth was temporarily sealed with two layers of resin-modified glass-ionomer cement (Photac Fil Quick Apical; 3M ESPE, St Paul, MN). Occlusal contacts were adjusted to minimize pressure on the tooth. The medication period with chlorhexidine ranged from 7 days to 15 days (mean, 9.5 days), as determined by the patient’s availability for the second treatment session.

Second Treatment Session

The tooth was isolated and disinfected, and the disinfectants inactivated in the same manner as in the first session. The temporary resto-
ration was partially removed by using sterile burs under sterile saline irrigation. The access cavity was disinfected, the disinfectant inactivated, and an access cavity sample (AC2) obtained. The remainder of the temporary restoration was then removed with a new sterile bur. The root canal system containing CHX was rinsed with 2 mL filter-sterilized lecithin mixed with Tween 80 to inactivate chlorhexidine (29), followed by 3 mL of sterile saline. A postmedication root canal sample (2A) was obtained. The canal was irrigated again with 2.5% sodium hypochlorite followed by 5% sodium thiosulfate, flushed with 3 mL sterile saline, and dried with sterile paper points. In the last 11 subjects only, a final sample (2B) was obtained. The root canal was then filled with either AH Plus sealer (Dentsply/DeTrey, Konstanz, Germany) and laterally condensed gutta percha or Pulp Canal Sealer (Kerr, Romulus, MI) and vertically condensed warm gutta percha. The access cavity and the tooth crown were permanently restored, and a final periapical radiograph was exposed.

Sample Volume

The preweighed microtube with 1.4 mL RTF was reweighed after addition of the sample. Weighing was repeated three times and the average weight calculated. The volume of the sample was calculated as the difference between the weight of the microtube before and after addition of the sample, assuming a specific gravity of 1.0.

Root Canal Volume

An Eppendorf pipette with a gel-loading tip was filled with 100 µL sterile water, and the contents dispensed slowly into the previously dried root canal until full. The balance of the water left in the pipette tip was placed in an empty preweighed microtube, and the filled microtube was reweighed. The root canal volume in microliters was calculated as 100 less the weight difference of the microtube, assuming a specific gravity of 1.0.

Sample Processing for Culture

Each sample was divided into 3 subsamples: one for plate culture and two for epifluorescence microscopy. For the plate culture, 52 µL was inoculated on blood agar (Difco; Becton Dickinson, Sparks, MD) in a 15-cm petri dish (Becton Dickinson, Franklin Lakes, NJ) by using a Spiral Plater (Spiral System, Cincinnati, OH). Samples AC1 and AC2 were each inoculated on two plates and incubated, respectively, in an aerobic or anaerobic atmosphere at 37°C for 14 days. The plates with the root canal samples were incubated anaerobically in sealed jars with a gas mixture (10% hydrogen, 10% carbon dioxide, and 80% nitrogen) at 37°C for 14 days. All incubated plates were counted for CFUs after 7 days and also after 14 days to account for slow growing bacteria.

Sample Processing for Microscopy

Each of the two subsamples designated for epifluorescence microscopy were diluted by mixing 300 µL with 700 µL RTF for a total of 1 mL. The diluted subsamples were processed for two independent procedures as follows. The first subsample was stained with 10 µL dihydroethidium (DHET) solution, prepared by mixing 1 mg DHET in 500 µL dimethyl sulfoxide (Sigma-Aldrich, Oakville, Canada). The microtube was vortexed and incubated at 37°C for 20 minutes in the dark. The second diluted subsample was stained with 3 µL LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR); the microtube was vortexed and incubated at room temperature for 15 minutes in the dark.

Vital Microscopy

Immediately after the staining procedures described earlier, aggregates of bacteria were dispersed by pumping the sample through a sterile 18-G needle and 3-mL syringe, and then 800 µL of the sample was filtered through a black polycarbonate filter (0.2-µm porosity; Whatman, Maidstone, England). Bacteria on the surface of the polycarbonate filters were counted by epifluorescence microscopy using a 100× lens (NPL Fluotar; Leitz, Wetzlar, Germany) and interchangeable optical filters. For the BacLight-stained bacteria, broad band excitation (BP450-490) and emission (BP520-560) filters were used with a FT510 chromatic beam splitter. These filters allowed detection of both green-emitting (live) and red-emitting (dead) bacterial cells (Fig. 1). Only the green-emitting cell counts were used for this study. For the DHET-stained bacteria, green excitation (BP546/12) and red emission (LP590) filters were used with a FT580 chromatic beam splitter, allowing visualization of red-emitting live bacterial cells (Fig. 2). Images of randomly selected microscope fields were captured by a charge-coupled device camera. Numerical densities of cells were established by visual inspection of digital images for low densities or with an interactive image analysis package (Optimas 6.5; Media Cybernetics, Silver Spring, MD) for densities exceeding 20 to 30 cells per field.
TABLE 1. Proportion of Samples Showing No Bacterial Growth after 7 and 14 Days of Incubation.

<table>
<thead>
<tr>
<th>Sample†</th>
<th>n</th>
<th>No growth (%)</th>
<th>7 d</th>
<th>14 d</th>
<th>p values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1‡</td>
<td>21§</td>
<td>90.5</td>
<td>71.4</td>
<td></td>
<td>0.0625</td>
</tr>
<tr>
<td>AC1</td>
<td>22</td>
<td>77.3</td>
<td>63.6</td>
<td></td>
<td>0.1250</td>
</tr>
<tr>
<td>1A</td>
<td>21§</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1B</td>
<td>22</td>
<td></td>
<td>68.2</td>
<td>52.4</td>
<td></td>
</tr>
<tr>
<td>AC2‡</td>
<td>20¶</td>
<td>65.0</td>
<td>55.0</td>
<td></td>
<td>0.2500</td>
</tr>
<tr>
<td>AC2</td>
<td>22</td>
<td>63.6</td>
<td>50.0</td>
<td></td>
<td>0.0625</td>
</tr>
<tr>
<td>2A</td>
<td>22</td>
<td>45.5</td>
<td>31.8</td>
<td></td>
<td>0.1250</td>
</tr>
<tr>
<td>2B</td>
<td>11#</td>
<td>72.7</td>
<td>70.0</td>
<td></td>
<td>0.5000</td>
</tr>
</tbody>
</table>

*McNemar’s chi-square analysis of differences in proportion of negative cultures (no growth) between 7 and 14 days of incubation for each sample, using the 1-sided test of the Exact McNemar statistic (exact p value).
†Anaerobically incubated.
‡Aerobically incubated.
§One plate was excluded because of contamination.
¶One plate was excluded because of contamination; another was excluded from the analysis because it was an outlier.
#One plate was excluded because of contamination. 

Bacterial Concentration

Bacterial counts were calculated as the number of bacteria per 1 μL of sample. Bacterial concentrations in the figures and tables were expressed as bacteria per 1 μL of root canal volume. However, to facilitate comparisons with previous studies, these bacterial concentrations were converted to numbers per tooth; the concentration for each sample was multiplied by the specific volume of the root canal from which the sample was obtained.

Analysis

All statistical analyses were performed by using SAS 8.2 (Cary, NC) at the 0.05 alpha level of significance. Because of a wide range of values, CFU and microscopic vital counts were transformed to the natural logarithm (ln) so as to normalize the data before analysis. Individual sample data and differences between samples were assessed for normality and outliers. Because of the dependent nature of the bacterial samples taken from the same tooth, a paired t test analysis was used to detect significant differences in bacterial counts between the initial and subsequent treatment steps. For the culture method, McNemar’s chi-square analysis was used to analyze differences between samples in the proportion of plates without detectable growth (negative culture). In addition, the data obtained with the three methods of enumeration (CFU, BacLight, and DHET staining) were compared by using the analysis of variance general linear models procedure followed by Duncan’s multiple range test.

Results

Percentage of Negative Cultures

The proportions of samples showing no bacterial growth on agar plates (percentage negative culture) are summarized in Table 1. The statistical analysis of differences between samples is presented in Table 2. Not all of the access cavity (AC) samples were negative. The 1A samples all showed bacterial growth (positive culture). There was a significant increase (p < 0.001) in the percentage of negative cultures from the 1A to the 1B samples. The percentage of negative cultures decreased significantly (p < 0.05) from the 1B to the 2A samples and increased again (p < 0.04, 14d incubation) in the 2B samples to a level not significantly different from the 1B samples. A 3% to 19% reduction in negative cultures was observed between the 7 days and 14 days incubation periods; however, the differences were not statistically significant.

CFU Counts

The root canal volumes after chemomechanical preparation ranged from 10 to 30 μL, with a mean volume of 17.6 μL. The mean and standard deviation CFU counts per 1 μL of canal volume are presented for all root canal samples in Figure 3. The mean CFU count of the 1A samples was significantly higher (p < 0.0001) than that of all other samples. The mean count of the 2A samples was more than one order of magnitude and significantly (p < 0.05) higher than that of the 1B samples; however, it was still 1/2,000 of the mean count of the 1A samples. The mean CFU count of the 2B samples was more than one order of magnitude lower than that of the 2A samples and not significantly different from the count of the 1B samples. The difference between the CFU counts after 7 days and 14 days incubation was significant for the 1B sample (p < 0.05) and 2A sample (p < 0.03) but not for the 1A and 2B samples.

Vital Microscopy

All root canal samples stained with BacLight were positive for viable bacteria, whereas four samples stained with DHET were negative. The mean and standard deviation counts of viable bacteria are presented in Figure 4, and the statistical analysis of differences between samples is presented in Table 3. The mean viable bacterial count (ln) of

![Figure 3](image)

**Figure 3.** Mean and SD. CFU counts per microliter of root canal volume obtained in different steps of root canal treatment, and paired t test analysis of differences between these samples (n = 22, sample 2B n = 11). Statistically significant differences between samples are represented by different letters.
To avoid sampling through a contaminated field, in the present study, the teeth and rubber dam were disinfected and their juncture thoroughly sealed. The degree of disinfection of the field was then determined by obtaining the access cavity samples. Demonstrated sterility of access cavity samples is essential when paper points are used for root canal sampling (3) because bacteria collected by the paper point from contaminated cavity walls are added to the canal sample when the paper point is transferred into the culture fluid (31). However, positive access cavity samples may be less critical when the aspiration sampling technique is used, as in this study. Each root canal sample was acquired with a very fine needle and care was taken, using an operating microscope, that the needle did not contact the access cavity walls. The aspirated sample was then dripped into the RTF without the needle contacting the microtube, reducing the risk of bacterial transfer from the needle surface to the transport fluid. This study also differed from previous ones in the manner the access cavity samples were acquired. The invariably negative control samples in the majority of studies (5, 7, 9, 12) were acquired by swabbing the access cavity with paper points or cotton pellets (3), thus limiting the chance of recovering bacteria. In the present study, the access cavity was flooded with RTF, which was then aspirated. The transport fluid was likely to contact undercut areas and margins of fillings that would be inaccessible to paper points or cotton pellets.

Considering all of this, teeth with positive access cavity samples were not excluded from the analysis. On the contrary, these positive samples could be significant for the interpretation of the root canal samples because they underscored the limitations of the topical application of antibacterial agents. If the readily accessible and morphologically regular access cavity could not be disinfected, the challenge of disinfecting the morphologically complex root canal system might be insurmountable. In this context, it was considered that the low bacterial counts observed in the access cavity samples represented the best result that could realistically be achieved in the root canal samples. Indeed, the bacterial counts in both root canal samples obtained after chemomechanical preparation (1B and 2B) did not differ significantly from the respective access cavity samples (anaerobic AC1 and AC2).

The antibacterial agents used were inactivated before samples were acquired to avoid carry over into the culture media and potential false-negative results. As suggested by Möller (3), 5% thiosulfate was used to inactivate the iodine in access cavities and the sodium hypochlorite in root canals. In addition, 3% Tween 80 and 0.3% L-α-lecithin were used in the canals to inactivate the chlorhexidine (29).

To further reduce the risk of false-negative results, an effort was made to maximize the accuracy of both the sampling and enumeration procedures. Samples were acquired by fluid aspiration as suggested by Le Goff et al. (24), rather than with paper points as in the majority of the studies (2, 4–14). Dagher (25) compared the two sampling methods in an in vitro model and concluded that aspiration afforded higher recov-

**Table 3. Paired t-Test Analysis of Differences between Treatment Steps for Live Bacterial Counts (ln) after Staining with BacLight and Dihydroethidium (DHET)**

<table>
<thead>
<tr>
<th>Samples</th>
<th>DF</th>
<th>BacLight p*</th>
<th>DHET p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A – 1B</td>
<td>21</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>1A – 2A</td>
<td>21</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>1A – 2B</td>
<td>10</td>
<td>0.0002</td>
<td>0.0036</td>
</tr>
<tr>
<td>1B – 2A</td>
<td>21</td>
<td>0.0695</td>
<td>0.0971</td>
</tr>
<tr>
<td>1B – 2B</td>
<td>10</td>
<td>0.6256</td>
<td>0.4086</td>
</tr>
<tr>
<td>2A – 2B</td>
<td>10</td>
<td>0.2071</td>
<td>0.3820</td>
</tr>
</tbody>
</table>

*Values of p < 0.05 indicate significant difference. Boldface highlights statistical significance.*
ery of bacterial cells than paper points that might entrap bacteria and thus underestimate their counts. Enumeration by epifluorescence microscopy was added to the CFU method used in the previous studies (3–14), as the former has been shown to yield higher counts of bacteria (26, 27). When dental-unit water system contamination was studied, only 5% of the live bacteria detected by fluorescence microscopy could be cultured on agar plates (26). Similarly, in an endodontic in vitro study (27), direct enumeration of viable bacteria with fluorescence labeling provided valuable information about the vitality status of the bacteria compared with the CFU counts. Indeed, in the present study, significant differences were detected among the 3 enumeration methods used, and higher bacterial counts were obtained with microscopic enumeration than with the culture method.

To facilitate detection of live bacterial cells by microscopy, samples were stained with BacLight and dihydroethidium (DHET). The different modes of action of these staining methods may result in differences in live bacterial counts. BacLight uses a combination of two fluorescent dyes: Syto 9 stains both live and dead bacterial cells, detected by green fluorescence, whereas propidium iodide stains only cells with membrane damage (considered to be dead cells), detected by red fluorescence. Nonfluorescent DHET is metabolized by the dehydrogenases of live cells, and the ethidium bromide released is detected by red fluorescence. Thus, a dead bacterial cell that does not metabolize, but still has an intact membrane, can be identified as live by BacLight staining.

For the culture method, all the samples were incubated anaerobically considering the predominance of obligate anaerobes in the endodontic microflora (1, 6). However, the access cavity samples were also incubated aerobically to detect aerobic and facultative contaminants from the oral cavity.

In previous studies (2–14, 27), the bacterial concentration was expressed as the total number of bacteria recovered from a tooth/canal per unit volume of transport fluid, usually 1 mL. Such calculation does not take into consideration differences in the size and volume of the sampled canals. Therefore, in this study, in order to more realistically express the bacterial density within the root canals, the volume of each canal was measured and the bacterial concentration expressed per 1 μL of canal volume. The mean root canal volume measured was 17.6 μL with a range from 10 to 30 μL. This becomes more important when considering whether the bacteria form a biofilm or are in planktonic suspension or when the dentin surface area to canal volume is considered to determine the effects of the substantivity of chlorhexidine.

In this study, samples were incubated for 14 days, although counts were made at 7 days to facilitate comparison with the 4 to 10 days incubation periods used in previous studies (4–14). Several of the samples yielded higher counts at 14 days, probably containing slow-growing strains that could not be counted after 7 days. The differences between the 7-day and 14-day mean CFU counts were indeed significant in the 1B and 2A samples but not in the 1A and 2B samples.

Use of stricter inclusion criteria for selection of the study cohort might have reduced the variation of the results. For example, teeth associated with clinical signs and symptoms could have been excluded. In this study cohort, almost one third of the teeth presented with a sinus tract, consistent with chronic apical abscess. Such teeth may host greater bacterial concentrations than asymptomatic teeth (9), and they may have periapical exudates seeping into the root canal between treatment sessions, resulting in increased bacterial counts at the second session. The inclusion criteria could also be limited to teeth without any coronal restorations. It has been shown that teeth restored with temporary cement are more difficult to disinfect than teeth without restorations (5). In this study, all selected teeth presented with a permanent coronal restoration, and bacteria harbored at the restoration margins might have contaminated the access cavity and the root canal during the medication period between treatment sessions.

A control group in which canals would be left without antibacterial medication was not established. The rapid regrowth of bacteria reported in previous studies (4, 5, 7) highlighted the ethical concern for leaving canals without medication and was used as the historic control in lieu of a control group.

The antibacterial efficacy of chemomechanical preparation and intracanal medication of infected root canals was assessed in the present study using two outcome measures: (1) an increase in the proportion of samples with no detectable bacterial growth (percentage negative culture) and (2) a decrease in the bacterial concentration in root canal samples. In the following section, both measures are discussed concurrently, as two facets of the same outcome. The results before and after the chemomechanical preparation stage are discussed first, followed by those after the intracanal medication stage. For the culture method, data from 7 days incubation are discussed only so as to facilitate comparison with other studies.

Importantly, all initial pretreatment samples (1A) in the present study showed positive cultures consistent with the infective etiology of apical periodontitis (1). The mean CFU count of the initial sample (1A), expressed per tooth to allow comparison with previous studies, was 3.85 × 10^6. This value was 3 × (14) and 16 × (15) lower than in two studies but 4 × higher than in another study (12). The initial counts in most other studies (9–11) are 10× to 100× lower than those in this study. As suggested earlier, the higher counts in the present study could
be attributed to the aspiration sampling method, which might be more sensitive than the paper point method used in the previous studies (25). The increase to 68% negative cultures after irrigation with 2.5% sodium hypochlorite and apical enlargement to size 35 to 60 (1B sample) was within the range of 28% to 92% reported in previous studies (5, 6, 8, 11–14). In earlier studies from one group of researchers (5, 6, 8), irrigation with 0.5% or 5.25% sodium hypochlorite, alone or in combination with EDTA (6) and apical enlargement to size 8, yielded negative cultures in 50% to 55% of teeth. More recent studies from another group (11, 13, 14) reported 47% to 62% negative cultures after canal irrigation with 1% sodium hypochlorite and apical enlargement to size 36 to 60, and over 89% after extensive apical enlargement up to size 100. In another recent study (12), 76% negative cultures were reported after irrigation with 2% sodium hypochlorite and apical enlargement to size ≥35. The mean CFU count of 9.74 × 10^3 per tooth at the end of the first session in this study (1B sample) was 16× lower than the 1.58 × 10^5 count reported by Ørstavik et al. (9), 5× higher than the 1.86 × 10^3 count reported by Shuping et al. (11), and 67.76× lower than the 6.6 × 10^5 count reported recently by McGurkin-Smith et al. (14). Calculating the mean counts from positive samples only, Peters et al. (12) reported 1.8 × 10^3 CFU per tooth. When adjusted for positive cultures only, the mean CFU count in the present study was 3.06 × 10^2, 6.6× lower than that reported by Peters et al. (12). These differences in bacterial counts after root canal preparation may be related to several factors including the concentration, frequency, and volume of irrigation with sodium hypochlorite; the extent of canal enlargement; and the sampling method. Because data on the volume of canals and exposure time to sodium hypochlorite are not consistently available, and the reported apical enlargement sizes cannot be interpreted as reflecting the actual enlargement of canals, any suggestions regarding the differences in results among the various studies would be speculative.

The study hypothesis was that intracanal medication with 2% chlorhexidine gluconate liquid would further increase the number of negative cultures and reduce the bacterial concentration beyond that achieved by chemomechanical preparation of the root canal in the first session. However, after medication for 7 days to 15 days there was a decrease in negative cultures from 68.2% after chemomechanical preparation (1B sample) to 45.5% (2A sample), and an increase in the mean bacterial counts as obtained by the three enumeration methods (Table 4), from 181/μL canal volume (1B sample) to 655/μL canal volume (2A sample). Thus, the hypothesis was not supported and under the in vivo conditions of this study, the potential benefits of CHX, suggested by in vitro studies (17–21), could not be fully realized. Nevertheless, the bacterial regrowth observed was less than that reported in historic controls (4, 5, 7), where canals were left empty without any intracanal medication.

In the absence of other data on the efficacy of intracanal medication with chlorhexidine in vivo, it was intended to compare the results with those of the previous studies in which calcium hydroxide was used for intracanal medication (7, 9, 11, 12, 14). However, the results of these studies have varied considerably. The percentage of negative cultures after dressing canals with calcium hydroxide for 1 to 4 wks has ranged from 97.1% without inactivation before sampling (7), to 92.5% (11) and 65.2% (9) after inactivation with 0.5% citric acid, to an alarmingly low 28.6% without inactivation but with removal of calcium hydroxide verified by microscopic examination (12). The latter represented a decrease in the percentage of negative cultures from the end of the first session, similar to that observed in the present study after intracanal medication with CHX. An increase in mean CFU counts was also shown after intracanal medication with calcium hydroxide, from 1.8 × 10^3 to 9.5 × 10^3 (12) and from 1.58 × 10^3 to 2.51 × 10^3 (9).

Other studies have shown opposite results for medication with calcium hydroxide, with a decrease in mean CFU counts from 6.6 × 10^5 to 8.25 × 10^4 (14) and from 18.6 to 1.62 (11). These contrasting reports preclude comparison of the efficacy of intracanal medication with CHX, as reported herein, and calcium hydroxide, as reported previously. CHX may be inactivated or buffered by dentin and other substances present in root canals (32–34). This could partially explain the poorer-than-expected performance of CHX as an intracanal medication in the present study. Alternatively, the liquid form of CHX might not be well suited for use as an intracanal medication. The choice of liquid form was based on its excellent performance in previous in vitro experiments (18–20) and the premise that the intracanal medication should be easy to place and to remove from the root canal system (19). Care was taken to completely fill each canal, yet often at the start of the second session the canals were not filled with liquid. The liquid may have escaped through the apical foramen or diffused into the root dentin (20). In either case, the root canal may not have been completely medicated, allowing some bacterial regrowth between the first and the second treatment sessions. This shortcoming of the CHX liquid might be overcome by alternative forms of delivery, such as a gel (19–21, 35) or a controlled-release device (17, 19–21). Although the gel may be more stable than the liquid over the period of time of canal dressing, the ability to totally remove it has not been confirmed, raising a concern about potential interference of any gel residue with the seal of the root filling (19, 35). This concern has only been addressed in an in vitro leakage study (35); clearly, it must be alleviated before the gel can be applied clinically (20).

According to the original study design, the canals were to be filled immediately after the 2A samples were obtained, without further canal irrigation at the second session. However, the increased postmedication bacterial counts (2A sample) obtained in the first 11 subjects warranted a further disinfecting procedure in the second session. To assess its potential benefit, additional sampling was performed after further irrigation of the canals in the second session in the remaining 11 subjects (2B sample). At 72.7%, the proportion of negative cultures at the end of the second session was higher (14 days incubation) than at the start (2A sample). The converted mean CFU count of 1.40 × 10^2 per tooth was 23× lower than the 3.16 × 10^5 count reported by Ørstavik et al. (9), whereas the converted 5.13 × 10^5 count in the positive samples only was 4× higher than the 1.4 × 10^5 count reported by Peters et al. (12). Notably, the mean CFU counts at the end of the first session (1B sample) and the second session (2B sample) did not differ significantly.

In conclusion, 2% chlorhexidine gluconate liquid applied in vivo as intracanal medication for 7 days to 15 days did not increase the proportion of teeth with negative cultures or reduce the bacterial counts beyond that achieved after chemomechanical preparation in the first treatment session. Although the bacterial regrowth was less than in historic controls, the potential benefits of CHX indicated by in vitro studies were not fully realized. Thus, further research is warranted on different delivery forms of chlorhexidine, as well as alternative intracanal medications. Expressing the bacterial concentration per root canal unit volume, use of the aspiration technique for sample acquisition and the use of vital microscopic counts substantially improved the sensitivity by which the dynamics of canal disinfection can be studied.

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References