Chlorhexidine substantivity in root canal dentin

Sidney Rosenthal, DDS, MDentSci,[a] Larz Spångberg, DDS, PhD,[b] and Kamran Safavi, DDS, MEd,[c]
Farmington, Conn
UNIVERSITY OF CONNECTICUT

Objective. The purpose of this investigation was to evaluate the substantivity of chlorhexidine (CHX) within a root canal system and to assess how long the CHX remains antimicrobially effective.
Study design. Bovine roots were sectioned and standardized to 8 mm. Sections, which served as controls, were treated with 1% sodium hypochlorite and 1 mol/L EDTA, then obturated with gutta percha and AH26 sealer. Experimental sections were treated similarly except they were placed in 2% CHX for 10 minutes prior to obturation. Control specimens were divided into 4 control groups and stored in saline for 1 day, 3 weeks, 6 weeks, and 12 weeks. Experimental specimens were divided into 4 groups and stored in saline for 1 day, 3 weeks, 6 weeks, and 12 weeks. After their respective storage periods, all specimens were halved and canal wall dentin was ground out with Peeso reamers. Dentin specimens were agitated in 700 μl of saline for 5 hours to release CHX. After centrifugation the supernatants were analyzed with UV spectrophotometry at 253 nm. To determine whether the CHX from dentin samples remained antimicrobial, the extracts from experimental and control groups were mixed with cultures of *Enterococcus faecalis*.
Results. After 1 day of storage, the dentin extract contained approximately 0.0048% CHX. After 3, 6 and 12 weeks, dentin extracts contained approximately 0.0023%, 0.0016%, and 0.0010% CHX respectively. Extracts from the storage groups were found to be highly antimicrobial corresponding to the CHX concentration.
Conclusion. The results of this study indicate that CHX is retained in root canal dentin in antimicrobially effective amounts for up to 12 weeks.
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Viable microorganisms remaining after root canal preparation and disinfection contribute significantly to failure in endodontic therapy.[1] Methods to reduce root canal microorganisms have been described in numerous studies and include thorough instrumentation, the use of an effective irrigating solution, and intracanal medicaments. Coronal microleakage through temporary and permanent restorations may also contribute to treatment failures.[2] After root canal filling and during final coronal restoration, there are circumstances in which the root canal space may become reinfected. Of significance is the period of time when the pulp space is only temporarily restored. Zinc oxide and eugenol products are popular temporary restorations for endodontically treated teeth. However, there are many studies using dye, bacteria, and radioisotopes, which have shown these restorations to provide relatively poor seals.[3-7] Cavit, another popular endodontic temporary restorative material, provides a relatively acceptable seal but it is still prone to leakage and it is mechanically weak.[8-10] In general, coronal leakage has received a great deal of attention in the endodontic literature. It has been stated that the coronal seal of endodontically treated teeth may be as important as the apical seal in the ultimate success or failure of root canal therapy.[2] The current standard irrigant, sodium hypochlorite (NaOCl), possesses many desirable properties including the ability to dissolve necrotic debris as well as a potent antimicrobial action.[11-14] However, it is not known to exhibit any degree of substantive activity. Intracanal medicaments such as the phenolic compounds are highly toxic. While somewhat effective as antiseptics, they are known to dissipate rapidly from the canal space.[15] Calcium hydroxide has become a standard intracanal medicament owing to its ability to predictably disinfect the root canal space.[16,17] After mechanically removing the calcium hydroxide there is no residual antimicrobial effect, however. Other studies have suggested that calcium hydroxide is ineffective against *Enterococcus faecalis* as well as *Candida albicans*.[18,19] This is important because endodontically treated teeth with persistent apical periodontitis are frequently found to be infected with *E faecalis*[20-22] and *C albicans*.[23] Chlorhexidine digluconate (CHX) is a broad-spectrum antibacterial agent that has been shown to be effective against many strains of bacteria found in infected root canals.[24] Studies in vitro have shown CHX to exhibit sustained antimicrobial activity in the root canal for some time after being used as

[a]Former Postdoctoral Student, Department of Endodontology, University of Connecticut School of Dental Medicine, Farmington.
[b]Associate Professor, Department of Endodontology, University of Connecticut School of Dental Medicine, Farmington.
[c]Professor and Head, Department of Endodontology, University of Connecticut School of Dental Medicine, Farmington.
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an endodontic irrigant.[25] Therefore, CHX has been suggested as a root canal irrigant owing to its unique ability to bind to dentin, its effectiveness as an antimicrobial agent, and its substantivity in the root canal system.[25-30]

The purpose of this investigation was to evaluate the substantivity of CHX within a root canal system and to assess how long after the root filling the CHX remains antimicrobially effective.

MATERIAL AND METHODS

Preparation of root segments

Sixty bovine incisor roots were extracted from sectioned mandibles procured from a slaughterhouse. Crowns and apices were sectioned off and the root sections were standardized to 8 mm length. The root sections were instrumented using Peeso reamers sizes 4 through 6 depending on size of the root canal space. Instrumentation was occasionally supplemented by a size 120 K-file (Dentsply Maillefer, Ballaigues, Switzerland) to remove any remaining pulp tissue. The root canals were irrigated copiously with a fresh solution of 1% NaOCl buffered with sodium bicarbonate. Instrumentation was considered complete when the canal wall was visually confirmed as smooth and free of debris. All specimens were then treated with a 1 mol/L solution of EDTA for 10 minutes, rinsed well with sterile saline, and then steam autoclaved.

Following sterilization, the root sections were randomly assigned to two treatment groups: experimental (n = 40) and control (n = 20). Experimental sections were treated by immersion in a 2% solution of CHX for 10 minutes. This solution was prepared by dilution of a 20% stock CHX (Sigma Scientific, St. Louis, Mo) with sterile deionized water. All solutions of CHX were prepared fresh, stored in dark bottles, and used within approximately two weeks. Control sections were treated by immersion in sterile saline for 10 minutes. Both control and experimental sections were then blotted dry with sterile gauze and canals were dried with extra-large paper points. AH26® (DeTrey, Dentsply, Konstanz, Germany) root canal sealer was mixed according to the manufacturer's instructions. In addition, an Obtura® (Spartan USA, Fenton, Mo) warm gutta percha device was loaded with pellets of gutta percha and temperature set to 200°C. The canal walls of each root section were evenly coated with a layer of sealer using a size 120 K-file. While holding the open apical end of the root segment against a sterile glass slab, the root canals were obturated with 2 separate aliquots of warm gutta percha and condensed with a #12 Schilder plugger (Dentsply Maillefer). Aseptic technique was used in all work.

After experimental and control group root sections were obturated, each group was further randomly divided into four separate "storage" groups to be placed in sterile saline for various time periods. These groups were identified as follows.

Experimental: E1 (n = 10), storage for 1 day; E2 (n = 10), storage for 3 weeks; E3 (n = 10), storage for 6 weeks; E4 (n = 10), storage for 12 weeks.

Control: C1 (n = 5), storage for 1 day; C2 (n = 5), storage for 3 weeks; C3 (n = 5), storage for 6 weeks; C4 (n = 5), storage for 12 weeks.

Eight 400 ml dark glass storage jars were sterilized by ethylene oxide gas sterilization. Each jar was then filled with 150 ml of sterile saline. Experimental and control group samples were placed in their respective storage jars.

Processing of specimens: Quantification of CHX

After their respective storage periods root segments were removed from the saline, blotted dry with sterile gauze, and prepared for quantification analysis of CHX. The sides of each root section were scored with a #2 round bur and split in half with a pair of orthodontic wire cutters. Gutta percha remaining in one half of the specimen was removed by teasing it out with a dental explorer. Using a #6 Peeso reamer, the canal walls of each specimen were ground so that the dentin shavings would fill a 1 ml Eppendorf tube approximately halfway. Eppendorf tubes were weighed separately and then together with specimens to determine the exact weight of the dentin shavings. Seven hundred microliters of sterile saline was added to each tube and specimens were agitated for five hours on a rotary agitator (Roto-torque, Cole Parmer, Vernon Hills, Ill). Immediately after agitation, specimens were centrifuged for 20 minutes at 5000 rpm in an Eppendorf centrifuge (Brinkman Instruments, Hamburg, Germany). Supernatants were aspirated and these dentin extracts were submitted for spectrophotometric analysis to determine the quantity of retained CHX.

The quantification of CHX was done using a spectrophotometer (Ultraspec 4050, LKB Biochem, Cambridge, England) to read absorbency at 253 nm. Absorbency was referenced to zero using the same sterile saline used to extract CHX from dentin shavings by placing 500 μl of saline into a 500 μl quartz cuvette. Five hundred microliters of dentin extract was placed into a 500 μl quartz cuvette and ultraviolet absorbence was recorded as optical density at 253 nm (OD_{253}). Cuvettes were washed thoroughly with sterile saline between samples. The samples were recovered and stored in a freezer at −10°C.

Processing of specimens: Qualification of CHX

To determine whether the residual CHX detected from dentin samples remained antimicrobial, the extracts were

mixed with cultures of *E faecalis* (now reclassified as *E hirae* ATCC 9790) that were kept in log growth phase. The culture was diluted with sterile saline to have the same absorbence as the #1 MacFarland standard, resulting in a final concentration of approximately 3×10^8 colony-forming units (CFU) per ml. The standardized culture was diluted with sterile saline to 3×10^4 CFU/ml. An additional control dilution of 300 CFU/ml was made to analyze and ensure precision of procedures. One milliliter of this dilution was plated on blood agar.

Six samples each of the following CHX dilutions were made from a 20% stock solution of CHX (Sigma Scientific) and used as controls: SD1 at 2% concentration, SD2 hat 0.002%, SD3 at 0.0002%, SD4 at 0.00002%, and SD5 at 0.000002%. One hundred microliters of the 3×10^4 CFU/ml culture was added to 900 μl of each concentration of CHX.

In order to determine the antimicrobial effectiveness of CHX remaining in the dentin extracts, the dentin extracts were mixed with 3×10^4 CFU/ml culture. One hundred microliters of culture was added to 900 μl of three random samples each of experimental (E1-E4) and control dentin extracts (C1-C4). In addition, serial dilutions of CHX and saline controls were also mixed with culture. After 30 minutes of interactions, 400 μl of this mixture was added to 400 μl of an aqueous solution of 3% Tween 80® (Sigma Scientific), 0.3% lecithin, and 0.5% sodium thiosulfate for 10 minutes in order to neutralize any remaining CHX.[31,32] Six hundred microliters of this mixture was plated on blood agar (Sigma Scientific) and incubated at 37°C for 24 hours. After incubation the number of colonies was counted on all plates and recorded.

RESULTS

Quantification of CHX

CHX was recovered from root canal dentin in all specimens at all storage periods. Based on a "standard curve" developed from pilot experiments, the approximate concentration of CHX was calculated. After 1 day of storage, the dentin extract contained approximately 0.0048% CHX. After 3, 6, and 12 weeks, dentin extracts contained approximately 0.0023%, 0.0016%, and 0.0010% CHX respectively.

Qualification of CHX

Extracts from the one-day storage group (E1) were found to be highly antimicrobial with only 1 CFU being found among the three samples. After 3 weeks, group E2 remained antimicrobial with a mean CFU of 41.33 ± 18.23. Groups E3 and E4 displayed progressively less antimicrobial activity with mean CFU's of 169 ± 79.64 and 577.33 ± 177.78 respectively. Control groups C1, C2, C3, and C4 displayed no antimicrobial activity; their mean CFU's were, respectively, 1026.33 ± 73.58, 925.67 ± 26.95, 1026 ± 54.37, 955.33 ± 73.87.

Serial dilutions of CHX were highly antimicrobial until groups SD4 (0.00002% CHX) and SD5 (0.000002% CHX). These lowest concentrations had mean CFU's of 992.83 ± 104.6 and 1040.33 ± 136.4. The saline control group, SAL, displayed a mean CFU count of 1000.83 ± 87.58.

The "300 CFU control plate," described in Materials and Methods, had a mean CFU count of 300.33 ± 89.13, which assured reasonably accurate laboratory methods.

DISCUSSION

The results of this study indicate that CHX is retained in root canal dentin in antimicrobially effective amounts for at least 12 weeks. Previous studies that have investigated the substantive properties of CHX in the root canal have only tested for its presence for up to 3 weeks.[25,29,30] In addition, these previous studies only analyzed the antimicrobial activity of CHX.[33,34] In the current study, UV spectrophotometry was successfully used to estimate the amount of CHX that is retained in the dentin of the root canal wall.

AH26 sealer used in obturating root segments released a soluble constituent that weakly absorbed UV light. Therefore, OD_{253} readings from controls were subtracted from OD_{253} readings from experimental groups to give a true resultant concentration of CHX.

The effective concentrations in the antimicrobial experiments were E1 0.0043% CHX, E2 0.0021% CHX, E3 0.0014% CHX, and E4 0.0009% CHX.

Although the approximate concentration of CHX from the 12-week dentin sample was 0.001%, the antimicrobial activity was clearly less than expected based on serial dilution samples. This may be due to the presence of small amounts of dentin powder, which have been shown to affect the antimicrobial activity of disinfectants.[35]

In some studies investigating the effects of CHX in the root canal, results may have been affected by the lack of antimicrobial inactivation before culturing.[27,29,30,36-38] Owing to its affinity to many substrates, CHX may easily be transferred from antimicrobial test assays and result in false negative test results. This would tend to overestimate any effect CHX may have. Therefore, it is essential to neutralize remaining amounts of CHX in the sample before culturing.

The obturated bovine root specimens in the current study were lacking apical constriction and coronal restoration. This allowed for a potentially faster and

more complex washout of CHX during the storage period than allowed for in a clinical case. The fact that antimicrobial amounts of CHX remained bound to canal dentin after these long time periods is testament to the substantivity of CHX.

The dentin from which CHX was extracted presumably still contained high concentrations of CHX after the extraction process. However, assuming that equilibrium was reached between the dentin and the saline after being agitated for five hours, CHX in the dentin was estimated based on the weight of the dentin samples. These estimated dentin concentrations were for the E1 group 0.057% CHX, for the E2 group 0.025%, for the E3 group 0.018%, and for the E4 group 0.011%. It is likely, however, that owing to the substantivity of CHX the true dentin concentration of CHX may be substantially greater.

CHX has been suggested as an endodontic intracanal irrigant by a number of authors.[24,25,27-30,37,38] CHX is known to be particularly effective against many strains of bacteria found in infected root canals, including *E faecalis*.[24] In a study comparing common endodontic disinfectants, 0.5% CHX was also significantly more effective at killing *C albicans* than $Ca(OH)_2$, 5% and 0.5% NaOCl, and 2% IKI.[18] While these substantive and antimicrobial properties of CHX found here are promising, it does not have the tissue-dissolving properties of NaOCl.[39] Although NaOCl is still considered the irrigant of choice, the use of CHX may be considered advantageous as a treatment prior to obturation, an alternate irrigant during retreatments, or even incorporated into antimicrobial dressings.

REFERENCES

1. Sjögren U, Figdor D, Persson S, Sundqvist G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. Int Endod J 1997;30: 297-306.
2. Madison S, Wilcox LR. An evaluation of coronal microleakage in endodontically treated teeth, Part III in vivo study. J Endod 1988; 14:455-8.
3. Marosky JE, Patterson SS, Swartz M. Marginal leakage of temporary sealing materials used between endodontic appointments and assessed by calcium 45—an in vitro study. J Endod 1977;3:110-3.
4. Keller DL, Peters DD, Setterstrom J, Bernier WE. Microleakage of softened temporary restorations as determined by microorganism penetration. J Endod 1981;7:413-7.
5. Blaney TD, Peters DD, Setterstrom J, Bernier WE. Marginal sealing quality of IRM and Cavit as assessed by microbial penetration. J Endod 1981;7:453-7.
6. Anderson RW, Powell BJ, Pashley DH. Microleakage of three temporary endodontic restorations. J Endod 1988;14:497-501.
7. Chohayeb AA, Bassouny MA. Sealing ability of intermediate restorative used in endodontics. J Endod 1985;11:241-4.
8. Barthel CR, Strobach A, Briedigkeit H, Gobel UB, Roulet JF. Leakage in roots coronally sealed with different temporary filling materials. J Endod 1999;25:731-4.
9. Deveaux E, Hildelbert P, Neut C, Romond C. Bacterial microleakage of Cavit, IRM, TERM, and Fermit. J Endod 1999;25:653-9.
10. Kazemi RB, Safavi KE, Spångberg LS. Assessment of marginal stability and permeability of an interim restorative endodontic material. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1994;78:788-96.
11. Spångberg LSW, Acierno TG, Cha BY. Influence of entrapped air on the accuracy of leakage studies using dye penetration methods. J Endod 1989;15:548-51.
12. Byström A, Sundqvist G. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. Int Endod J 1985;18:35-40.
13. Byström A, Sundqvist G. Bacteriologic evaluation of the effect of 0.5% sodium hypochlorite in endodontic therapy. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1983;55:307-12.
14. Spångberg L, Engström Langeland K. Biologic effects of dental materials. 3. Toxicity and antimicrobial effect of endodontic antiseptics in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1973;36:856-70.
15. Messer HH, Chen RS. The duration of effectiveness of root canal medicaments. J Endod 1984;10:240-5.
16. Byström A, Claesson R, Sundqvist G. The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals. Endod Dent Traumatol 1985;1:170-5.
17. Sjögren U, Figdor D, Spångberg L, Sundqvist G. The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. Int Endod J 1991;24:119-25.
18. Waltimo TM, Ørstavik D, Siren EK, Haapasalo MP. In vitro susceptibility of *Candida albicans* to four disinfectants and their combinations. Int Endod J 1999;32:421-9.
19. Haapasalo M, Ørstavik D. In vitro infection and disinfection of dentinal tubules. J Dent Res 1987;66:1375-9.
20. Sundqvist G, Figdor D, Persson S, Sjögren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998;85:86-93.
21. Molander A, Reit C, Dahlen G, Kvist T. Microbial status of root filled teeth with apical periodontitis. Int Endod J 1998;31:1-7.
22. Winkler KC, Van Amerongen J. Bacteriologic results from 4,000 root canal cultures. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1959;12:857-75.
23. Nair PN, Sjögren U, Krey G, Kahnberg KE, Sundqvist G. Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a long term light and electron microscopic follow-up study. J Endod 1990;16: 580-8.
24. Ohara PK, Torabinejad M, Kettering JD. Antibacterial effects of various endodontic irrigants on selected anaerobic bacteria. Endod Dent Traumatol 1993;9:95-100.
25. White RR, Hays GL, Janer LR. Residual antimicrobial activity after canal irrigation with chlorhexidine. J Endod 1997;23:229-31.
26. Rölla G, Löe H, Schiøtt CR. The affinity of chlorhexidine for hydroxyapatite and salivary mucins. J Periodont Res 1970;5: 90-9.
27. Vahdaty A, Pitt Ford TR, Wilson RF. Efficacy of chlorhexidine in disinfecting dentinal tubules in vitro. Endod Dent Traumatol 1993;9:243-8.
28. Jeansonne MJ, White RR. A comparison of 2.0% chlorhexidine gluconate and 5.25% sodium hypochlorite as antimicrobial endodontic irrigants. J Endod 1994;20:276-8.
29. Komorowski R, Grad H, Wu XY, Friedman S. Antimicrobial substantivity of chlorhexidine-treated bovine root dentin. J Endod 2000;26:315-8.
30. Leonardo MR, Filho MT, Silva LAB, Filho PN, Bonaf 171 cio, Ito IY. In vivo antimicrobial activity of 2% chlorhexidine used as a root canal irrigating solution. J Endo 1999;25:167-71.
31. Sheikh W. Development and validation of a neutralizer system for in vitro evaluation of some antiseptics. Antimicrob Agents Chemother 1981;19:429-34.

32. Fitzgerald KA, Davies A, Russell AD. Uptake of ^{14}C-chlorhexidine diacetate to *Escherichia coli* and *Pseudomonas aeruginosa* and its release by azolectin. FEMS Microbiol Lett 1989;60:327-32.
33. Ørstavik D, Haapasalo MP. Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. Endod Dent Traumatol 1990;6:142-9.
34. Emilson GG. Susceptibility of various microorganisms to chlorhexidine. Scand J Dent Res 1977;85:255-65.
35. Haapasalo HK, Siren EK, Waltimo TM, Ørstavik D, Haapasalo MP. Inactivation of local root canal medicaments by dentin: an in vitro study. Int Endod J 2000;33:126-31.
36. Parsons GJ, Patterson SS, Miller CH, Katz S, Kafrawy AH, Newton CW. Uptake and release of chlorhexidine by bovine pulp and dentin specimens and their subsequent acquisition of antibacterial properties. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1980;49:455-9.
37. Ringel AM, Patterson SS, Newton CW, Miller CH, Mulhern JM. In vivo evaluation of chlorhexidine gluconate solution and sodium hypochlorite solution as root canal irrigants. J Endo 1982; 8:200-4.
38. Delaney GM, Patterson SS, Miller CH, Newton CW. The effect of chlorhexidine gluconate irrigation on the root canal flora of freshly extracted necrotic teeth. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1982;53:518-23.
39. Gordon TM, Damato D, Christner P. Solvent effects of various dilutions of sodium hypochlorite on vital and necrotic tissue. J Endod 1981;7:466-9.

Reprint requests:

Sidney Rosenthal, DDS, MDentSci
4300 Bayou Boulevard, Suite 11
Pensacola, FL 32503