Minimum Contact Time and Concentration of Sodium Hypochlorite Required to Eliminate *Enterococcus faecalis*

Bonnie Retamozo, DDS,MSD, Shahrokh Shabahang, DDS, MS, PhD, Neal Johnson, DDS, PhD, Raydolfo M. Aprecio, OD, and Mahmoud Torabinejad, DMD, MSD, PhD

Abstract

Introduction: The purpose of this investigation was to determine the concentration of sodium hypochlorite and the irrigation time required to disinfect dentin cylinders infected with *Enterococcus faecalis.* **Methods:** Four hundred fifty dentin cylinders (5 mm in diameter and 4 mm in height) with a lumen (2–3 mm in width) were prepared from freshly extracted bovine incisors. The cementum and predentin were then removed. The tubules were opened by using a 4-minute application with 17% ethylenediaminetetraacetic acid and 5.25% NaOCl and then exposed to *E. faecalis* (ATCC 4082) for 3 weeks in brain-heart infusion broth. The cylinders were then divided into 3 groups, and a 1.3%, 2.5%, or 5.25% concentration of NaOCl was applied in 5-, 10-, 15-, 20-, 25-, 30-, 35-, and 40-minute intervals for a total of 30 subgroups including positive and negative controls. Each test sample was placed into a tube of 2 mL brain-heart infusion broth and incubated for 72 hours. Absence of turbidity demonstrated no bacterial growth, whereas turbidity indicated presence of remaining viable bacteria. **Results:** The most effective irrigation regimen was 5.25% at 40 minutes, whereas irrigation with 1.3% and 2.5% NaOCl for this same time interval was ineffective in removing *E. faecalis* from infected dentin cylinders. **Conclusions:** High concentration and long exposure to NaOCl are needed for elimination of E. *faecalis* contaminated dentin. *(J Endod 2010;36:520–523)*

Key Words

Bovine teeth, *Enterococcus faecalis*, root canal disinfection, sodium hypochlorite

From the Department of Endodontics, School of Dentistry, Loma Linda University, Loma Linda, California.

Address requests for reprints to Mahmoud Torabinejad, DMD, MSD, PhD, Professor and Director, Endodontic Residency Program, Department of Endodontics, School of Dentistry, Loma Linda University, Loma Linda, CA 92350. E-mail address: mtorabinejad@llu.edu.

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Bacteria have long been recognized as the primary etiologic factors in the development of pulp and periapical lesions (1–3). The purpose of root canal treatment is the removal and eradication of bacteria from the root canal system and the prevention of reinfection. Reinfection or continued periapical inflammation can be caused by any number of bacteria found within the root canal system and dentinal tubules (4), although the actual number of viable bacteria required for this process is unknown. In failed endodontic cases, *Enterococcus faecalis* is the dominant species recovered (5–8). On the basis of *in vitro* studies, *E. faecalis* can remain viable in root-filled canals 12 months after surgery, and it is capable of penetrating into the dentin tubules up to 100 μm from the canal lumen (9, 10).

Among the procedures involved in the control of root canal infections, irrigation is an important process in eliminating microorganisms from the root canal system. Intracanal cleaning and disinfection procedures are highly dependent on the mechanical instrumentation and chemical effects of the irrigants used. The desirable qualities of an irrigant include the ability to dissolve pulp tissue, ability to remove the smear layer, and low toxicity while providing a bactericidal/bacteriostatic effect (11). Sodium hypochlorite (NaOCl) dissolves tissue (12–14) and is antibacterial (15). However it is unable to remove the smear layer (16).

NaOCl is the most commonly used root canal irrigant during chemomechanical debridement of root canals. Numerous publications have addressed the antimicrobial ability of NaOCl; however, the actual time necessary to completely eradicate *E. faecalis* with commonly used concentrations of NaOCl has not been determined (Table 1). Siqueira et al (17) evaluated the chemomechanical reduction of the bacteria by using 1%, 2.5%, and 5.25% concentrations of NaOCl. Baumgartner et al (18) compared the antimicrobial efficacy of 1.3% NaOCl/BioPure MTAD with 5.25% NaOCl/15% ethylenediaminetetraacetic acid (EDTA) as a root canal irrigant, and Berber et al (19) evaluated the efficacy of 0.5%, 2.5%, and 5.25% concentrations of NaOCl at reducing *E. faecalis* within root canals and dentinal tubules by using various instrumentation techniques. Clegg et al (20) examined the effect of exposure to 1%, 3%, and 6% concentrations of NaOCl on apical dentin biofilms, and Vianna and Gomes (21) assessed the efficacy of 1%, 2.5%, and 5.25% NaOCl alone and combined with chlorhexidine gluconate against *E. faecalis in vitro* by using an agar diffusion method. The irrigation or contact time of NaOCl used in these studies ranged from 2–30 minutes. The concentration of NaOCl in these same studies ranged from 0.5%–5.25%. To date, no general agreement exists regarding the optimal concentration of NaOCl or irrigation time necessary to eliminate bacteria from the canal system. The purpose of this study was to determine the minimum contact time and concentration necessary to effectively remove *E. faecalis* from dentinal tubules of infected bovine teeth.

Materials and Methods

Specimen Preparation

The infected dentin model used in this experiment was modified from that developed by Haapasalo and Orstavik (10). Freshly extracted bovine incisors were kept in 0.5% NaOCl overnight for surface disinfection. To standardize the size of the test specimens, dentin cylinders were prepared from the bovine teeth by removing the crown at

TABLE 1. Previous studies on the antimicrobial activity of NaOCl by using various concentrations and irrigation times

Study	Year	Concentration of NaOCl	Time
Siqueira et al	2000	5.25%	NA
Gomes et al	2001	5.25%	<30 sec to 30 min
Shabahang et al	2003	5.25%	10 min
Abdullah et al	2005	3.00%	2 min
Haapasalo et al	2005	1.00%	5 min
Berber et al	2006	0.5%, 2.50%, 5.25%	10 min
Clegg et al	2006	1.0%, 3.00%, 6.00%	15 min
Kho et al	2006	1.3%, 5.25%	2 min
Baumgartner et al	2007	5.25%, 1.3%	2 min
Metzger et al	2007	5.00%	10 min
Gomes et al	2009	1.0%, 2.25%, 5.25%	30 sec, 3 min, 5 min

the cementoenamel junction and the apical 3 mm by using a diamond disk rotating at 700 rpm under water cooling to create cylinders 5 mm in diameter and 4 mm in height. The cementum was removed with a cylindrical sandpaper disk at low speed. The predentin was removed with a high-speed diamond instrument, and the canal was enlarged to create a lumen 2–3 mm in diameter. The teeth and dentin cylinders were maintained in tap water during all procedures to prevent dehydration. The smear layer was removed by treatment in an ultrasonic bath with17% EDTA for 4 minutes followed by a 4-minute soaking in 5.25% NaOCl. The cylinders were then autoclaved in water for 20 minutes at 121°C.

Bacterial Inoculation

A single strain of *E. faecalis* (ATCC 4082), which is a gram-positive cocci, was cultured overnight in brain-heart infusion (BHI) broth. The dentin cylinders were then inoculated with this strain of *E. faecalis* in BHI broth. The dentin cylinders remained in aerobic conditions at 37 °C for 3 weeks, but the media were replaced weekly to ensure viability of bacteria.

Specimen Irrigation

All specimens were handled with sterile gloves and forceps under aseptic conditions. Fifteen cylinders were randomly assigned to 1 of the 30 subgroups (Table 2). The positive control consisted of 15 cylinders for each concentration irrigated with distilled water. The negative control was composed of 15 cylinders for each concentration irrigated with distilled water and autoclaved. On the day of the experiment, fresh NaOCl solution was diluted to yield accurate concentrations of 1.3%, 2.5%, and 5.25%. The remaining specimens were divided into 24 groups with 15 specimens in each group. Each concentration of NaOCl was tested at 5, 10, 15, 20, 25, 30, 35, and 40 minutes. The test irrigant was removed from the test samples, and residual NaOCl was inactivated by rinsing the dentin cylinders with 2 mL of a prepared 5% sodium thiosulfate solution. Each test sample was then removed, placed on sterile gauze, and allowed to air-dry under sterile conditions.

Growth Determination

Each test sample was placed in a tube of 2 mL BHI broth and incubated for 72 hours. Absence of turbidity demonstrated no bacterial growth, whereas turbidity demonstrated remaining viable bacteria. All samples were visually examined by 2 examiners to determine turbidity. All non-turbid test samples were vortexed and plated 25 μL onto BHI plates. Three positive and 3 negative control specimens were also plated on BHI plates. The plates were incubated for 24 hours and visually examined for the presence or absence of bacterial growth. Presence of *E. faecalis* was confirmed by visualization of individual white pinpoint colonies on the agar plates and microscopic observation of Gram stain to confirm the presence of gram-positive cocci.

Results

All positive controls showed turbidity, whereas none of the negative controls showed any. All positive controls demonstrated growth with gram-positive cocci, whereas none of the negative control demonstrated growth on the BHI plates. All 120 samples tested with 1.3% NaOCl demonstrated turbidity regardless of the application time. One hundred thirteen of 120 samples tested with 2.5% NaOCl demonstrated turbidity when used for less than 40 minutes, whereas only 8 of 15 (53.3%) tested at 40 minutes demonstrated turbidity. When samples were tested with 5.25% NaOCl, turbidity was found in all samples tested at 5-, 10-, and 15-minute application times. Samples tested at 20, 25, 30, and 35 minutes demonstrated turbidity in 11 of 15, 9 of 15, 6 of 15, and 4 of 15, respectively. All samples tested with 5.25% for 40 minutes remained clear and showed no evidence of turbidity (100%). None of the non-turbid samples demonstrated growth when plated on BHI agar. The data were analyzed by using the SPSS statistical software system (v. 17.0; SPSS Inc, Chicago, IL) by using χ^2, one-way analysis of variance (ANOVA). Statistically significant differences were found between the 5.25% NaOCl concentration and time frames used in this study. Statistically significant differences were also found between the 2.5% and 5.25% concentrations of NaOCl and the positive control group (saline solution). NaOCl at 5.25% showed a linear decrease in positive (turbid) samples as the contact time of the irrigant increased (Fig. 1).

TABLE 2. Irrigation regimen on the treatment groups

Contact time (min)	Treatment groups (n = 15)		
5	1.3% NaOCl	2.5% NaOCl	5.25% NaOCl
10	1.3% NaOCl	2.5% NaOCl	5.25% NaOCl
15	1.3% NaOCl	2.5% NaOCl	5.25% NaOCl
20	1.3% NaOCl	2.5% NaOCl	5.25% NaOCl
25	1.3% NaOCl	2.5% NaOCl	5.25% NaOCl
30	1.3% NaOCl	2.5% NaOCl	5.25% NaOCl
35	1.3% NaOCl	2.5% NaOCl	5.25% NaOCl
40	1.3% NaOCl	2.5% NaOCl	5.25% NaOCl
	Positive control	Positive control	Positive control
	Negative control	Negative control	Negative control

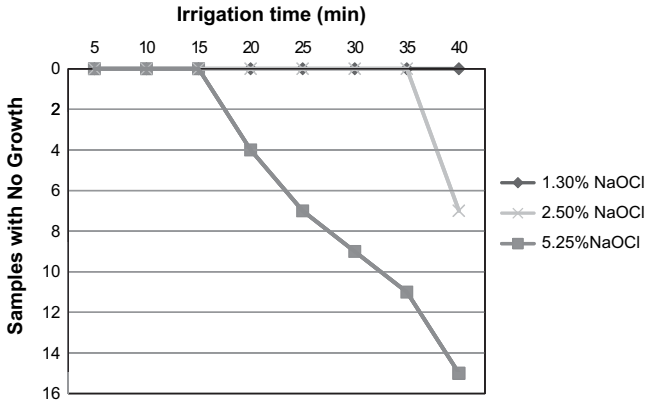

Figure 1. Results for 3 concentrations of NaOCl with irrigation times of 5–40 minutes.

Discussion

The intent of the current study was to determine the minimum application time and concentration of NaOCl required to remove *E. faecalis* from the dentin cylinders prepared from bovine teeth. Our design allowed direct comparison of each concentration of NaOCl at specific time frames while eliminating other confounding variables such as root canal system complexities. The present model with bovine teeth has been proved effective, allowing standardized reproducibility (10). Also bovine incisors are readily available, and their morphology is similar to that of human teeth. Bovine dentinal tubules are of similar size and density to those of human teeth (22). However, human root dentin is mostly atubular in the apical area and impermeable to bacteria in that region (23, 24). An ideal model would be forming a biofilm in a protein-rich environment that would reflect the true nature of root canal infections (25). The disadvantage of such a model is the potential for introduction of some uncontrolled variables.

E. faecalis (ATCC 4082) was chosen as the primary test organism because it is the dominant species recovered in failed endodontic cases (5–8), and it has been used in previous experiments (26, 27).

The minimum irrigation time frame of 5 minutes was determined on the basis of previous studies with NaOCl (26, 27). The maximum irrigation time was determined after multiple pilot studies that demonstrated a minimum of 25 minutes was needed to achieve some nonturbid samples with 5.25% NaOCl.

An important limitation of many studies when evaluating the endodontic microbiota refers to sampling procedures. The visual turbidity model was used in this study to detect remaining viable bacteria. This is an effective method for the detection of remaining viable bacteria in the dentin tubules. However, strict aseptic techniques are required to prevent contamination (26, 27). Paper points have been widely used for sampling, but it is acknowledged that bacteria located in other regions of the entire root canal system (including ramifications, dentinal tubules, isthmi, irregularities, and some untouched areas of the main canal) can pass unnoticed by this identification method (11). Thus, bacterial sampling with paper points only detects the microorganisms that are present in the main root canal, whereas the bacteria that are located inside the dentin tubules are inaccessible (11). Direct contact of an irrigant with a microbe is another method used to test the irrigant's ability to kill the microbe. However, it does not take into account the buffering of dentin or the complex root canal system (28).

Contemporary sampling techniques currently being used such as molecular assays are able to detect uncultivable microbes but have difficulty quantifying them. This lack of quantification can make it challenging to assess the role played by a microorganism in its environment (29).

The results of the present study showed that irrigation with 5.25% NaOCl for 40 minutes was the only regimen able to completely eliminate *E. faecalis*. These results are in agreement with the findings of Siqueira et al (17), Shabahang et al (27), and Metzger et al (30), showing the difficulty in eliminating *E. faecalis*. Siqueira et al infected teeth with *E. faecalis* and instrumented and irrigated them with 1%, 2.5%, and 5.25% NaOCl. Sodium hypochlorite significantly reduced the bacterial load but did not completely eliminate *E. faecalis* contamination (17). Shabahang et al showed that 1.3% and 5.25% NaOCl irrigation failed to consistently eliminate *E. faecalis*. However, samples exposed to MTAD for 5 minutes in addition to either concentration of NaOCl irrigant showed no turbidity and no bacterial growth in the dentin shavings (27).

Many factors can allow survival of *E. faecalis* in teeth. These factors include bacterial starvation (30), short exposure to irrigants (28, 31, 32), lack of instrumentation after contamination, and reduction in the efficacy of the irrigant. The time and concentration of the irrigant play a significant role in the ability of an irrigant to eliminate bacteria (31). The present experiment tested each concentration of NaOCl with contact times ranging from 5–40 minutes. In the present model, the dentin cylinders were prepared before contamination, thus allowing the results to reflect the effects of chemical irrigation without the contribution of mechanical debridement. In bovine teeth, *E. faecalis* can penetrate to a depth of 800–1000 μm after 3 weeks of incubation when the cementum is absent (10). The presence of cementum prevents the penetration of bacteria from the cementum side (4, 10). The present study called for the removal of the cementum as well as the predentin. NaOCl might disinfect to a depth of 200–300 μm into the tubules (33). Thus, it is possible to have bacterial penetration into the tubules much deeper than NaOCl can reach. Further research has shown that the interaction of the irrigant and microbe not only kills the microbe but also weakens the irrigant (28). Some have theorized that the resistance of *E. faecalis* to the action of the medicament might depend in part on the denser tubular infection by the organism (33).

Results from previous studies have shown that short exposure times to NaOCl do eliminate *E. faecalis* entirely (28, 31, 32), which contradict the findings of the present study. Haapasalo and et al (28) showed that in the absence of dentin powder, 1% NaOCl kills *E. faecalis* in less than 5 minutes. By using cell culture plates, Gomes et al (31) inhibited the growth of *E. faecalis* with 0.5% NaOCl after 30 minutes of contact time and reduced this time to less than 30 seconds with 5.25% NaOCl. Abdullah et al (32) eliminated 48-hour-old *E. faecalis* biofilm on a membrane dipped into the test solution and in a cell dense with planktonic formations by using 3.0% NaOCl within 2 minutes. The differences found among these studies and the present investigation are most likely due to the inhibitory effects of dentin on NaOCl (28), reduction of direct contact between irrigant and *E. faecalis* as a result of tubule infection, and the lack of instrumentation. Another difference between this study and other published reports (28, 34, 35) was the use of 5% sodium thiosulfate to deactivate the antimicrobial activity of NaOCl. Also, the strain of *E. faecalis* used could have had a significant impact on the results of other studies. For instance, genetic mutations might occur as a result of serial *in vitro* passages in laboratory conditions, leading to the emergence of genotypic and phenotypic differences between laboratory reference strains and clinical isolates of the same species (36).

Conclusion

The current study model allowed for standardization of the procedure while eliminating confounding variables. A key finding of this study

was the evidence that irrigation with 1.3% or 2.5% NaOCl is ineffective in eliminating this strain of *E. faecalis* (ATCC 4082) in bovine teeth at less than 40 minutes. On the other hand, 5.25% NaOCl was 100% effective at 40 minutes. Clinically, actual root canal systems are more complex (37). In addition, technological advances have made procedures more efficient, leading to reduced irrigation contact time (38). On the basis of the findings of this study, it appears that high concentration and long exposure to NaOCl are needed for elimination of E. *faecalis* contaminated dentin.

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