Clinical and Experimental Health Sciences

Antimicrobial Effectiveness of Hypochlorous Acid in Infected Dentin Tubules: A Pilot Study

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ABSTRACT

Objective: To assess the disinfection capacity of HOCl, NaOCl, and CHX on dentinal tubules.

Methods: Enterococcus faecalis suspension was supplemented to the dentin blocks. The groups were created according to the irrigation solution, 2.5% NaOCl was used in Group 1, 2% CHX in Group 3, and 200 ppm HOCl in Group 3. All the irrigants (50 µL) were dropped on dentin for 30 seconds, 1 min, and 3 min. Four samples were selected for each solution group to form its control group. To observe the bacterial growth 10 ml of a sample taken from the tubes was cultivated on Mueller-Hinton agar. After the incubation, the total number of colonies was determined.

Results: In Group 1, the number of colonies in the samples taken for all three-time intervals was 0, and the solution efficiency was found to be 100%. In Group 2, the success rate was 97.4%, 99.2%, and 99,9% for 30 sec., 1 min, and 3 min, respectively. In Group 3, the success rate was 63%, 86.3%, and 93,4% for 30 sec., 1 min, and 3 min, respectively.

Conclusion: HOCl has a success rate of antimicrobial effect of more than 90% at the end of the 3 min duration on dentinal tubules.

Keywords: Antimicrobial effect, dentin tubules, hypochlorous acid, ırrigation solution.

1. INTRODUCTION

Biofilm formation was observed in the root canal system due to pulp necrosis, leading to dentin infection. (1). Mechanical methods are usually used to remove the biofilm during root canal treatment, however, the high complexity of the root canal anatomy such as lateral canals and isthmuses decreases the efficiency of endodontic instruments (1). To obtain a disinfected root canal system without bacterial accumulation, various endodontic irrigation solutions especially chlorine-containing solutions such as sodium hypochlorite (NaOCl) are used during root canal treatment (2).

Hypochlorous acid (HOCl) dissociated into H^+ and OCl^- ions in an aqueous solution is accepted as a powerful oxidizing compound. HOCl destroys viruses by denaturation and aggregating proteins with nitrogen-centered radicals (3). Inactivation or destruction of microbes exists with the disinfection mechanism including the breaking down of the cell wall of microbes or viruses (4,5). Currently, various forms of hypochlorous acid solutions are suggested as a disinfectant because of their ideal disinfectant properties such as being non-toxic to surface contact, non-corrosive and inexpensive for coronaviruses in oral and maxillofacial surgery offices (6). Therefore, hypochlorous acid-containing solutions as recommended by the United States Environmental Protection Agency (USEPA) against COVID-19 (7). HOCl solutions are considered one of the ideal disinfectants due to being inexpensive, easy to use, quickly effective in large areas, and having a wide range of bactericidal and viricidal effects based on concentrations above 50 ppm at least 3 min of contact time (6).

The in vitro replication of bacterial invasion in dentinal tubules usually evaluated with cultural methods has been investigated for more than a century (8). In these methods, the root canals of extracted teeth or prepared dentin blocks were used as media for the growth of bacteria. Bacterial penetration for up to 500 mm can be observed in dentinal tubules using the dentin block model for nearly 2 decades

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in antimicrobial efficacy studies (9). Recently, confocal laser scanning microscopy (CLSM) helped many researchers to visualize both live and dead bacteria within infected dentin, and it has been accepted as a suitable method (10-14). However, there are still some roadblocks to collecting specimens from dentin tissue containing heavy and equal bacterial load, as simulating clinical infection using traditional in vitro methods is known to be difficult (15).

To knowledge there is no previous study evaluating the antimicrobial activity of HOCl solution as an irrigation solution in endodontic treatment. The purpose of this study was to assess the disinfection capacity of HOCl, sodium hypochlorite (NaOCl), and chlorhexidine (CHX) on dentinal tubules.

2. METHODS

2.1. Preparation of Dentin Block

Thirty extracted human mandibular premolar teeth were used in this study according to the protocol approved by the Research Ethics Committee of the Faculty of Medicine Akdeniz University (KAEK-904). The teeth were stored in 0.01% NaOCl solution before use to prevent dehydration. The coronal part of the teeth was separated from the root by a diamond disk and root canal preparation was finished with Protaper Next X3 rotary file. Each cylindrical dentin block was sectioned vertically and the cement layer was removed to obtain standard dentin blocks with the size of 4x4x2 mm described as in the previous study (16). A total of 60 dentin blocks were obtained and four of them were excluded from the study due the distruption of the dentin blocks. Each sample was irrigated with 17% EDTA solution for 1 min for smear layer removal. After irrigation with sterile water for 10 min, the outer layer of dentin blocks was covered by resin composite. The samples were sterilized by autoclaving for 20 min at 121 ° C. The sterility of the dentin blocks was checked by incubating each sample for 24 hours at 37 ° C in 5 ml of brain heart infusion agar.

2.2. Dentin Infection with Enterococcus faecalis

Enterococcus faecalis (E. faecalis) ATCC 29212 was cultured (100 µL of seeding was done) on brain heart infusion agar (Becton-Dickinson, Sparks, MD) at 35°C ± 2°C for 18 to 20 h. After incubation, the bacteria were suspended in brain heart infusion broth (BHI) (Becton-Dickinson, Sparks, MD) and the spectrophotometric standardization method was used to provide $3X10^6$ colony-forming units (CFU)/mL (OD405=0.05).

E. faecalis ATCC 29212 suspension of 500 µl was supplemented to the dentin specimen in each eppendorf tube and centrifugation was repeated twice at 5600 g for 5 min. A fresh bacterial solution was added in tubes between each centrifugation. And after centrifugation, the solution that penetrated the dentin blocks was discarded to facilitate bacterial recovery. All dentin blocks were then incubated in BHI broth at 37 ° C for 18-24 hours. The same procedure was repeated 2 days later. All samples were incubated at 37 ° C for a total of 5 days. Randomly chosen 5 samples were separated for SEM examination to verify the presence of bacteria in dentin tubules. For the randomization, the random number generator was used.

2.3. SEM Examination

Before disinfection protocol, one dentin block from each group was selected for SEM examination to verify the presence of *E. faecalis* ATCC 29212 in dentin tubules. Glutaraldehyde (2.5%) and osmium tetroxide (1%) were used in the fixation procedure for 30 min and 1 hour, respectively. The samples were dehydrated using ethanol and sputter-coated with gold-palladium in a vacuum evaporator (Polaron SC7620, Quorum Technologies Ltd., UK). The penetration of *E. faecalis* in dentin tubules was observed by SEM (LEO-1430 VP SEM system, Carl Zeiss AG, Jena, Germany)) at a magnification of 5000-7000x operating at 15.00 kV**.** (Figure 1).

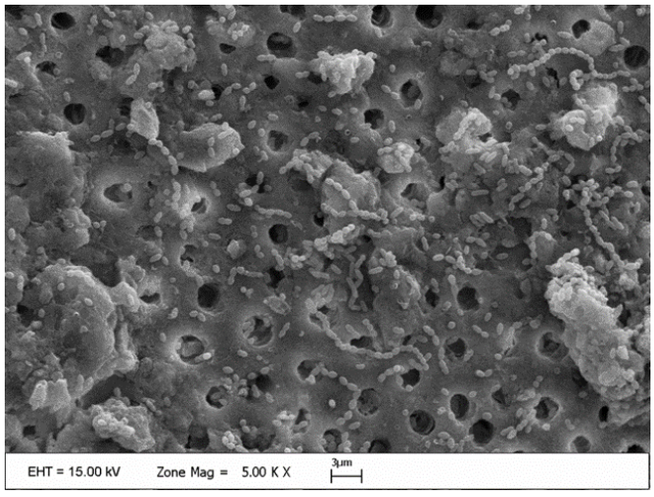

Figure 1. *SEM image of infected dentin surface and occluded by E. faecalis after centrifugation.*

2.4. Dentin Disinfection

At the end of the incubation period, each tooth was removed from Eppendorf tubes and washed with sterile distilled water for one min, and left to dry in sterile petri dishes. A total of 60 samples were divided into three groups according to the irrigation solution used for the disinfection procedure.

2.5. Application of Irrigation Solution

Four samples were selected for each group to form its control group. Only sterile saline solution was applied to these samples and the mean value was calculated. Sodium hypochlorite solution (2.5% NaOCl) was used as an irrigation solution in Group 1, and 0.2% Chlorhexidine (CHX) (Klorhex, Drogsan Pharmaceutical Ind. And Trade Inc), and 200 ppm HOCI were the other irrigation solutions for Group 2 and Group 3. HOCl solution was prepared by using Toucan

Eco Active (ECA Australasia Pty Ltd., Australia) according to manufacturer's recomendation. All the irrigants were dropped on the inner side of the dentin wall as a droplet of 50 µL for 30 sec, 1 min, and 3 min. 2.5% NaOCl solution was obtained from 5% NaOCl (Wizard, Wizard, RehberKimya, Istanbul, Turkey) by diluting 1:1 with distilled water.

The samples were then irrigated with sterile saline solution for 1 min and one sample was chosen from each group (sample with 3 min process) for confocal laser scanning microscope (CLSM) examination. The remaining samples are placed in tubes containing 1 ml of sterile saline to agitate the samples using a Vortex mixer (Onilab LCC, California, USA) for 1 min Then, 100 µl of bacterial suspension prepared in 1/100 dilution was added to tubes containing 4 ml Muller Hinton Agar (MHA), and kept in a 60 °C water bath, and were mixed and spread on MHA plates. The plates were incubated at 35°C ± 2°C for 18 to 20 h. At the end of the incubation period, the colonies that proliferated in each petri dish were counted. The results were multiplied by 10^3 to get the number in 1 ml. After the experiment, the numbers of colonies formed in the petri dishes for each disinfectant and the application period are indicated in the tables. The samples chosen for CLSM examination for visualization of damaged and survived bacteria cells were stained with the SYTO 9/propidium iodide (LIVE/DEAD, BacLigth; Invitrogen, Eugene, OR) as previously reported (1). The samples were observed using a confocal laser scanning microscope (ZEISS LSM 800, Jena, Germany) with 20X magnification at a resolution of 1024x1024 **(**Figure 2).

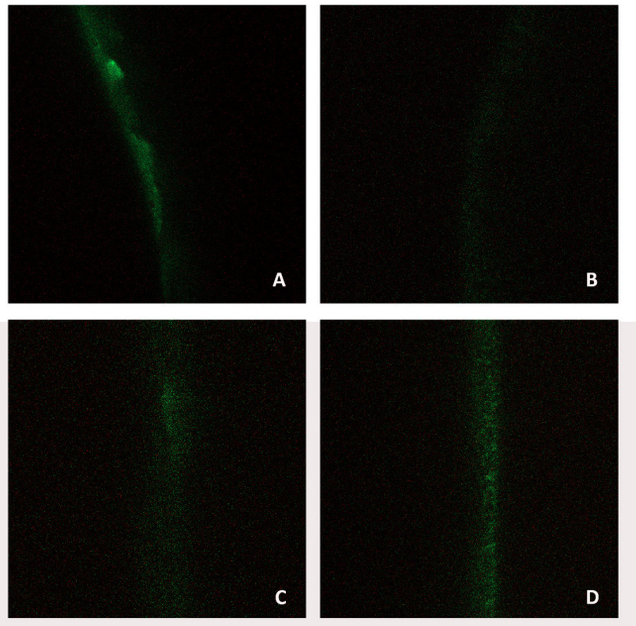

Figure 2. *Representative CLSM images obtained by staining with cell viability stain after the application of different solutions for 3 min.* ***(A)*** *Control (NaCl),* ***(B)*** *2.5% NaOCl,* ***(C)*** *2% CHX,* ***(D)*** *200 ppm HOCl.*

3. RESULTS

For each solution used in the study, 100 µL of seeding was done and the average colony values found at 30 seconds and first min, and the third min in 1 mL were shown in Table 1 in detail.

Table 1. *The mean and standart deviation values of colonies found at 30 second, first minute and third minute when 100 µL of seeding was done in 1 mL.*

	Group 1 (2.5% NaOCl)				Group 2 (2% CHX)				Group 3 (200 ppm HOCl)			
	Control	*30 sec.*	*1 min*	*3 min*	*Control*	*30 sec.*	*1 min*	*3 min*	*Control*	*30 sec.*	*1 min*	*3min*
Colony numbers x 10$^{3)}$	10.10^4	0	0	0	35.10^4	910^3 16710^2	2510^2 2710^2	$2,510^2$ 510^2	40.10^4	14810^3 11810^3	5510^3 39.210^3	26.310^3 13.310^3
Inactivated Factor (IF) Ncontrol / Nsample	-	100000	100000	100000	-	38,88	140	1400	-	2,7	7,27	15,2
inactivated	-	5	5	5	-	1,58	2,14	3,14	-	0,43	0,86	1,81
Efficency *100/Ncontrol	-	100%	100%	100%	-	97.4%	99.2%	99.9%	-	63%	86.3%	93.4%

**NaOCl: sodium hypochlorite, CHX: Chlorhexidine, HOCl: Hypochlorous acid, Sec.: Second, Min.: Minute.*

Number of colonies were determined at specific time intervals (30 seconds and first min, and the third min) following the application of the irrigation solution. The success rates of the solutions were calculated by comparing the number of colonies obtained in the experimental group with those in the control group. In Group 1 (%2.5 NaOCl), the number of colonies in the samples taken for all three-time intervals was 0, and the solution efficiency was found to be 100%. In Group 2 (2%CHX) the success rate was 97.4%, 99.2%, and 99,9% for 30 sec., 1 min, and 3min, respectively. In Group 3 (200 ppm HOCl) the success rate was 63%, 86.3%, and 93,4% for 30 sec., 1 min, and 3min, respectively. Because this study was a pilot study with an insufficient sample size for statistical analysis, no analyses were performed.

4. DISCUSSION

In this study the disinfection capacity of HOCl, NaOCl, and CHX on dentinal tubules was evaluated by using culture method. Furthermore, a singular biofilm model was preffered

in the current research to minimize any changes resulting from bacterial interactions and to guarantee consistency and uniformity (13). According to the findings, HOCl demonstrates an antibacterial efficacy of 90% after a 3-minute exposure on dentinal tubules.

HOCl solutions became popular because of their easy access and use, quick effect in many areas, and wide range of bactericidal and viricidal effects based on concentrations above 50 ppm at least 3 min of contact time (6). There are various studies evaluating the effectiveness of HOCl both in inert surfaces and in vivo conditions. It has been shown that HOCl can inactivate several types of viruses including coronaviruses in less than one min (19). To decontaminate inert surfaces containing enteric viruses, HOCl at a concentration of 200 ppm is successful in a one min contact time (6). In an animal study, the systemic and gastrointestinal effects of ingesting HOCl, from the perspective of its use in mouthwash, were evaluated and there are no abnormalities in histopathological and enamel roughness tests and systemic effects (18). Also the effectiveness of HOCl in cleaning biofilm-contaminated implant surfaces by reducing the lipopolysaccharide concentration of *P. gingivalis* (20). In this study, the disinfection capacity of HOCl, NaOCl, and CHX solutions on dentinal tubules were evaluated.

Wang et. al (21) showed that over 60% of E.faecalis cells in a 1-day-old biofilm inside dentin were eliminated by 6% NaOCl in 3 minutes. In contrast, less bacteria were killed when 3-week-old biofilms in dentin were exposed to the same solution (21). Furthermore, a prior study indicated that to replicate the clinical environment challenge, the bacteria were cultured for 3 weeks to establish mature biofilm formation (13). Ma et al.(16) suggested that a 24-hour incubation time following centrifugation allowed bacteria to potentially recover from any damage they had incurred. Furthermore, histologic sections stained using the Brown and Brenn method and scanning electron microscope (SEM) examinations have revealed that only a small number of dentinal tubules are infiltrated by bacteria even after an extended period of incubation (23). In the present study, all samples were incubated for a total of 6 days and the presence of bacteria in dentin tubules was verified by SEM examination.

Various methods have been used to evaluate the effectiveness of endodontic irrigation solutions. The dentin block model was one of the first and most widely used methods for an in-vitro examination to evaluate the antimicrobial efficacy of irrigation solutions (16, 22, 24). Ma et. al (16) evaluated the effectiveness of several disinfecting solutions by using a noninvasive CLSM method and found that NaOCl and Qmix had higher antimicrobial effects deep into dentin tubules than the other solutions. In a previous study, the antimicrobial activity of a 2.5% NaOCl/9% etidronic acid (HEBP) solution on *E. faecalis* was assessed and it was shown that HEBP did not affect the activity of NaOCl to eliminate *E. Faecalis* located in dentinal tubules (22). In the present study, the dentin blocks were contaminated with *E. faecalis* and the effectiveness of the solutions was evaluated by using culturing method.

The successful and long prognosis of the teeth with endodontic treatment is widely dependent on the disinfection of pathogen microorganisms and the elimination of bacterial biofilm from the root canal system. *E. faecalis* which is a facultative anaerobe is one the most commonly isolated bacteria from the root canal system of unsuccessful endodontic cases. *E. faecalis* in the form of biofilm The extracellular polysaccharide matrix produced by biofilm, makes the bacteria more resistant to antimicrobial agents (25). NaOCl is the most effective irrigating solution with its strong disinfection property and dissolution capacity of necrotic debris and other organic materials (26). However, its cytotoxic effect and causing metal corrosion, bad taste and smell noticed by the patient during root canal treatment, risk of injury to the oral mucosa, skin, and eye area, obstruction, and sensitivity of the airway are the main risks and disadvantages of NaOCl (26, 27).

Chlorhexidine is also a popular irrigation solution in endodontics with its property of broadspectrum antimicrobial effect. The previous study (28). showed that CHX exhibited a well antibacterial effect on *E. faecalis* followed by NaOCl. However, there was no agreement on the antimicrobial efficacy of CHX against NaOCl in recent studies because of the contradictory results (29). Therefore, although CHX has considerably lower toxicity than NaOCl (30), in the previous studies it was shown that CHX is a cytotoxic agent irrelevant to the cell type by disrupting the cell membranes (31, 32). The cytotoxic effects on stem cells from human exfoliated deciduous teeth (SHED) cells were also demonstrated by using CHX as an irrigation solution in different concentrations (32). It was known that, at an effective antimicrobial concentration range, HOCl which is produced by immune cells has a lower cytotoxic effect on mammalian cells when compared with NaOCl and H_2O_2 (33).

The efficacy of HOCl on *Staphylococcus aureus* growing as a biofilm on glass (34), *S. epidermidis and P. aeruginosa* biofilms (35), biofilms in chronic wounds (36) and bacteria in biofilms formed by tested ocular pathogens (37) were indicated in the previous studies. However, there is not any study that evaluates the disinfection capacity of HOCl on dentinal tubules. In the present study, at the end of the waiting period of 3 min, it was observed that HOCl showed similar antimicrobial activity to CHX. In the previous studies, the inactivation capacity of 200 ppm HOCl against coronavirus, noroviruses, and other enteric viruses in less than 1 min was shown (18, 38). This can be explained by the fact that biofilms reduce the effectiveness of antimicrobial agents.

In the previous review article, it was shown that although the neutral chlorine-containing solutions have an antimicrobial effect, this antimicrobial capability is lower than NaOCl concentrations used in daily practice in endodontic treatment (39). Furthermore, the modification of pH values below 7.5 decrease the dissolution capability of organic tissue (39). The other usage of HOCl in dentistry is as a chemotherapeutic

agent for the treatment of periodontitis (40). HOCl plays an important role that activating tyrosine kinase signaling cascades, generating an increase in the production of inflammatory mediators, and growth factors (40). Chen et. al (20) evaluated the antibacterial activity of HOCL, NaOCl, and CHX on the titanium alloy surfaces of biofilm-contaminated implants. It was indicated that all these irrigants showed an antibacterial effect and killed the majority of bacteria. However, HOCl significantly lowered the LPS concentration of P. gingivalis when compared with the other irrigants. Besides all the advantages of HOCL, the difficulty of storage, short expiration date, and having a proper generator to possess HOCl are the disadvantages of this strong oxidizing agent (41).

Various approaches, such as molecular techniques, colorimetric techniques, bioluminescence, , and microscopic techniques (SEM, and CLSM) are employed to assess the antibacterial efficacy of different disinfection protocols in endodontics (42). Nevertheless, these approaches have some limitations. Morphological and structural features of microbial biofilms can be identified by capturing high-resolution images of the root canal surface using SEM analysis, however it is not possible to ascertain the viability of the bacterial cells (38). Furthermore, SEM analysis does not allow for the visualization of the multi-layered biofilm in three dimensions. Fluorescent dyes in CLSM analysis enable visualization of the three-dimensional structure and geographical distribution of biofilm (14). They also facilitate the assessment of quantitative parameters including the ratio of live to dead bacteria and biofilm bio-volume (14). However, previous suggestions indicate that the contents of root canal samples may contain autofluorescent and detrital components that could be mistaken for bacteria. Additionally, background fluorescence was sometimes seen in the canal lumen (16). In addition, using culture methods to evaluating the antimicrobial effect of an irrigation solutions on dentin blocks have some limitations such as that the exact part of the root canal space in which the recovered bacteria comes from can not be determined; the survived bacteria remained from residual biofilms attached to dentinal tubules on root canal walls.

5. CONCLUSION

Within the limitations of this in vitro model, HOCl has a success rate of antimicrobial effect of more than 90% at the end of the 3 min duration on dentinal tubules that biofilm-contaminated dentin discs. Further studies evaluating the disinfection capacity and antimicrobial effect of HOCl for endodontic treatment are needed. Therefore, the use of HOCl in regenerative endodontic treatments, which are frequently applied in today's endodontics, should be investigated by searching the cytotoxic effect on stem cells.

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