


RESEARCH ARTICLE

Dentine tubule disinfection by different irrigation protocols

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Abstract

The purpose of this study was to test the antimicrobial activity and the smear layer removal of different irrigation protocols—sodium hypochlorite (NaOCl), NaOCl followed by ethylenediaminetetraacetic acid (EDTA), and NaOCl combined with etidronic acid (HEBP)—against infected dentine tubules during root canal preparation. Single rooted premolars contaminated with *Enterococcus faecalis* were chemomechanically prepared. Depending on the irrigation protocols, the roots were divided into the following groups: (1) distilled water during and after instrumentation; (2) 2.5% NaOCl during and after instrumentation; (3) 2.5% NaOCl/9% HEBP during and after instrumentation; and (4) 2.5% NaOCl during instrumentation followed by 17% EDTA after instrumentation. The percentage of dead cells and the biovolume in infected dentine tubules were measured using confocal laser scanning microscopy and the live/dead technique. Smear layer removal on root canal wall surfaces was observed by means of scanning electron microscopy. Results were compared through parametric tests ($p < .05$). The groups NaOCl, NaOCl/HEBP, and NaOCl+EDTA exerted the highest antimicrobial activity ($p > .05$), followed by the group irrigated with water. All the irrigation protocols—including water—significantly reduced the bacteria biovolume. No dentine tubules free of smear layer were found in the positive control or the 2.5% NaOCl group. With NaOCl/HEBP and NaOCl+EDTA, respectively, $90.41\% \pm 7.33$ and $76.54\% \pm 15.30$ of dentine tubules were free of smear layer ($p = .01$). NaOCl/HEBP and NaOCl+EDTA exerted an important antimicrobial activity against bacteria inside dentine tubules, lowering the bacteria biovolume and eliminating a high amount of the smear layer, particularly in the NaOCl/HEBP group.

KEYWORDS

dentine tubule disinfection, etidronic acid, root canal irrigants, sodium hypochlorite

1 | INTRODUCTION

Persistent endodontic infections are mainly caused by bacteria that survive the root canal treatment. The survival of bacteria may be partly attributed to their location in areas unaffected by instruments and antimicrobial agents, such as lateral canals, isthmuses and apical deltas (Nair, Henry, Cano, & Vera, 2005) or the bacterial invasion of dentine tubules (Vieira, Siqueira, Ricucci, & Lopes, 2012). For this reason, a main goal of endodontic treatment is to reduce the bacterial bulk in the entire root canal system to levels compatible with healing (Siqueira & Rôças, 2008).

Dentine tubule infection reportedly occurs in over 70% of teeth with primary apical periodontitis (Matsuo et al., 2003). In the clinical scenario, bacteria trapped in the smear layer generated by the action

of instruments tend to eventually become packed into these areas of difficult access. Antimicrobial strategies for intratubular disinfection should therefore be included in root therapy for long-term clinical success.

Traditional treatment involves the sequential use of root canal irrigants with different modes of action against the organic and inorganic matter. Although there is no clear consensus on the sequence of irrigation, the most common protocol is to use sodium hypochlorite (NaOCl) during mechanical preparation to dissolve the organic matter and kill microorganisms, then a strong chelating agent like ethylenediaminetetraacetic acid (EDTA) to remove the smear layer inorganic components and to promote sealer penetration into dentinal tubules (Baca, Junco, Arias-Moliz, González-Rodríguez, & Ferrer-Luque, 2011; Jardine et al., 2016; Mancini, Cerroni, Iorio, Dall'Asta, & Cianconi,

2017; Zehnder, 2006). Alternatively, the use of a weak chelating agent such as etidronic acid (HEBP) (Tartari et al., 2017; Ulusoy, Zeyrek, & Çelik, 2017; Zehnder, Schmidlin, Sener, & Waltimo, 2005) in combination with NaOCl has been proposed as a single irrigating solution to be used during root canal preparation and as the final solution. The antimicrobial and organic dissolution properties of this combined solution are similar to those of NaOCl alone (Arias-Moliz, Ordinola-Zapata, Baca, Ruiz-Linares, & Ferrer-Luque, 2014; Tartari et al., 2015; Zehnder et al., 2005), while its advantages during instrumentation are reduced smear layer formation (Lottanti, Gautschi, Sener, & Zehnder, 2009) and debris accumulation (Paque, Rechenberg, & Zehnder, 2012).

In order to study the antimicrobial activity of irrigants against infected dentinal tubules, a dentine infection model entailing centrifugation was introduced to create a homogeneous presence of bacteria inside tubules (Ma, Wang, Shen, & Haapasalo, 2011). Using this methodology, different irrigation protocols that include NaOCl followed by a chelating agent and NaOCl combined with HEBP have demonstrated adequate antimicrobial activity against infected dentine tubules (Morago et al., 2016; Wang, Shen, & Haapasalo, 2013). This technique does not account for the action of instruments during endodontic preparation, however. In fact, to date there are no published data regarding the effect of different irrigation protocols employed during mechanical treatment on the bacteria inside dentine tubules. The aim of this study was, therefore, to test the antimicrobial activity and the smear layer removal of irrigation protocols including NaOCl, NaOCl followed by EDTA, and NaOCl combined with HEBP, against infected dentine tubules during root canal preparation. The null hypothesis was that there is no difference in the intratubular disinfection achieved by the different experimental irrigation protocols, and the smear layer does not influence their activity.

2 | MATERIALS AND METHODS

Sixty-six freshly extracted, mature, non-carious, single-rooted human premolars were selected and stored in 0.1% thymol solution at 4 °C until use. Immature teeth and teeth with caries, previous restorations and curved roots were excluded. The Ethics Committee for the use of human extracted teeth approved the protocol performed in the study (UGR-438).

The teeth were decoronated using an Accuton-50 machine (Struers, Copenhagen, Denmark) and the coronal part was reduced until each root specimen measured 12 mm in length. The working length was determined by subtracting 1 mm from this measurement. The outer cementum was removed with silicon carbide papers and each root canal was enlarged using stainless K-files up to size 25 (Dentsply Maillefer, Ballaigues, Switzerland) and distilled water as the irrigant. To allow the handling of roots during the experiment, a customized model of each was fabricated with polyvinyl siloxane impression material (Zhermack, Rovigo, Italy). Thereafter, cylindrical root segments 4 mm in length were obtained by sectioning the root horizontally from the coronal part. The root segments were then sectioned vertically into halves using a low-speed handpiece with a diamond disk (355,514,220 HP; Edenta AG, Au, St. Gallen, Switzerland). The smear layer formed during preparation of the specimens,

including the two halves of the coronal segments and the rest of the root, was removed with 5.25% NaOCl and 17% EDTA in an ultrasonic bath, for 5 min each. Finally, the specimens were washed with distilled water and sterilized by autoclave.

2.1 | Dentine infection

To contaminate the dentine tubules of the coronal segments, each half was placed in the upper chamber of a filter tube (VWR International Eurolab SL, Barcelona, Spain) with the canal side up, and gaps with the inner wall of the tube were sealed with flowable light cured composite resin (Tetric EvoFlow; Ivoclar Vivadent, Schaan, Liechtenstein). For dentine tubule infection, 500 µL of an *Enterococcus faecalis* suspension ATCC 29212 in BHI broth (Scharlau Chemie SA, Barcelona, Spain) of approximately 1×10^7 colony-forming units per milliliter was added to the upper chamber of each filter tube with the dentine specimen inside. The tubes were centrifuged at 1,400g, 2,000g, 3,600g, and 5,000g in sequence (twice each for 5 min) (Arias-Moliz et al., 2014). Between the centrifugations, the suspension was replaced with 500 µL of a fresh one. The same procedure was repeated after 2 days. The samples were incubated for a total of 5 days at 37 °C.

To infect the rest of the root, each was contaminated with 1.2 mL of the *E. faecalis* suspension in an Eppendorf tube for 5 days at 37 °C, with broth refreshment performed every 2 days.

2.2 | Antimicrobial test

The two halves of the coronal segments and the rest of the root of each specimen were taken out of the tubes and washed with saline solution for 1 min. Afterward, they were re-approximated using utility wax with the aid of tweezers. The apical foramina of each root was also sealed to create a closed-end system. The assembled specimen was then placed in the customized model. The specimens were randomly divided into four experimental groups ($n = 10$ /group) according to the irrigation protocol: Group 1: distilled water during and after instrumentation; Group 2: 2.5% NaOCl (Panreac Química SA, Castellar del Vallés, Spain) during and after instrumentation; Group 3: 2.5% NaOCl/9% HEBP (Cublen K8514 GR; Zschimmer & Schwarz, Mohsdorf, Germany) during and after instrumentation; and Group 4: 2.5% NaOCl during instrumentation followed by 17% EDTA (Merck, Darmstadt, Germany) after instrumentation. For the 2.5% NaOCl/9% HEBP association, both irrigants were prepared at double concentration and mixed in a 1:1 ratio. Ten specimens neither irrigated nor instrumented served as the positive control group.

The assembled specimens were instrumented using a series of Protaper Universal SX to F3 rotary files (Dentsply Maillefer, Ballaigues, Switzerland) to the working length, according to the manufacturer's instructions. The canals were irrigated at each file change with 1 mL of the irrigating solution for 1 min. After instrumentation the roots were irrigated with 3 mL of the final irrigating solution during 1 min. When the protocol included NaOCl, the canals were irrigated with 5 mL of 5% sodium thiosulfate for 5 min in order to inactivate it. All the root canals were then rinsed with 5 mL of 0.9% saline solution. The irrigation procedures were performed with a 27 G side-

vented needle attached to a 3-mL Luer lock syringe (Monoject, Covidien, Mansfield, MA).

After preparation, the coronal segments were removed from the customized model and the rest of the root was discarded. Each half was vertically cut into two quadrants through the root canal and they were washed with saline solution, stained, and observed under confocal laser scanning microscope (CLSM).

2.3 | CLSM analysis

For dentine disinfection analysis, the Syto-9/Propidium iodide (PI) technique (Live/Dead, BacLight, Invitrogen, Eugene, OR) was used (Ordinola-Zapata et al., 2012). Syto-9 is a green-fluorescent stain that labels both live and dead microorganisms. PI is a red-fluorescent nucleic acid stain and penetrates only the cells with damaged membranes (dead microbes). All the samples were observed using a CLSM (Nikon Eclipse Ti-E, Nikon Canada, Mississauga, Canada). Four microscopic confocal volumes from random areas were obtained from each sample (total of 40 operative fields per group) using a 40 \times oil immersion objective, 1 μ m step-size and a format of 512 \times 512 pixels. Each picture represented an area of 317 \times 317 μ m. The scanning was performed inside the dentine structure, 5–10 μ m from the subsurface level of the dentine, in order to obtain the fluorescence of bacteria unaffected by the sectioning procedure. For quantification purposes, *bioimage_L* (<http://www.bioimage.com>) software was used (Chávez de Paz, 2009). The parameters evaluated in each group were the percentage of red population (dead cells) and the total biovolume.

2.4 | Smear layer analysis

Four additional samples from each experimental group were contaminated and prepared chemomechanically as explained above. Thereafter, the halves of the coronal segments were separated from the specimens, fixed in 2.5% glutaraldehyde, desiccated, sputter-coated with gold, and viewed with scanning electron microscopy (SEM, Hitachi S-510; Hitachi Ltd., Tokyo, Japan) operated at 25 Kv. Three digital photomicrographs at \times 1,500 were taken of the canal walls of each sample for a total of 12 operative fields per group. The number of tubules without any smear layer were counted by two examiners. The

TABLE 1 Mean \pm standard deviation of the percentage of dead cells and the total biovolume of bacteria inside dentine tubules after irrigation with water, 2.5% NaOCl, 2.5% NaOCl mixed with 9% HEBP and 2.5% NaOCl followed by 17% EDTA

	Death percentage (%) ^a	Total biovolume (μ m ³) ^{**}
Positive control	20.75 \pm 13.85 ^a	3606.18 \pm 3234.95 ^a
Water	31.96 \pm 18.07 ^b	1517.73 \pm 981.80 ^b
2.5% NaOCl	72.52 \pm 16.99 ^c	1461.40 \pm 1593.89 ^b
2.5% NaOCl/9% HEBP	70.69 \pm 17.13 ^c	1344.68 \pm 1615.09 ^b
2.5% NaOCl +17% EDTA	79.86 \pm 14.62 ^c	940.30 \pm 943.84 ^b

Positive control: uninstrumented and unirrigated.

Read vertically, the same letters show differences that are not statistically significant by Tukey test, previously subjecting data to Anscombe ($\arcsin\sqrt{p}$)^a and Poisson^{**} ($\sqrt{x + 3/8}$) transformations.

two examiners were previously trained and acted in blinded fashion with respect to the groups, assigning each dentine tubule to one of the two categories (without or with smear layer). When there was no concordance between the examiners, the decision was made by consensus. The mean percentage of cleaned tubules per group was calculated and they were compared.

2.5 | Statistical analysis

An ANOVA test followed by the Tukey post-hoc test were used for comparisons of the death percentages and the total biovolume, previously subjecting the data to Anscombe ($\arcsin\sqrt{p}$) (Robert, 2000) and Poisson ($\sqrt{x + 3/8}$) normalization transformations, respectively. The Student t-test was used for comparisons among the percentages of cleaned tubules. The level of significance was $p < .05$. Statistical analyses were performed by means of SPSS 20.0 software (SPSS Inc., Chicago, IL).

3 | RESULTS

The mean global comparison by ANOVA showed significant differences among groups in bacteria biovolume as well as death percentages ($p < .001$ in both cases). The irrigating protocols that included NaOCl—that is, NaOCl, NaOCl/HEBP and NaOCl+EDTA—exerted the highest antimicrobial activity, with no significant differences in their death percentages. When water was used as the irrigating solution, a significant reduction was observed for the bacterial viability as compared to the control group, although it was significantly lower than the groups that included NaOCl.

Regarding the bacteria biovolume, all the irrigation protocols, even water, significantly reduced the bacteria without statistical differences; this reduction was significant with respect to the uninstrumented and unirrigated control group. Results of the intratubular infection test are given in Table 1. Representative CLSM images are shown in Figure 1.

No smear-layer-free dentine tubules were observed by SEM in the water and the 2.5% NaOCl groups. In the NaOCl/HEBP group, 90.41% \pm 7.33 of dentine tubules were free of smear layer, and 76.54% \pm 15.30 in the NaOCl+EDTA group, this difference being statistically significant ($p = .01$). Representative SEM images are shown in Figure 2.

4 | DISCUSSION

In the present study, the antimicrobial activity of different irrigating solutions containing NaOCl alone or combined with a chelating agent was evaluated against bacteria inside dentine tubules in an ex vivo root model. Smear layer removal was also evaluated to determine its influence on intratubular disinfection. For the antimicrobial activity, a modification of a previously described methodology was used (Arias-Moliz et al., 2014). The coronal 4 mm of the roots were used for antimicrobial evaluation because the number and size of dentine tubules are greater at this location. Furthermore, the bias regarding confusion

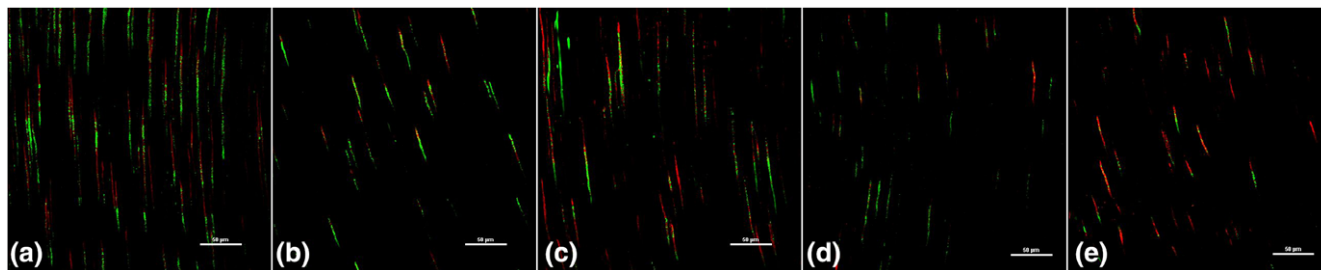


FIGURE 1 Representative CLSM images of the positive control (a) and the groups irrigated with distilled water (b), 2.5% NaOCl (c), 2.5% NaOCl/9% HEBP (d) and 2.5% NaOCl +17% EDTA (e) [Color figure can be viewed at wileyonlinelibrary.com]

between sclerotic dentine and smear layer was minimized (Vasiliadis, Darling, & Levers, 1983). Once two halves of the coronal segments had been contaminated by centrifugation, they and the rest of the root were reassembled in the customized model for chemomechanical treatment to have the entire root canal during canal preparation and to simulate, *ex vivo*, the clinical situation. Although endodontic infections are polymicrobial (Tzanetakis et al., 2015), a single species culture of *E. faecalis* was selected given its frequent involvement in treatment-resistant cases (Rõças, Siqueira, & Santos, 2004) and in assessing materials' antimicrobial properties against endodontic infections; moreover, it resists centrifugations (Morago et al., 2016). The methodology used in this study marks a step forward in dentine tubule disinfection in light of previous studies (Arias-Moliz et al., 2014; Ma, Wang, Shen, & Haapasalo, 2011) as it considers volumes and contact times resembling the clinical situation. However, limitations regarding the polymicrobial nature of infections, root canal anatomy variability and the presence of pulp debris should be acknowledged.

A group irrigated with water was included to evaluate the action of instrumentation inside dentine tubules. Mechanical debridement without an antimicrobial irrigant is known to reduce the bacterial load in the main root canal, but complete eradication is not possible (Aydin et al., 2007; Ferrer-Luque, Bejarano, Ruiz-Linares, & Baca, 2014; Siqueira et al., 2002). In this study, when water was used as an irrigant, a significant reduction of the intratubular biovolume was obtained, similar to the groups that included NaOCl. According to these results, the reduction of the intratubular biovolume would depend more on the action of the instruments than on the irrigants. However, the fact that most of the residual bacteria were alive in the group irrigated with water supports the use of antimicrobial irrigants during chemomechanical preparation (Siqueira et al., 2002).

All the irrigating protocols that included NaOCl showed a similar behavior against bacteria inside the dentine tubules. They all exerted antimicrobial activity (Morago et al., 2016; Neelakantan et al., 2015; Wong & Cheung, 2014) and reduced the bacteria biovolume, and this activity was not dependent on the smear layer removal. Hence, the null hypothesis was accepted. Previous authors have demonstrated that the antimicrobial activity of NaOCl inside tubules is reduced by the presence of smear layer (Morago et al., 2016; Wang et al., 2013), although the methodology differs: in their studies the dentine was treated with only a few microliters of the irrigating solution, and no chemomechanical sequence of the root canal treatment was performed. Dentine penetration by an irrigant is known to be independent of smear layer presence, instead being a function of tubular sclerosis, though possibly depending on the molecular size of the agent (Paqué, Luder, Sener, & Zehnder, 2006). It is also important to consider that NaOCl dissolves the organic components of the smear layer, thereby leaving a less densely adhered layer that could allow irrigant penetration (Aktener, Cengiz, & Pişkin, 1989), as seen in Figure 2. Despite the antimicrobial results obtained with NaOCl alone, there is a clear consensus regarding smear layer elimination, because it acts as a bacterial reservoir and also affects the sealing of obturation materials (Clark-Holke, Drake, Walton, Rivera, & Guthmiller, 2003).

It is important to highlight that the behavior of the irrigating solutions is different on root canal walls where biofilms were completely or almost completely destroyed when NaOCl was tested in combination with a chelating agent (Ferrer-Luque et al., 2014; Neelakantan et al., 2015). Most studies focus on the bacterial viability of surface biofilms (Arias-Moliz et al., 2015; Guerreiro-Tanomaru et al., 2014) and very few consider areas of difficult access such as dentine tubules (Morago et al., 2016; Wang et al., 2013). The results presented here show that the irrigation protocols NaOCl/HEBP and NaOCl+EDTA exert a significant antimicrobial activity against infected dentinal

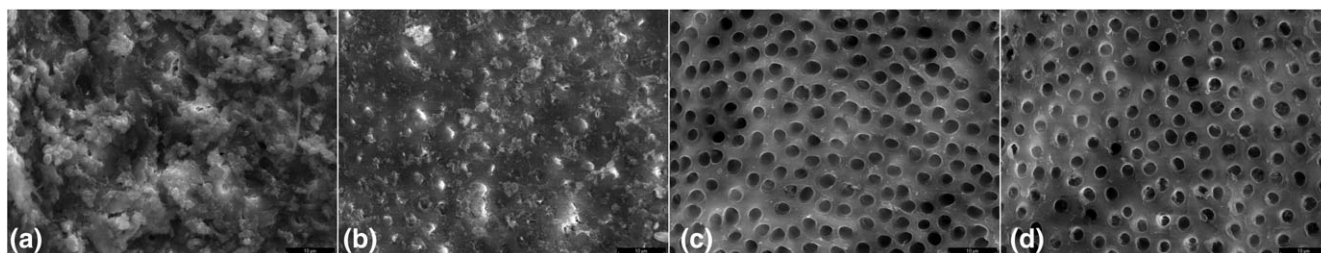


FIGURE 2 Representative SEM photomicrographs at $\times 1,500$ after treatment with distilled water (a), 2.5% NaOCl (b), 2.5% NaOCl/9% HEBP (c) and 2.5% NaOCl +17% EDTA (d)

tubules in the coronal third of the roots. Still, none of the protocols rendered dentine tubules free of bacteria, which implies a risk of persistent infection. It is nonetheless important to bear in mind that dentine tubule disinfection per se may not be so relevant to achieve periapical healing, in comparison to disinfection of apical deltas or main and accessory canals. Besides, to some extent the antimicrobial activity of these irrigation protocols might be expected to vary throughout the root canal, due to a greater difficulty in reaching the apical third and higher chlorine consumption. Future research to explore such conditioning factors should therefore be undertaken.

5 | CONCLUSIONS

NaOCl/HEBP and NaOCl+EDTA exerted an important antimicrobial activity against bacteria inside dentine tubules, lowering the bacteria biovolume and eliminating a substantial amount of the smear layer, particularly in the NaOCl/HEBP group.

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CONFLICT OF INTEREST

The authors have nothing to disclose and have no conflict of interest to declare.

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