Effects of Dentin Debris on the Antimicrobial Properties of Sodium Hypochlorite and Etidronic Acid

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Abstract
Introduction: The purpose of this study was to determine the influence of dentin powder on the concentration, pH, and antimicrobial activity of sodium hypochlorite (NaOCl) alone and combined with etidronic acid (HEBP). Methods: Biofilms of Enterococcus faecalis were grown on the surface of dentin blocks for 5 days and then exposed to 1% and 2.5% NaOCl alone or combined with 9% HEBP for 3 minutes in the absence and presence of dentin powder. The biovolumes of the biofilm were measured using confocal microscopy and the live/dead technique. The available chlorine and pH of the solutions were also measured. Nonparametric tests were used to determine statistical differences (P < .05).

Results: The presence of dentin powder resulted in a reduction of the free available chlorine and pH in all the irrigating solutions; 1% NaOCl lost its antimicrobial activity completely in the presence of dentin powder. The antimicrobial activity was significantly reduced in the 2.5% NaOCl and 1% NaOCl/HEBP groups, and it was not affected in the 2.5% NaOCl/HEBP group.

Conclusions: The presence of dentin powder significantly decreased the available chlorine and antimicrobial activity of 1% NaOCl, 2.5% NaOCl, and 1% NaOCl/HEBP irrigating solutions. The antimicrobial activity of 2.5% NaOCl/HEBP was not affected by the dentin powder after a 3-minute contact time against E. faecalis biofilms. (J Endod 2016;42:771–775)

Key Words
Antimicrobial activity, dentin debris, etidronic acid, root canal irrigants, sodium hypochlorite

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with 9% HEBP. For the NaOCl/HEBP association, both irrigants were prepared at a double concentration in sterile distilled water and mixed in a 1:1 ratio.

The dentin powder and specimens were obtained from human noncarious teeth (ethics committee UGR-438). For the dentin powder, the outer cementum was eliminated by polishing with silicon carbide papers, and the pulp tissue was removed using K-files. To avoid any contaminants such as metal particles that could modify the pH and chloride measurements, the radicular dentin was ground using an agate ball mill (Spex 8000M Mixer Mill; Spex Certiprep Industries Inc, Metuchen, NJ) for 10 minutes. The resultant dentin powder was filtered in order to obtain a particle size $\leq 190\ \mu m$, sterilized in a glass flask using an autoclave (121°C for 15 minutes), and stored at 4°C until use. To evaluate the effect of dentin on the biological properties of the solutions, 10 mg dentin powder was suspended in 100 μL of the irrigating solutions (wt/vol).

Determination of the Concentration and pH in NaOCl Solutions

The concentration of the NaOCl solutions in the presence or absence of dentin powder was determined by measuring the free available chlorine using a standard iodine/thiosulfate titration method (19). The pH values of the solutions were recorded with a pH meter (micropH 2001; Crison, Alella, Spain). Measurements were performed in triplicate immediately after the solutions were prepared as well as after 1, 3, and 10 minutes. Between measurements, the solutions were stored in darkness at 4°C.

Antimicrobial Activity Test

Sixty dentin blocks (2 × 2 × 1.2 mm) were prepared from 12 noncarious freshly extracted teeth (20). The smear layer was removed using 17% EDTA for 5 minutes. After sterilization, they were kept in sterile saline solution until use.

For Enterococcus faecalis biofilm formation, a previous methodology was used (16). The dentin blocks were fixed with fluid resin to the tips of modified pegs of the MBEC-high-throughput (HTP) device (Innovotech, Edmonton, AB, Canada). The trough was then inoculated with approximately $1 \times 10^7\ \text{CFU/mL}$ E. faecalis ATCC29212 suspended in 22 mL brain-heart infusion medium (Scharlau Chemie, Barcelona, Spain) supplemented with 1.3% glucose. The device was placed on a rocking table (Swing Sw 8 10000-00015; OVAN, Badalona, Spain) and incubated at 37°C for 5 days at 5 rocks per minute. The brain-heart infusion broth was refreshed every 2 days, and the purity of the inoculum in the trough was evaluated by Gram staining and colony morphology in agar plates after 5 days of incubation.

The infected dentin blocks were detached from the pegs and rinsed with 0.9% saline solution for 2 minutes. The specimens were randomly divided into 12 groups ($n = 5$) according to the irrigating solutions and the presence or absence of dentin powder in the solution: group 1, 1% NaOCl; group 2, 1% NaOCl/9% HEBP; group 3, 2.5% NaOCl; group 4, 2.5% NaOCl/9% HEBP; group 5, 9% HEBP; and group 6, distilled water (control). Groups 7 through 12 consisted of the same solutions listed in groups 1 through 6 mixed with dentin powder (10 mg/100 μL). The dentin blocks were submerged in 100 μL of the irrigating solutions for 3 minutes. Then, the NaOCl of the solutions was inactivated by adding 5% sodium thiosulfate for 5 minutes to enhance the quality of the staining process for NaOCl-treated dentin (21). After exposure, the biofilms were rinsed with saline solution, stained, and observed under confocal laser scanning microscopy. Five samples per study group were used in 2 independent experiments.

For disinfection analysis, the SYTO 9/propium iodide technique (Live/Dead BacLight; Invitrogen, Eugene, OR) was used (21). SYTO 9 is a green fluorescent stain, labeling both live and dead microorganisms;

Figure 1. The mean values of the available chlorine and pH of the 1% and 2.5% NaOCl solutions alone and combined with 9% HEBP immediately after the solutions were prepared and after 1, 3, and 10 minutes. D, dentin powder.

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propidium iodide is a red fluorescent nucleic acid stain and penetrates only the cells with damaged membranes (dead microorganisms). After staining the samples for 15 minutes, they were rinsed with saline solution and observed using an inverted confocal laser scanning microscope (Eclipse Ti-E; Nikon, Mississauga, Canada). Four microscopic confocal volumes from random areas were acquired from each sample using the 40× oil lens, 1-μm step size, and a format of 512 pixels. Each picture represented an area of $317 \times 317 \mu m$. For quantification purposes, bioimage L software (Luis Chavez de Paz, Malmö, Sweden) was used (22). The parameters evaluated in each group were the total and green (live) biovolumes ($\mu m^3$). Statistical analysis of these parameters was performed using the nonparametric Kruskal-Wallis and Dunn tests ($P < .05$) because of the absence of normal distribution confirmed in the preliminary analysis. Prisma 5.0 (GraphPad Software Inc, La Jolla, CA) was used as the analytic tool.

**Results**

The mean values of the available chlorine and pH of the NaOCl solutions immediately after the solutions were prepared and after 1, 3, and 10 minutes in the absence and presence of dentin powder are presented in Figure 1. The presence of dentin powder resulted in a reduction of the free available chlorine (this reduction was higher during the first minute) and the pH in all the irrigating solutions and contact times.

All the NaOCl solutions reduced the total and green biovolumes significantly in comparison with the control ($P < .05$), except for 1% NaOCl in the presence of dentin powder (Table 1). HEBP did not show antimicrobial activity (green biovolume) with respect to the control regardless of the presence of dentin; yet, it was able to reduce the total biovolume. The presence of dentin powder significantly decreased the total and green biovolumes of the solutions 1% NaOCl alone and combined with HEBP and 2.5% NaOCl. The antimicrobial activity of 2.5% NaOCl/HEBP was not affected by the dentin powder after a 3-minute contact time against *E. faecalis* biofilms. No reduction in the biovolumes was detected in the positive control in the presence of dentin powder. Representative images of the treated biofilms are provided in Figure 2A–D.

**Discussion**

The present study aimed to evaluate the inhibitory effect of dentin powder on the chemical and biological activity of NaOCl alone and associated with HEBP. NaOCl presents a well-recognized in vitro antimicrobial activity although in some cases this solution fails to achieve a bacteria-free root canal under clinical conditions (23, 24). The in vitro performance may be traced to a failure of the irrigant to reach complex areas or the presence of a complex chemical environment containing a mixture of organic and inorganic products, such as necrotic pulp, dentin debris, and biofilms.

Previous studies using microcomputed tomographic imaging reported that dentin debris produced during instrumentation actively accumulates within the isthmus level of the root canal of posterior teeth (8, 9, 25). In the first part of this study, NaOCl was noted to react progressively with the dentin powder, leading to a higher reduction of the chlorine concentration and pH during the first minute for all the solutions and less consumption in the rest of the time intervals. Camps et al (26) showed that the 2.5% NaOCl solution reduced its chlorine level to 1.38% after 10 minutes in the presence of pulp tissue, which is higher than the 0.5% found after the reaction of this solution with dentin powder. Macedo et al (7) showed a significant reduction of the available chlorine from 2% to 1.65% after 1 minute of contact with the dentinal root canal walls. Although differences in the methodologies, substrate, and volumes of NaOCl make it

| TABLE 1. Median (range) of the Total Biovolume and the Green (live) Biovolume ($\mu m^3$) of *Enterococcus faecalis* Biofilms after 5 Minutes of Treatment with the Irrigating Solutions in the Absence and Presence of Dentin. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Power           | Total biovolume | Green biovolume |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Control         | No dentin powder | 3.54 (1.62–8.78)×10^5 | 3.6 (1.9–6.3)×10^4 |
| 1% NaOCl        | No dentin powder | 2.0 (1.9–5.3)×10^4 | 3.4 (1.7–6.0)×10^4 |
| 2.5% NaOCl      | No dentin powder | 2.0 (2.0–4.1)×10^4 | 1.9 (1.2–3.1)×10^4 |
| 2.5% NaOCl/HEBP | No dentin powder | 2.0 (2.0–4.1)×10^4 | 1.9 (1.2–3.1)×10^4 |
| 1% NaOCl        | 1% HEBP         | 2.0 (2.0–4.1)×10^4 | 1.9 (1.2–3.1)×10^4 |
| 2.5% NaOCl      | 2.5% NaOCl/HEBP | 2.0 (2.0–4.1)×10^4 | 1.9 (1.2–3.1)×10^4 |
| 2.5% NaOCl/HEBP | 9% HEBP         | 2.0 (2.0–4.1)×10^4 | 1.9 (1.2–3.1)×10^4 |

HEBP, etidronic acid; NaOCl, sodium hypochlorite. Read vertically, the same superscript letters show differences not statistically significant by Kruskal-Wallis test. Read horizontally, the same superscript numbers show differences not statistically significant by the Mann-Whitney test.
difficult to compare the results, all studies indicated a reduction of available chlorine in the presence of the different substrates. Yet, even if the concentration of NaOCl in the main root canal remains high (27), it is very possible that the chlorine concentration decreases in the lateral areas and irregularities filled with dentin debris after instrumentation under in vivo conditions.

The antimicrobial effect of NaOCl solutions depends on their free available chlorine, which consists of hypochlorous acid and the hypochlorite ion (5). Our results indicate an effective activity of standard (without dentin powder) NaOCl and NaOCl/HEBP solutions against *E. faecalis* biofilms after 3 minutes, a time commonly used to show the eradication of *E. faecalis* biofilms by NaOCl solutions (15, 16, 28). Despite the fact that the presence of dentin powder significantly decreased antimicrobial activity in the 1% NaOCl/HEBP and 2.5% NaOCl solutions, it did not imply a loss of their antimicrobial effect in comparison with the control. After 3 minutes, 2.5% NaOCl mixed with dentin powder maintained a concentration over 0.5% that can be considered antimicrobial (29) although its effect was also significantly lower compared with the nonmixed solution. Longer contact times were not used because standard solutions were highly effective after 3 minutes; moreover, the chlorine and pH levels rapidly decreased when dentin powder was added to the solution and then stabilized after the 1–3 minute period, thus diminishing the chance of bacteria killing without using fresh solution (30).

Regarding the 1% NaOCl concentration, Haapasalo et al (17) showed that its antimicrobial effect was reduced but not totally eliminated by the presence of dentin powder. When NaOCl was exposed to an *E. faecalis* planktonic culture, it was able to kill all the bacteria, whereas preincubation of the solution with dentin greatly delayed the killing process. In the present experiment, an interesting observation was made with the 1% NaOCl concentration. Despite a progressive reduction of the available chlorine to 0.28%–0.40% in the 1% NaOCl solutions, respectively, with and without HEBP, after 3 minutes of contact in the presence of dentin powder, there was significant antimicrobial activity in the 1% solution containing HEBP as opposed to the solution without the chelating agent (which completely lost its activity). This phenomenon could be explained by the chemical interaction of the inorganic superficial layer of the infected dentin block with the chelator (13), probably allowing detachment of bacteria from the dentin surface even when a sublethal chlorine concentration was present in the solution, and by the effect of HEBP on the biofilm structure. Indeed, the HEBP alone was found to reduce significantly only the total biovolume of the biofilms in comparison with the control. The incorporation of HEBP to 2.5% NaOCl was beneficial in the presence of dentin powder, helping to avoid NaOCl inactivation by dentin, although the differences were not significant with 2.5% NaOCl alone.

In the interpretation of results, it is important to keep in mind the in vitro methodology applied in this study. Results related to the

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**Figure 2.** Representative confocal laser scanning microscopic images after treatment with distilled water in the (A) absence of dentin powder, (B) distilled water in the presence of dentin powder, (C) 1% NaOCl in the absence of dentin powder, and (D) 1% NaOCl in the presence of dentin powder. Scale bars = 50 μm.
inactivation processes of irrigating solutions inside the root canal system appear to become diverse and more complex when different amounts of debris and smear layer as well as the organic tissue are present, most likely influencing the biological activity of the solutions (1, 18, 31). Further studies are needed to evaluate the inactivation of irrigating solutions simulating clinical conditions as well as to assess the efficacy of other irrigating solutions such as EDTA and NaOCl at higher concentrations. In summary, the presence of dentin powder significantly decreased the antimicrobial activity of the 1% NaOCl, 2.5% NaOCl, and 1% NaOCl/HEBP solutions studied. The antimicrobial effect of 2.5% NaOCl/HEBP was not affected by the presence of dentin powder after a 3-minute contact time. Consequently, the null hypothesis was partially rejected. This serves to underline the importance of a continuous refreshment of solutions to compensate the loss of chemical efficiency (32, 33) plus the use of new strategies such as HEBP to reduce the accumulation of hard tissue debris.

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References