**Influence of dentine debris and organic tissue on the properties of sodium hypochlorite solutions**

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**Keywords:** dentine debris; dissolution; etidronic acid; organic tissue; root canal irrigants; sodium hypochlorite.

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**Abstract**

**Aim** To determine the free available chlorine of 2.5% sodium hypochlorite (NaOCl) alone and combined with 9% etidronic acid (HEDP) in the presence of inhibitors, organic tissue and organic tissue plus dentine debris; to evaluate the influence of dentine debris on the tissue-dissolving capacity of both NaOCl solutions; and to determine the antimicrobial action of these solutions when in contact with organic tissue and organic tissue plus dentine debris.

**Methodology** The available chlorine of the solutions over time in the absence and presence of the inhibitors was measured using a titration method. The organic tissue dissolution by the solutions alone and in the presence of dentine powder was evaluated by weighing bovine tissue specimens before and after exposure to the solutions for 3 and 10 minutes. For the antimicrobial activity, biofilms of *Enterococcus faecalis* were exposed to the solutions for 3 minutes in the absence and presence of organic tissue and organic tissue + dentine debris. The biovolume and percentage of damaged membrane cells of the biofilm were measured by means of confocal microscopy and the live/dead technique. Non-parametric tests were used to determine statistical differences (*p*<0.05).

**Results** Both inhibitors consumed the free available chlorine of the solutions over time. The presence of dentine debris significantly reduced the tissue-dissolution capacity of the NaOCl solutions (*p*<0.05). The percentages of biovolume reduction were not affected by the presence of the inhibitors in the two NaOCl solutions, whereas the percentage of damaged membrane cells was significantly reduced (*p*<0.001). Overall, a similar behaviour was observed in the NaOCl and NaOCl/HEDP groups.

**Conclusions** The presence of organic tissue and organic tissue + dentine debris favoured rapid consumption of the free chlorine of NaOCl and NaOCl/HEDP. This resulted in a decreased ability to dissolve organic tissue without affecting the short-term antimicrobial activity.

**Keywords:** dentine debris; dissolution; etidronic acid; organic tissue; root canal irrigants; sodium hypochlorite.

**Introduction**

Irrigation plays an important role in root canal treatment since it helps to clean the root canal system by killing microorganisms and removing inorganic and organic tissue (Siqueira & Roças 2018). To date the most common irrigation protocol includes the use of NaOCl during root canal instrumentation, followed by a chelating agent such as EDTA (Zehnder 2006). NaOCl has strong biological properties that include antimicrobial activity and organic tissue dissolution capacity. Its activity depends on the free available chlorine, which consists of hypochlorite ions and hypochlorous acid (Baker 1947). These molecules are consumed as the NaOCl exerts its biological activity, indicating that it has a rapid and effective action. Given the instability of NaOCl, in recent years, a new concept of continuous chelation irrigation during root canal instrumentation has been introduced (Neelakantan *et al*. 2012). It entails the use of a single irrigating solution during root canal instrumentation: NaOCl mixed with a weak chelating agent such as HEDP or tetrasodium EDTA (Zehnder *et al*. 2005, Tartari *et al*. 2017). The former mixture maintains the properties of both solutions during a short period of time (Zehnder *et al*. 2005, Lottanti *et al*. 2009, Paqué *et al*. 2012, Tartari *et al*. 2015, Arias-Moliz *et al*. 2014, Arias-Moliz *et al*. 2015).

It is important to bear in mind that the root canal system is an environment characterized by a complex anatomy and the presence of organic and inorganic matter comprising bacteria biofilms, pulp tissue and dentine. The action of instruments also generates the smear layer, a layer of organic and inorganic residue on root canal walls, as well as the accumulation of hard tissue debris in non-instrumented areas (Paqué *et al*. 2009). These components may have an effect on the biological action of NaOCl solutions. It has been reported (Slutzky-Goldberg *et al*. 2013) that the presence of dentine in contact with pulp decreases the tissue dissolution capacity of NaOCl. Furthermore, the smear layer and dentine debris act as a physical barrier that significantly affect the antimicrobial activity of NaOCl solutions against infected dentinal tubules and surface biofilms (Wang *et al*. 2013, Arias-Moliz *et al*. 2016, Morago *et al*. 2016). When NaOCl was tested in combination with HEDP in the absence and presence of smear layer and dentine debris, however, these properties were not affected (Arias-Moliz *et al*. 2016, Morago *et al*. 2016). To date, no studies have been conducted to discern the effect of the organic/inorganic matter on the biological properties of NaOCl. Thus, the objective of this study was threefold: to determine the free available chlorine of NaOCl alone and combined with HEDP in the presence of organic tissue and organic tissue plus dentine debris; to evaluate the influence of dentine debris on the tissue-dissolving capacity of both NaOCl solutions; and to determine the antimicrobial action of these solutions when in contact with organic tissue and organic tissue plus dentine debris. The null hypothesis was that the dentine debris does not affect the dissolution capacity and that the organic tissue alone or together with the dentine debris does not affect the antimicrobial activity of the NaOCl solutions.

**Material and Methods**

The irrigating solutions evaluated were 2.5% NaOCl (Panreac Química SA, Castellar del Vallès, Spain), 9% HEDP (wt/vol) (Cublen K8514GR; Zschimmer & Schwarz, Mohsdorf, Germany) and a combination of both. All the solutions were prepared by mixing the chemicals with distilled water. For the 2.5% NaOCl/9% HEDP association, both irrigants were prepared at double concentration and mixed in a 1:1 ratio.

Bovine meat was employed for the experiments to simulate pulp tissue (Haapasalo *et al*. 2014). The tissue samples were standardized in size (2×2×4 mm) and weight (30.0 ± 1.8 mg). They were preserved in 0.9% saline solution at -20° C until use. For each millilitre of irrigating solution, one organic tissue sample was used.

The dentine powder was obtained from human non-carious teeth (approved by the Ethic Committee of the institution) as previously described (Arias-Moliz *et al*. 2016). Briefly, the teeth were decoronated and outer cementum was eliminated with silicon carbide papers, and the pulp tissue using K-files. The radicular dentine was ground using an agata ball mill (Spex 8000M Mixer Mill; Spex Certiprep Industries Inc., Metuchen, NJ, USA) for 10 minutes. The resultant dentine powder was filtered in order to obtain a particle size ≤ 190 µm, sterilized in a glass flask by autoclave (121ºC, 15 minutes) and stored at 4°C until use. To evaluate the effect of dentine on the biological properties of the solutions, two concentrations of dentine powder were prepared: a low concentration where 10 mg of dentine powder was suspended in 1 mL of the irrigating solutions (wt/vol), and a high concentration of 100 mg/mL (wt/vol).

Available chlorine in NaOCl solutions

The 2.5% NaOCl and 2.5% NaOCl/9% HEDP solutions were iodometrically titrated (American Public Health Association, 1989) in triplicate to determine their available chlorine content in the absence or presence of organic tissue, and of organic tissue plus dentine powder, at 10 mg/mL and 100 mg/mL. They were titrated immediately after mixing the solutions and again after 1, 3 and 10 min. Between measurements, the solutions were stored in the dark at room temperature.

According to the results obtained for available chlorine (Figure 1), the higher concentration of dentine powder, 100 mg/mL, was used for the rest of the experiments.

Tissue dissolution test

For the dissolution test, the organic tissue samples were threaded, dried for 5 minutes on absorbent paper and weighed on a precision balance (HM 202, AND, Tokyo, Japan) to determine the initial weight of each sample. The specimens were randomly divided into twelve groups (n=6/group) according to the irrigating solution (2.5% NaOCl, 2.5% NaOCl/9% HEDP or 9% HEDP); the time of exposure (3 and 10 min); and the absence and presence of dentine powder.

The organic tissue samples were immersed for 3 and 10 min in tubes containing 1 mL of the test solutions with or without the dentine debris. After this time they were blotted dry and reweighed for comparison with the initial values. The percentage of weight loss in each group was calculated.

Antimicrobial activity test

Sixty dentine blocks (2×2×1.2 mm) were prepared from ten non-carious freshly extracted teeth (Baca *et al*. 2011). The smear layer was removed using 17% EDTA for 4 minutes. After sterilization, the blocks were kept in sterile saline solution until use. For the *Enterococcus faecalis* biofilm formation, a previous methodology was used (Arias-Moliz *et al*. 2015). The dentine blocks were fixed with fluid resin to the tips of modified pegs of the MBEC-HTP device (Innovotech, Edmonton, Canada). The trough was then inoculated with approximately 1×107 CFU/mL of *E. faecalis* ATCC 29212 suspended in 22 mL of BHI (Scharlau Chemie, Barcelona, Spain) supplemented with 1.3% of 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 BHI broth was refreshed every two days.

The specimens were again randomly divided into 12 groups (n=5) according to the irrigating solution and the absence or presence of organic tissue and of organic tissue + dentine powder: 2.5% NaOCl, 2.5% NaOCl/9% HEDP, 9% HEDP and 0.9% saline solution (positive control) all tested alone, in the presence of organic tissue, and of organic tissue + dentine powder.

The dentine blocks were removed from the pegs with sterile tweezers and washed with saline solution to remove weakly adhered planktonic bacteria. They were then submerged in 100 µL of the irrigating solutions in the absence and presence of organic tissue and of organic tissue + dentine powder for 3 minutes. In order to keep the proportions of the organic tissue and dentine debris with the volume of irrigating solution, each 100 µL of the irrigating solution was exposed to samples of approximately 3 mg of organic tissue alone or together with 10 mg of dentine debris. When the solutions were tested in the presence of organic tissue, the organic tissue sample was placed in a glass bottle, and immediately afterwards 100 µL of solution and the contaminated dentine block were put inside the glass bottle and left in contact for 3 minutes. When the solutions were tested in the presence of organic tissue + dentine debris, both inhibitors were placed in the bottle at the same time and the rest of the procedure was the same as explained above. The NaOCl groups were inactivated by adding 5% sodium thiosulfate for five minutes. Thereafter, all the specimens were washed with saline solution, stained, and observed under confocal laser scanning microscopy (CLSM, Nikon Eclipse Ti-E, Mississauga, Canada).

The stain used was the LIVE/DEAD BacLigth (Invitrogen, Eugene, OR, USA), which includes Syto 9 and propidium iodide (PI). Both dyes target the nucleic acids and discriminate viable from dead cells on the basis of the membrane integrity: Syto 9 is a green fluorescent stain labeling cells with intact membranes which can be both live and dead microorganisms, while PI is a red fluorescent stain that penetrates only the cells with damaged membranes. The PI has a stronger affinity to nucleic acids than Syto 9; thus, when both stains are present within a cell, Syto 9 will be displaced from the nucleic acids and the cells will show fluorescence in red (Netuschil *et al*. 2014). After staining the samples for 15 min, they were rinsed with saline solution and observed using an inverted CLSM (Nikon Eclipse Ti-E, 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 317×317 µm. For quantification purposes *bio*image\_L software was used (Chavez de Paz *et al*. 2009). The parameters evaluated in each group were the total biovolume (µm3) and the percentage of apparently dead cells that are damaged membrane cells (red).

Statistical analysis

All the results were expressed as percentages: percentage of organic tissue dissolution, percentage of total biovolume reduction with respect to the control, and damaged membrane cell percentage. Global comparisons were performed by means of the Kruskal Wallis test, and the pairwise comparisons using the Mann-Whitney test. The level of significance was *p*<0.05. Statistical analyses were performed with SPSS 20.0 software (SPSS Inc., Chicago, IL).

**Results**

The organic tissue alone and combined with dentine powder reduced the free available chlorine of the solutions over time, the behaviour being similar for both solutions. The greatest chlorine reduction was obtained when the solutions were exposed to the organic tissue plus dentine debris at the higher concentration (Figure 1).

The presence of dentine powder produced a significant reduction of the tissue-dissolution capacity of NaOCl and NaOCl/HEDP at 3 and 10 minutes (Table 1). At 3 minutes the behaviour of both solutions was similar in presence of dentine powder; yet after 3 minutes in the absence of dentine powder and after 10 minutes with or without dentine powder, the NaOCl/HEDP dissolved significantly less tissue (*p*<0.05). An increase in the tissue weight was observed in all HEDP groups at either time (comparisons with the other groups were not performed).

The percentages of bacteria biovolume reduction were significantly higher (*p*<0.001) in the NaOCl and NaOCl/HEDP groups than in the HEDP, with no statistical differences between the two NaOCl groups in the absence or presence of organic tissue but not in the presence of organic tissue + dentine powder (Table 2). Overall, a higher percentage of damaged membrane cells was obtained with the solutions as opposed to the control group (Table 3). These percentages were significantly higher (*p*<0.001) in the NaOCl and NaOCl/HEDP groups, without significant differences between them, and the percentages were reduced when in presence of organic tissue and of organic tissue + dentine debris (*p*<0.05). In the HEDP group, the percentage of biovolume reduction and the percentage of damaged membrane cells were significantly higher (*p*<0.001 and *p*=0.006, respectively) in the presence of organic tissue. Figure 2 shows representative confocal laser scanning microscopic images of the infected dentine treated with 2.5% NaOCl and 2.5% NaOCl/9% HEDP in the absence and presence of the organic tissue and of organic tissue + dentine debris.

**Discussion**

This study evaluated the influence of two inhibitors present in root canals, organic tissue and dentine debris, on the properties of NaOCl alone and combined with HEDP. The impact of dentine powder on the antimicrobial effect of different root canal disinfectants was previously described under laboratory conditions (Haapasalo *et al*. 2000, Portenier *et al*. 2001). The null hypothesis was partially accepted, as results revealed that the dissolution capacity was significantly reduced when the solutions were in contact with dentine debris, whereas the antimicrobial activity was hardly affected by the inhibitors after 3 minutes of exposure. At this time, a similar behaviour was observed in the NaOCl and NaOCl/HEDP groups, confirming that HEDP scarcely influences the short-term loss of NaOCl properties (Zehnder *et al*. 2005, Arias-Moliz *et al*. 2015, 2016, Ulusoy *et al*. 2018). Bovine meat was used as an organic tissue sample rather than pulp because it is widely available, has a uniform composition that resembles pulp tissue, and can be cut into sections of very similar size and weight, allowing for sample standardization (Stojicic *et al*. 2010, Tartari *et al*. 2015, 2017).

The free available chlorine was determined as it can shed light on the changes involved in the biological effect of NaOCl solutions when exposed to organic tissue and to organic tissue plus two different concentrations of dentine debris (Guneser *et al*. 2015, Arias-Moliz *et al*. 2016). As expected, the organic tissue alone and together with dentine debris consumed the free available chlorine of the solutions. This reduction was greater when in contact with the higher concentration of dentine debris (100 mg/mL) as compared to the lower one (10 mg/mL), which highlights that dentine debris has a strong influence on chlorine consumption. One explanation may be that the free chlorine consumption of NaOCl depends not only on the organic matter but also on the contact surface, which is greater in the case of dentine powder (Stojicic *et al*. 2010, Slutzky-Goldberg *et al*. 2013).

The progressive chlorine reduction when the solutions were in contact with the dentine debris was reflected in a reduced dissolution activity of both NaOCl solutions, at 3 and 10 minutes. Although this effect was previously reported for NaOCl (Guneser *et al*. 2015), no studies have been performed with the NaOCl/HEDP. Tartari *et al.* (2015) observed a progressive reduction of the dissolution activity of the combined solution over time, up to 26% after 15 minutes, but in the absence of dentine debris. Their results contrast with the ones obtained in the present study, where higher percentages of tissue dissolution were found with both NaOCl solutions. These discrepancies may be traced to differences in the methodology used, e.g. the organic sample size and the volume of the solution.

The presence of organic tissue and of organic tissue + dentine debris did not affect the capacity of the NaOCl and the NaOCl/HEDP to reduce the biofilm biovolume. The percentage of biovolume reduction was very high, over 98%, and similar with both solutions. Within the NaOCl/HEDP solution, though the percentages of biovolume reduction were very similar, respectively, 98.95%, 99.53% and 99.01%, the statistical comparisons revealed differences when in presence of organic tissue as compared with the absence and presence of both inhibitors, probably due to the low dispersion of the data. In view of the tissue dissolution results, the activity of both NaOCl solutions on the biofilm biovolume could be expected, since the biofilm surface is smaller to that of the organic tissue, making it easier for the NaOCl to react with the bacteria. In contrast to the biovolumes, the inhibitors reduced the killing effect of the NaOCl solutions. Nevertheless, it is important to consider that the percentage of damaged membrane cells was evaluated on a residual biovolume that suffered an approximately 99% reduction after being exposed to the solutions. A reduced activity of the NaOCl solutions would be expected under *in vivo* conditions, where the dentine walls and smear layer are also present. Future studies should be performed to overcome these limitations.

With respect to the HEDP group, an increase of the tissue weight and a higher antimicrobial activity in the presence of organic matter (alone or together with dentine debris) were observed. This may be traced to the organic tissue hydration as a consequence of sodium ions depositing on its surface, thereby favouring water adsorption (Hand *et al*. 1978, Tartari *et al*. 2017). When tested in the absence of organic tissue, such a deposition could have occurred on the organic layer that conforms the biofilm, interfering with the chelating activity of the HEDP, and therefore implying the detachment of bacteria from the dentine surface.

**Conclusions**

In summary, organic tissue and organic tissue + dentine powder favoured a rapid consumption of the free chlorine of NaOCl solutions alone and combined with HEDP. Specifically, the greater the amount of dentine powder, the greater the loss of free chlorine from the solutions. This leads to a decreased ability to dissolve organic tissue without affecting the short term antimicrobial activity.

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| **Table 1.** Percentage of organic tissue dissolution after its exposure to the solutions for 3 and 10 minutes in the absence and presence of dentine powder. Mean (standard deviation). |
|  | 3 min |  | 10 min |
|  | No dentine powder | Dentine powder |  | No dentine powder | Dentine powder |
| 2.5% NaOCl  | 35.69 (6.34)1,a | 5.93 (3.70)2,a |  | 66.01 (1.74)3,a | 9.28 (1.61)2,a |
| 2.5% NaOCl/9% HEBP  | 24.90 (5.79)1,a | 7.17 (3.95)2,a |  | 53.22 (7.45)3,b | 4.69 (3.14)2,b |
| 9% HEBP\* | -31.52 (20.85)1 | -35.51 (24.36)1 |  | -24.08 (5.97)1 | -28.68 (2.94)1 |
| \* No organic tissue dissolution. An increment of the weight was observed (- sign). Read horizontally, the same numbers show that there are no statistically significant differences determined by the Kolmogorov-Smirnov test. Previous global comparisons by Kruskal Wallis test showed a *p*<0.001 in NaOCl and NaOCl/HEBP groups and *p*=0.829 in HEBP group.Read vertically, the same letters show that there are no statistically significant differences determined by the Kolmogorov-Smirnov test. |

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| **Table 2.** Percentage of total biovolume reduction respect to the control of the *E. faecalis* biofilms after exposure to the solutions for 3 minutes in the absence of organic tissue and dentine powder or in the presence. Mean (standard deviation). |
|  | No organic tissueNo dentine powder | Organic tissue No dentine powder | Organic tissue Dentine powder | Comparison*p* value\* |
| 2.5% NaOCl  | 99.72 (4.73)1,a | 99.27 (0.76)1,a | 99.35 (0.79)1,a | 0.251 |
| 2.5% NaOCl/9% HEBP | 98.95 (1.26)1,a | 99.53 (0.73)2,a | 99.01 (0.65)1,a | 0.003 |
| 9% HEBP  | 61.37 (15.44)1,b | 85.33 (9.04)2,b | 74.59 (20.41)2,b | <0.001 |
| Comparison *p* value\* | <0.001 | <0.001 | <0.001 |  |
| \* Global comparisons by Kruskal Wallis test.Read horizontally, the same number show that there are no statistically significant differences determined by the Kolmogorov-Smirnov test.Read vertically, the same letters show that there are no statistically significant differences determined by the Kolmogorov-Smirnov test. |

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| **Table 3.** Death percentage of the *E. faecalis* biofilms after exposure to the solutions for 3 minutes in the absence vs. presence of organic tissue and dentine powder. Mean (standard deviation). |
|  | No organic tissueNo dentine powder | Organic tissue No dentine powder | Organic tissue Dentine powder | Comparison*p* value\* |
| Positive control | 10.59 (9.75)1,a | 3.11 (3.30)2,a  | 11.07 (8.38)1,a  | <0.001 |
| 2.5% NaOCl | 83.77 (14.13)1,b | 50.29 (27.26)2,b | 55.79 (31.35)2,b | <0.001 |
| 2.5% NaOCl/9% HEBP | 85.13 (19.67)1,b | 55.49 (32.69)2,b  | 47.45 (17.92)2,b | <0.001 |
| 9% HEBP | 19.54 (12.65)1,a | 34.71 (14.83)2,c | 30.38 (17.09)2,c | 0.006 |
| Comparison *p* value\* | <0.001 | <0.001 | <0.001 |  |
| \* Global comparisons by Kruskal Wallis test.Read horizontally, the same number show that there are no statistically significant differences determined by the Kolmogorov-Smirnov test.Read vertically, the same letters show that there are no statistically significant differences determined by the Kolmogorov-Smirnov test. |

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| Figure 1. Available chlorine of the 2.5% NaOCl and 2.5% NaOCl/9% HEDP in the absence and presence of organic tissue and of organic tissue plus dentine debris. Means and standard deviations (bars). |

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| Figure 2. Representative CLSM images of the positive control (A), positive control in the presence of organic tissue (B) and in the presence of organic tissue + dentine powder (C); NaOCl (D), NaOCl in the presence of organic tissue (E) and in the presence of organic tissue + dentine powder (F); NaOCl/HEDP (G), NaOCl/HEDP in the presence of organic tissue (H) and in the presence of organic tissue + dentine powder (I); and HEDP (J), HEDP in the presence of organic tissue (K) and in the presence of organic tissue + dentine powder (L).  |