Biofilm Dissolution and Cleaning Ability of Different Irrigant Solutions on Intraorally Infected Dentin

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Abstract

Introduction: The aim of this study was to evaluate the biofilm dissolution and cleaning ability of different irrigant solutions on intraorally infected dentin. Methods: One hundred twenty bovine dentin specimens were infected intraorally by using a removable orthodontic device. Thirty samples were used for each irrigant solution: 2% chlorhexidine and 1%, 2.5%, and 5.25% sodium hypochlorite (NaOCl). The solutions were used for 5, 15, and 30 minutes and at 2 experimental volumes, 500 μL and 1 mL. The samples were stained by using acridine orange dye before and after the experiments and evaluated by using a confocal microscope. The percentage of biofilm, isolated cells, and noncolonized dentin was measured by using a grid system. Differences in the reduction or increase of the studied parameters were assessed by using nonparametric methods (P < .05). Results: The higher values of biofilm dissolution and noncolonized dentin were found in the 30-minute NaOCl group and in the 5-minute and 15-minute groups of 5.25% NaOCL. The use of 2% chlorhexidine solution did not improve the biofilm dissolution or increase the cleaning of the dentin in comparison with the NaOCl solutions (P < .05). Conclusions: Two percent chlorhexidine does not dissolve the biofilms. Thirty minutes of NaOCl are necessary to have higher values of biofilm dissolution and to increase the cleaning of the dentin independently of the concentration in comparison with the 5-minute and 15-minute contact times. (J Endod 2011;37:1134–1138)

Key Words

Acridine orange, bacteria, confocal laser scanning microscopy, dentin, irrigant solutions, oral biofilms

Bacteria and their products in avascular and necrotic root canal systems are the main etiologic factor of apical periodontitis (1). Biofilms can be defined as cells attached to a surface embedded in an exopolysaccharide matrix that fills the space between cells (2). Biofilms can resist alkali stress (3) and have increased resistance to antibacterial agents (4). Because of the irregularities of the root canal anatomy, antimicrobial irrigant solutions are used to dissolve necrotic tissue and to assist in the microbial control stage (5).

The results of clinical studies have shown that at least 50% of root canals can retain bacteria after endodontic procedures with sodium hypochlorite (NaOCl)—based or chlorhexidine-based protocols (6, 7). Clinical advantages of chlorhexidine are antimicrobial activity (8), substantive effect (9), and low toxicity. Despite the fact that NaOCl and chlorhexidine have shown no differences in their antimicrobial activity in clinical research, the cleaning ability of these compounds and direct comparisons by using microscopic methods are scarce. Residual organic biofilm can act as a source of toxins and might also interfere with the obturation process in noninstrumented areas such as fins (10).

Confocal laser scanning microscopy (CLSM) for the study of oral biofilms has been reported in earlier studies (11, 12). A potential advantage is the absence of dehydration or sputter-coating of the sample. This advantage allows for analysis of an infected sample before and immediately after antimicrobial treatment. Thus, this study aimed to evaluate under controlled conditions of the biofilm dissolution and cleaning dentin, the effect of different concentrations of NaOCl and 2% chlorhexidine on intraorally developed biofilm. The influence of time was also studied. The hypothesis of the study was that biofilm dissolution depends on the irrigant solutions and contact time.

Materials and Methods

The irrigant solutions used in this study were 1% and 2.5% NaOCl (CloroRio, São José do Rio Preto, SP, Brazil), 5.25% NaOCl (Farmacia Específica, Bauru, SP, Brazil), and 2% chlorhexidine (Villevie, Joinville, SC, Brazil). One hundred twenty dentin sections (4 × 4 × 2 mm) were obtained from previously sterilized bovine radicular dentin. The samples were treated with 2.5% NaOCl for 15 minutes and 17% ethylenediaminetetraacetic acid for 3 minutes. Three samples were examined under the scanning electron microscope to verify the cleaning protocol. Eight to 12 dentin samples were fixed in each experimental procedure on a removable orthodontic device to allow the biofilm development (13). To standardize the rate of biofilm development, one healthy volunteer (heavy plaque-former) used the intraoral device for 72 hours. The subject maintained his routine consumption of food and drink.
The evaluation was performed by using the Adobe Photoshop (Adobe Systems, San Jose, CA) software, as previously described (17). The following terminology was used to quantify the content of the confocal pictures: biofilm, the presence of cells embedded in an extracellular matrix attached to the dentin; isolated cells, very sparse individual bacteria not embedded in an extracellular matrix; noncolonized dentin, the dentin presents absence of microbial structures.

For quantification purposes the confocal pictures were divided into 100 squares by using a digital grid (Fig. 1). The percentage of biofilm, isolated cells, or noncolonized dentin was calculated by placing the digital grid over the maximum projection confocal images to allow counting the points in the picture that coincided with either biofilm areas or areas showing dentin (17). Each digital square represented an area of $27.5 \times 27.5 \, \mu m^2$. Two single evaluators with experience in confocal/biofilm research classified the content of each of the 100 squares individually in each preirrigation and postirrigation picture. Appropriate interobserver reproducibility was confirmed by using the Pearson correlation coefficient. The values of $r$ ranged from 0.89–1.00, showing high correlation between the measurements.

The preliminary analysis of the raw pooled data did not show a normal distribution. Thus, the Kruskal-Wallis and Dunn tests were used for multiple comparisons among the groups and time. The influence of volume was determined by using the Mann-Whitney $U$ test. The level of significance was set at $P < .05$, and Prisma 5.0 (GraphPad Software Inc, La Jolla, CA) was used as the analytical tool.

**Results**

A total of 720 experimental pictures were evaluated. The Mann-Whitney $U$ test showed the absence of differences between the 500-$\mu$L and 1-$\mu$L groups. It means that the results were not dependent on the irrigant volume. Consequently, the data were pooled to provide a single median of 10 samples (30 pictures) per group.

Overall, the preoperative biofilm thickness ranged from 30–50 $\mu$m, without statistical significances among the groups (data not shown). The medians and 25%–75% percentiles of the studied parameters of biofilm, isolated cells, and noncolonized dentin are presented in Table 1. No significant differences in the preoperative parameters as a percentage of area covered by biofilm, isolated cells, or dentin were found among the groups ($P > .05$) (Table 1). The higher values of biofilm dissolution were found in the 30-minute NaOCl group and in the 5-minute and 15-minute groups of 5.25% NaOCl. The 2% chlorhexidine–treated samples showed the highest values for the biofilm parameter in comparison with all the NaOCl groups ($P < .05$), except the 5-minute group of 1% NaOCl ($P > .05$). The higher increment of noncolonized dentin was found after 30 minutes of NaOCl ($P > .05$). There was a significant increment of the isolated cells in 1% NaOCL–15-minute group as a result of incomplete biofilm disorganization ($P < .05$).

An intragroup time comparison showed that time does not have an effect on 2% chlorhexidine or 5.25% NaOCl in all the parameters tested ($P > .05$). In the 1% and 2.5% NaOCl groups, time significantly altered the parameters of biofilm and noncolonized dentin ($P < .05$). In both cases, the 5-minute period was significantly different from the 15-minute period, and this group was significantly different from the 30-minute groups with one exception; 5 minutes of 1% NaOCl had the same effect on the noncolonized dentin parameter as 15 minutes ($P > .05$). Representative pictures of the experimental groups are shown in Figure 2.

**Discussion**

Dissolution of biofilms by irrigant solutions is crucial because a significant area of the root canal system is inaccessible to endodontic


TABLE 1. Medians and 25% and 75% Percentiles of the Percentage of Biofilm, Isolated Cells, and Noncolonized Dentin before and after Contact with 2% Chlorhexidine, 1% NaOCl, 2.5% NaOCl, and 5.25% NaOCl

<table>
<thead>
<tr>
<th></th>
<th>5 Minutes</th>
<th>Treatment</th>
<th>15 Minutes</th>
<th>Treatment</th>
<th>30 Minutes</th>
<th>Treatment</th>
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<tbody>
<tr>
<td><strong>Biofilm</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>2% Chlorhexidine</td>
<td>92.00 (84.00–98.00)</td>
<td>89.50 (83.00–95.00)</td>
<td>93.50 (87.00–98.00)</td>
<td>96.00 (84.00–99.00)</td>
<td>92.00 (81.50–98.00)</td>
<td>86.50 (64.50–94.00)</td>
</tr>
<tr>
<td>1% NaOCl</td>
<td>94.00 (87.00–98.00)</td>
<td>71.50 (32.00–92.00)</td>
<td>30.50 (9.50–58.00)</td>
<td>95.50 (92.00–100)</td>
<td>3.00 (0.00–6.50)</td>
<td>1.50 (0.00–7.50)</td>
</tr>
<tr>
<td>2.5% NaOCl</td>
<td>95.00 (89.00–99.50)</td>
<td>60.50 (14.00–78.50)</td>
<td>12.00 (8.00–39.00)</td>
<td>96.00 (86.00–100)</td>
<td>1.50 (0.00–7.50)</td>
<td>2.00 (1.00–7.50)</td>
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<tr>
<td>5.25% NaOCl</td>
<td>92.00 (82.50–97.00)</td>
<td>3.00 (0.00–8.50)</td>
<td>5.00 (2.00–12.00)</td>
<td>93.50 (87.00–98.00)</td>
<td>5.00 (2.00–12.00)</td>
<td>2.00 (1.00–7.50)</td>
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| **Isolated cells** |           |           |            |           |            |           |
| 2% Chlorhexidine | 1.00 (0.00–5.00) | 2.50 (0.00–7.00) | 0.00 (0.00–1.00) | 0.00 (0.00–1.00) | 0.00 (0.00–1.00) | 0.00 (0.00–1.00) |
| 1% NaOCl       | 1.00 (0.00–5.00) | 1.00 (0.00–3.00) | 1.00 (0.00–3.00) | 1.00 (0.00–3.00) | 1.00 (0.00–3.00) | 1.00 (0.00–3.00) |
| 2.5% NaOCl     | 1.00 (0.00–4.50) | 7.50 (0.00–21.00) | 1.00 (0.00–10.00) | 1.00 (0.00–6.50) | 1.00 (0.00–6.50) | 1.00 (0.00–6.50) |
| 5.25% NaOCl    | 2.50 (0.00–10.50) | 0.00 (0.00–4.00) | 0.00 (0.00–4.00) | 0.00 (0.00–4.00) | 0.00 (0.00–4.00) | 0.00 (0.00–4.00) |

| **Dentin**     |           |           |            |           |            |           |
| 2% Chlorhexidine | 3.50 (0.50–9.50) | 6.00 (1.00–10.50) | 2.50 (0.00–13.00) | 4.50 (1.00–12.00) | 7.00 (0.00–17.50) | 10.50 (4.00–31.00) |
| 1% NaOCl       | 3.00 (0.50–8.50) | 24.50 (5.00–58.00) | 0.50 (0.00–5.00) | 36.00 (16.00–74.50) | 2.00 (0.00–11.50) | 96.00 (90.00–99.00) |
| 2.5% NaOCl     | 2.00 (0.00–5.50) | 31.00 (7.50–67.00) | 1.50 (0.00–12.00) | 83.50 (54.00–89.00) | 3.00 (0.00–8.00) | 96.00 (86.50–100) |
| 5.25% NaOCl    | 3.00 (0.00–7.00) | 96.00 (82.00–100) | 2.00 (0.00–5.50) | 93.00 (86.00–98.00) | 3.00 (1.50–9.50) | 94.00 (87.50–99.50) |

Different superscript letters in each column represent significant differences. Kruskal-Wallis/Dunn test (P < .05). (n = 10).
can allow a more accurate volumetric quantification of the residual biomass. As stated by Bryce et al (12), “the use of dentin to grow biofilms can result in difficulties with background fluorescence from the dentin, which autofluoresces, potentially obscuring software based bacterial counting”. For this reason, volumetric analysis was discarded, and 2-dimensional semiquantification was used. The methodology used is not so different from scanning electron microscope evaluation (10), with the advantage that the samples can be observed before the experimental protocol, allowing verification of an adequate standardization of the dentin infection before contact with the irrigant solutions. One limitation of this method is the possibility of overlapping, mainly between the categories, biofilm/isolated cells. However, the proportion of isolated cells was very low, between 0%–7% of the total area evaluated, and they were only more prevalent in the 1% NaOCl 15-minute group as a result of incomplete biofilm dissolution. In addition, the cleaning ability of the irrigation solutions on dentin can be clearly confirmed by evaluating the statistical increment of noncolonized dentin in the different experimental periods, which is also a good indicator that the biofilm/bacteria dissolution has occurred, because dentin can be easily differentiated from biofilm or cells.

The advantage of direct observation by using microscopic techniques is clear; the sample is not dispersed as in culture technique, and this allows information on the proportion of the colonized surface in the infected tissue. Future studies with the live/dead technique can allow studying the vitality of persistor cells on dentin specimens. Moreover, the effect of the biofilm thickness appears to be important with this model. In addition, the influence of anatomical variables needs to be further determined.

Under the conditions of this study, it can be concluded that NaOCl solutions were more effective than 2% chlorhexidine to eliminate biofilms. Thirty minutes of NaOCl are necessary to have the higher values of biofilm dissolution and to increase the cleaning of the dentin mainly in 1% and 2.5% concentrations, thus accepting the tested hypothesis. Under clinical conditions, a lower contact time in the apical third might be insufficient to allow a total disintegration of intraradicular biofilm.

**Acknowledgments**

The authors deny any conflicts of interest related to this study.

**References**


**Figure 2.** (A and B) Representative confocal pictures before and after treatment with 2% chlorhexidine (15 minutes); biofilm structure is not affected. The effect of 5 and 15 minutes of 1% NaOCl on biofilm dissolution can be seen in (D) and (F). Preoperative pictures are shown in (C) and (E). After 5 minutes there is partial dissolution of the biofilm structure and visible loss of the biomass. However, deep biofilm layers are still covering the dentin structure (D). After 15 minutes of 1% NaOCl, dentin structure is visible, but residual biofilm structures are persistent (F). After 5 minutes of 5.25% NaOCl, no biofilm structure could be found in this sample (H); only remnant isolated cells are visible (*). Preoperative picture is shown in (G). All bars represent 20 µm.