ABSTRACT

Background: Multi-specie biofilms are highly resistant to antimicrobials due to cellular interactions found in them. The purpose of this study was to evaluate, by confocal laser scanning microscopy, the biofilm dissolution effectiveness of different irrigant solutions on biofilms developed on infected dentin in situ.

Materials and Methods: A total of 120 bovine dentin specimens infected intraorally (30/group) were treated by the following solutions: 2% of chlorhexidine digluconate, 1%, 2.5% and 5.25% of sodium hypochlorite (NaOCl). The solutions were utilized for 5, 15 and 30 min with 2 experimental volumes 500 μL and 1 mL. All the samples were stained using an acridine orange and the biofilm thickness before (control group) and after the experiments were evaluated, utilizing a confocal microscope at ×40. The Mann-Whitney U and the non-parametric Kruskal-Wallis Dunns tests were utilized to determine the influence of the volume and to perform the comparisons among the groups respectively. The significance level was set at P < 0.05.

Results: Statistical differences were not found among the control and the 2% chlorhexidine digluconate groups at any experimental period (P > 0.05). The biofilm dissolution treated with 1% NaOCl was directly proportional to the exposure time (P < 0.05). The higher values of biofilm dissolution were found in 2.5% and 5.25% NaOCl groups (P > 0.05).

Conclusion: The higher exposure times and concentrations of NaOCl were not sufficient to dissolve 100% of the biofilm. However, all NaOCl solutions were more effective than 2% chlorhexidine digluconate to dissolve organic matter.

Key Words: Biofilm, chlorhexidine, confocal laser scanning microscopy, dentin, sodium hypochlorite

INTRODUCTION

The main objective of endodontic therapy is to remove pulp debris and bacterial populations from the root canal system. However, due to the complex anatomy of the root canal system, more than 50% of its walls remain uninstrumented during instrumentation.[1] The study of the relationship between endodontic therapy and microbial biofilms involves the observation of bacterial condensation in the root canal system, with or without endodontic therapy[2] and the ability of irrigant solutions or endodontic procedures to dissolve or eradicate them.[3]

The persistence of infection in the root canal with endodontic treatment may occur due to the existence of bacteria in the dentinal tubules or even by bacteria introduced into the root canal.[4,5] Bacteria and their products in avascular and necrotic root canal systems are the main etiological factor of apical periodontitis.[6,7]

Conventionally, root canal disinfection is performed using procedures that include chemomechanical
cleaning, shaping and the applications of chemical disinfectant solutions.[8] Although this technique is the standard procedure to disinfect root canals with necrotic pulp, in many occasions, it may fail to completely eliminate bacterial biofilms, mainly due to microbiological and anatomic factors.[2,9] Several studies have shown the antimicrobial effectiveness of sodium hypochlorite (NaOCl) and chlorhexidine,[10-13] but currently there are limited information concerning toxicity and relative biocompatibility.[15]

Although NaOCl is the irrigant commonly used in endodontic therapy, it is highly toxic to periapical tissues.[14] In order to overcome this disadvantage, the chlorhexidine gluconate was suggested as an alternative endodontic irrigant solution, due to its low toxicity and relative biocompatibility.[15]

NaOCl has great organic tissue dissolution ability by saponification reaction and also, has a wide-spectrum antimicrobial efficacy produced by the acid neutralization and chloramination reactions that occur in the presence of microorganisms and organic matter.[16]

Chlorhexidine gluconate is an antiseptic solution belonging to the biguanide group. It possesses a broad spectrum against Gram-positive and negative bacteria.[17] Its bactericide effect is caused by the disruption of the microbial cell membrane of the bacteria. However, the main limitation of chlorhexidine is its inability to dissolve organic matter.[13]

Confocal laser scanning microscopy (CLSM) presents advantages for the study of biofilms without the necessity of a specific treatment applied to the sample, such as dehydration or sputter coating, which are usually necessary when a conventional scanning electron microscope (SEM) is used.[18,19] This advantage allows for the analysis of infected dentin samples both before and after the treatment with antimicrobial compounds. The aim of this study was to evaluate the biofilm dissolution of different concentrations of NaOCl and 2% chlorhexidine digluconate on biofilms developed on infected dentin in situ. The influence of volume and contact time was also studied. The null hypothesis of this study is as follows: The biofilm dissolution is affected by different variables, such as, exposure time, concentration and volume of the solution.

**MATERIALS AND METHODS**

This research was approved by the Human Ethical Committee of the Bauru Dental School (Protocol 064/2009). The irrigant solutions used in this study were 1% and 2.5% NaOCl (Cloro Rio, São José do Rio Preto, SP, Brazil), 5.25% NaOCl (Farmacia Específica, Bauru, SP, Brazil) and 2% chlorhexidine digluconate (Villelev, Joinville, SC, Brazil). Freshly extracted bovine teeth were used. The crowns were sectioned using an Isomet saw (Buehler Ltd., Evanston, IL). The roots were then cut parallel to the tooth axis and the segments obtained were then cut perpendicular to the tooth axis. Through this dentin blocks were obtained (approximately 3 mm × 3 mm × 3 mm). The root canal remained intact and was discarded. The samples were autoclaved and treated with 2.5% NaOCl for 15 min and 17% ethylenediaminetetraacetic acid for 3 min.

In order to induce bacterial infection, 12 dentin samples were fixed on a removable orthodontic device with cavities. Each sample was attached into these cavities using sticky wax. The dentin surface in contact with the oral cavity was fixed approximately 1-2 mm above the surface in order to facilitate the accumulation of plaque and to avoid the “sweeping effect” produced by the tongue. This procedure was repeated until all the samples were completed. A healthy single volunteer used the intraoral device for 72 h to try to standardize the biofilm thickness as much as possible. During this time, the volunteer received a controlled diet and also maintained recommended oral hygiene practices. After this time period, the samples were removed and stained with 1 mL of 0.01% acridine orange for 15 min. Afterwards, the samples were rinsed with 100 mL of distilled water. The pre-irrigation samples were used as a control group.

One drop of oil objective (CLSM Leica TCS-SPE; Microsystems GmbH, Mannheim, Germany) was used to facilitate the biofilm visualization. This means that a thin layer of oil covered the biofilm. This oil lens application did not vary the biofilm morphology nor did it interfere with the action of the experimental solutions. This statement was previously demonstrated in a pilot study.

The specimens were immediately analyzed at ×40 via the CLSM technique. The biofilm was evaluated in several locations, to find the greatest points of thickness. Three segments per sample were analyzed. The Z-Stack of Leica Application Suite-Advance Fluorescence Software (LAS AF, Leica, Mannheim, Germany) was utilized to measure the thickness of the biofilm. The samples were scanned at intervals...
of 1 μm, from the upper biofilm level to the dentin surface.

When the height of pre-irrigation biofilm was recorded, the samples were randomly divided into 3 groups (n = 10) according to the contact time (5, 15 and 30 min) and subdivided into 2 subgroups (n = 5) according to the volume of the solution (500 μL or 1 mL), totaling to thirty samples per irrigant. The samples were immersed in 24-well tissue culture plates, containing the experimental solutions. In the 15-min and 30-min groups the irrigants were refreshed every 5 min in order to simulate clinical conditions.

After the experimental periods, the samples treated with NaOCl were washed with 100 mL of 5% sodium thiosulfate for 5 min. Chlorhexidine-treated samples were washed with 100 mL of distilled water. The samples were then immediately stained with the acridine orange dye and the post-treated biofilm was measured for thickness. Representative images of the samples both before and after treatment with the experimental solutions can be observed in Figure 1.

The Mann-Whitney U-test was utilized to determine the influence of the volume. The non-parametric Kruskal-Wallis and Dunns tests were utilized for comparisons among the groups and times because the data did not show a normal distribution. The level of significance was set at $P < 0.05$. The Prisma 5.0 (GraphPad Software Inc., La Jolla, CA) was utilized as the analytical software.

RESULTS

A total of 120 samples were evaluated. The Mann-Whitney’s U test showed statistically, that there were no significant differences between the volumes (1 mL and 500 μL) of the experimental solutions ($P > 0.05$). As a consequence, the data was combined to provide a single mean of 10 samples (30 scans) per group.

The medians and 25-75% percentiles of the percentage values of the thickness of the biofilm in μm, both before and after contact with the irrigating solutions, are shown in Table 1.

Nearly 2% chlorhexidine digluconate showed no effect on biofilm thickness, in comparison to the other evaluated irrigant solutions. No significant statistical differences were found between the 2% chlorhexidine digluconate groups and the pre-irrigation samples (control) at any time ($P > 0.05$). The biofilm thickness treated with 1% NaOCl for 5 min, was significantly higher than the 15 and 30 min groups. The 15 min group was significantly higher than the 30 min group ($P < 0.05$).

No significant statistical differences were found between the 2.5% NaOCl groups, at any time ($P > 0.05$). Almost 5% NaOCl groups showed the same results.

DISCUSSION

In the present study, the CLSM technique was used because it allows an optical sectioning of the sample. This eliminates the possibility of physical sectioning, as in conventional light and electron microscopic techniques. In addition, the optical sectioning can be used to record data from three axes (x,y and z), allowing to analyze the biofilm in depth or laterally.[20]

Regarding to in vitro studies, they are important to determine bacterial interaction that occurs in biofilm,[21] but are not able to accurately simulate bacterial growth conditions in the oral cavity such as, the different varieties of nutrients, saliva, pH and temperature changes. In order to solve these limitations, an in situ model developed to study tooth decay,[22] was modified for this kind of endodontic methodology.[23-25]

Currently, there is not enough substantial information, indicating that the bacterial growth within the root
Table 1: Medians and (25-75%) percentiles of the percentage of biofilm thickness in μm before/after contact with 2% chlorhexidine (A), 1% NaOCl (B), 2.5% NaOCl (C), 5.25% NaOCl (D)

<table>
<thead>
<tr>
<th>Groups</th>
<th>5 min</th>
<th>15 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Treatment</td>
<td>Baseline</td>
</tr>
<tr>
<td>A</td>
<td>36.0 (30.0-49.0)a</td>
<td>30.5 (23.5-49.0)a</td>
<td>30.0 (25.0-44.5)a</td>
</tr>
<tr>
<td>B</td>
<td>28.0 (23.5-60.0)a</td>
<td>18.0 (14.0-24.0)b</td>
<td>39.0 (28.0-45.5)a</td>
</tr>
<tr>
<td>C</td>
<td>31.0 (28.0-41.0)a</td>
<td>15.5 (13.0-21.5)b</td>
<td>30.5 (26.0-36.0)a</td>
</tr>
<tr>
<td>D</td>
<td>32.0 (28.0-37.0)a</td>
<td>13.0 (10.0-16.0)b</td>
<td>37.5 (30.0-50.0)a</td>
</tr>
</tbody>
</table>

Different superscript letters in each column represent significant differences (P < 0.05); (n = 10)

Table: Medians and (25-75%) percentiles of the percentage of biofilm thickness in μm before/after contact with 2% chlorhexidine (A), 1% NaOCl (B), 2.5% NaOCl (C), 5.25% NaOCl (D)

The percentage of biofilm thickness was measured before and after treatment with different concentrations of NaOCl and chlorhexidine. The results showed that 6% NaOCl was more effective than 2% chlorhexidine, while 1% NaOCl partially removed the biofilm. The biofilm remained intact when it was irrigated with 2% chlorhexidine.

Moreover, in the present study, we observed that NaOCl, in its lower concentration and exposure time (1% for 5 min), was more effective than the 2% chlorhexidine digluconate for biofilm thickness reduction. These results are in agreement with the results found by Bryce et al.\(^{33}\) and Chavez de Paz et al.\(^{10}\)

Finally, although several studies have shown that chlorhexidine has antibacterial capacities, there is
evidence to show its lack of dissolution capacity.[10,11,13,34] This statement is similar to the results found in the present study. Similarly, Shen et al.,[35] using a CLSM, observed that 2% chlorhexidine digluconate was not able to dissolve the biofilm in any of the 3 time periods studied (1, 3 and 10 min). In accordance with this statement, the major implication of residual biofilms is that they can act as a protective shield for bacteria within the dentinal tubules.[36] Therefore, residual biofilms can be considered as an organic layer, which may interfere with the adaptation and intratubular penetration of sealing materials.

CONCLUSION

Although the NaOCl solutions showed significant ability to dissolve biofilm, 30 min of exposure time was insufficient to completely remove organic matter regardless of the irrigant concentration. 2% chlorhexidine was not able to dissolve the biofilm at any time.

REFERENCES


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