UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

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# RESPOSTA DO BIOFILME DE *ENTEROCOCCUS FAECALIS* FRENTE A DIFERENTES PROTOCOLOS DE IRRIGAÇÃO DURANTE O PREPARO DO CANAL RADICULAR: ANÁLISE EM MICROSCOPIA CONFOCAL

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Orientadora: Profa Fabiana Soares Grecca

Co-orientadora: Daiana Elisabeth Bottcher

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*"Cada experiência é um degrau para o progresso da alma. Não fique preso ao passado. Você está, agora, diante de uma nova experiência. Dedique-se a ela de corpo e alma, e verá surgir o próximo degrau de evolução."*

Masaharu Taniguchi

## RESUMO

HOCHSCHEIDT, Gabriela Luiza. **Resposta do biofilme de *Enterococcus faecalis* frente a diferentes protocolos irrigação durante o preparo do canal radicular: análise em microscopia confocal.** 2013. 19 f. Trabalho de Conclusão de Curso (Graduação em Odontologia) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2013.

O objetivo deste estudo *in vitro* foi avaliar o efeito de diferentes soluções irrigadoras endodônticas auxiliares sobre um biofilme de *Enterococcus Faecalis (Ef)* através de Microscopia Confocal de Varredura a Laser (MCVL). Quarenta e cinco incisivos bovinos foram infectados com *Ef* por 21 dias. Os dentes foram divididos em 5 grupos: grupo 1: hipoclorito de sódio (NaOCl) 2.5% + EDTA, grupo 2: clorexidina (CHX) 2% gel + EDTA, grupo 3: CHX 2% líquida + EDTA, grupo 4: NaOCl 2.5% + EDTA + CHX 2% gel, grupo 5: NaOCl 2.5%+ EDTA + CHX 2% líquida e um grupo controle negativo e um positivo (NCG; PCG). As amostras foram coradas com SYTO9 e iodeto de propídeo e analisados em MVLC. A viabilidade bacteriana foi analisada quantitivamente pela proporção de bactérias vivas e mortas no biofilme remanescente. Escores foram padronizados de acordo com a carga bacteriana total – 1: ≤ 25%, 2: > 25 ≤50%, 3: > 50 ≤75%, 4: > 75% e debris – 1: ausência de debris; 2: presença de debris. A análise estatística foi realizada através do teste de Kruskal-Wallis e os testes exato de Fischer (P = 0,05). Não foram observadas diferenças estatisticamente significativas entre carga bacteriana total, debris e viabilidade bacteriana. Nenhuma das substâncias testadas foi capaz de eliminar completamente *Ef* dos canais radiculares.



Palavras-chave: *Enterococcus faecalis*. Irrigantes endodônticos. Microscopia confocal. Viabilidade bacteriana.

## ABSTRACT

HOCHSCHEIDT, Gabriela Luiza. **Response of *Enterococcus faecalis* biofilms to different associations of auxiliary substances during root canal preparation.** 2013. 19 f. Final Paper (Graduation in Dentistry) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2013.

This *in vitro* study evaluated the effect of different endodontic auxiliary chemical substances over *Enterococcus faecalis (Ef)* biofilm through confocal laser scanning microscopy (CLSM). Forty-five bovine incisors were infected with *Ef* for 21 days. Teeth were divided into five groups: group 1: 2.5% sodium hypochlorite (NaOCl) + EDTA, group 2: 2% chlorhexidine (CHX) gel + EDTA, group 3: 2% CHX liquid + EDTA, group 4: 2.5% NaOCl + EDTA + 2% CHX gel, group 5: 2.5% NaOCl + EDTA + 2% CHX liquid and a negative and a positive control group (NCG; PCG). The samples were stained with SYTO9 and propidium iodide and analyzed by CLSM. Bacterial viability was quantitatively analyzed by the proportions of dead and live bacteria in the biofilm remnants. Scores were standardized according to the total bacterial load (TBL) – 1: ≤ 25%, 2: > 25 ≤50%, 3: > 50 ≤75%, 4: > 75% and debris – 1: absence of debris; 2: presence of debris. Statistical analysis was carried out through the Kruskal-Wallis and the Fischer Exact Tests (P=0.05). No statistical differences were observed to total bacterial load, debris and bacterial viability. None of the tested substances could completely eliminate *Ef* from the root canal space.



Keywords: *Enterococcus faecalis*. Endodontic irrigants. Confocal microscopy. Bacterial viability.

**SUMÁRIO**

## 1 INTRODUÇÃO

A infecção bacteriana possui papel primário na etiologia de desenvolvimento da necrose pulpar, patogênese periapical (KAKEHASHI et al., 1965). A partir disso, um dos fatores cruciais para o sucesso do tratamento consiste na erradicação de microrganismos e de seus produtos do sistema de canais radiculares (GOMES et al., 2004).

Apesar do preparo do canal com agentes antimicrobianos poder contribuir para a redução da microbiota endodôntica (ESTRELA et al., 2007), a escolha da solução irrigante ideal e de sua concentração é difícil (ESTRELA et al., 2003).

Características como atividade antimicrobiana, habilidade em dissolver tecidos orgânicos, propriedade lubrificante e baixa citotoxicidade são recomendáveis para que as soluções químicas sejam utilizadas juntamente ao preparo mecânico, especialmente em canais radiculares com complexidade anatômica (SAFAVI; SPANGBERG; LANGELAND, 1990).

Entre as substâncias químicas auxiliares estudadas em endodontia, o hipoclorito de sódio (NaOCl), em diferentes concentrações, tem sido o mais comumente usado. Esse irrigante endodôntico tem a habilidade de destruir um amplo espectro de microrganismos e de dissolver materiais orgânicos; todavia, é sabido que NaOCl tem efeitos citotóxicos e que alteram os componentes orgânicos da dentina, especialmente o colágeno (RING et al., 2008).

O digluconato de clorexidina (CHX) é uma substância que tem sido usada em endodontia como alternativa ao uso do NaOCl. Ela se encontra tanto na forma líquida quanto gel. Entre as suas propriedades está o amplo espectro antimicrobiano, sua substantividade e baixa toxicidade (VIANNA et al., 2004; FERRAZ et al., 2001). Comparada ao NaOCl, a desvantagem da CHX é que ela não age como um solvente tecidual (OKINO et al., 2004). Em função disso, não fica claro se essa substância é efetiva durante o preparo químico-mecânico como uma alternativa ao uso do NaOCl (OKINO et al., 2004).

Os estudos demonstram que estas substâncias exibem potencial em eliminar bactérias, porém com resultados semelhantes e controversos. Vianna et al. (2006), em estudo *in vivo*, sugerem que o NaOCl 2,5% possui maior capacidade de inviabilizar patógenos endodônticos e de suportar a remoção celular do que a CHX 2% gel. Em um

estudo *in vitro*, o NaOCl também demonstrou melhor eficácia antimicrobiana (ESTRELA et al., 2003). Já Vianna et al. (2004) e Ferraz et al. (2007), também em estudos *in vitro*, verificaram o contrário, maior potencial antimicrobiano por parte da CHX 2% gel e líquida em relação ao NaOCl 2,5%. Esse tipo de diferença, segundo Estrela et al. (2003), pode ter sido causada por diferentes métodos experimentais, concentração, tipo de solução irrigante, ou período de análise.

Adicionalmente, além do uso individual, a combinação de NaOCl e CHX também tem sido advogada com o intuito de elevar suas propriedades antimicrobianas. Como irrigação final, a CHX poderia prolongar esta atividade, devido a sua substantividade (VIANNA e GOMES, 2009). Outros achados também demonstraram que a irrigação acrescida da CHX 2% resultou em uma melhor desinfecção do sistema de canais radiculares (ZAMANY; SAFAVI; SPANGBERG, 2003). A desvantagem da associação estaria na formação de um precipitado que diminuiria a permeabilidade dentinária (AKISUE et al., 2010), potencial tóxico e carcinogênico (BASRANI et al., 2007)

Vários estudos tem utilizado o biofilme de *Enterococcus faecalis (Ef)* para a avaliação da atividade antimicrobiana de soluções irrigadoras, pois Ef é um microrganismo cocco Gram-positivo comum em infecções endodônticas secundárias, sendo de difícil eliminação (MOLANDER et al., 1998; SEDGLEY et al., 2006).

Para a avaliação de biofilmes, a Microscopia Confocal de Varredura a Laser (MCVL) é uma indicação pois proporciona a análise da viabilidade bacteriana dentro de túbulos dentinários *in situ* (ZAPATA et al., 2008) e permite reconstruções tridimensionais com acurácia (SHEN et al., 2009).

Diante do exposto, torna-se necessário investigar o efeito antimicrobiano de soluções irrigadoras no preparo químico-mecânico durante o tratamento endodôntico utilizando a MCVL como ferramenta de avaliação.

## 2 OBJETIVO

O objetivo do presente estudo foi avaliar *in vitro* o efeito de diferentes substâncias químicas auxiliares e suas associações no tratamento endodôntico sobre um biofilme de *Enterococcus faecalis* por meio de Microscopia de Varredura a Laser Confocal.

## 3 ARTIGO CIENTÍFICO

Este estudo encontra-se publicado e disponível em Microscopy Research and Technique, New York, v. 76, no. 6, p. 658–662, Jun. 2013.

# Response of E. faecalis Biofilms to Different Associations of Auxiliary Substances During Root Canal Preparation: A Confocal Laser Microscopy Analysis

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KEY WORDS Enterococcus faecalis; endodontic irrigants; confocal microscopy; bacterial viability

ABSTRACT Objective: This in vitro study evaluated the effect of different endodontic auxiliary chemical substances over Enterococcus faecalis (Ef) biofilm through confocal laser scanning microscopy (CLSM). Methods: Forty-five bovine incisors were infected with Ef for 21 days. Teeth were divided into five groups: group 1: 2.5% NaOCl 1 EDTA, group 2: 2% CHX gel 1 EDTA, group 3: 2% CHX liquid 1 EDTA, group 4: 2.5% NaOCl 1 2% CHX gel 1 EDTA, group 5: 2.5% NaOCl 1 2% CHX liquid 1 EDTA and a negative and a positive control group (NCG; PCG). The samples were stained with SYTO9 and propidium iodide and analyzed by CLSM. Bacterial viability was quantitatively analyzed by the proportions of dead and live bacteria in the biofilm remnants. Scores were standardized according to the total bacterial load (TBL)—1: ≤25%, 2: >25 ≤50%, 3: >50 ≤75%, 4: >75% and debris—1: absence of debris; 2: presence of debris. Statistical analysis was carried out through the Kruskal–Wallis and the Fischer exact tests (P 5 0.05). Results: No statistical differences were observed to CFU, debris and bacterial viability. Conclusion: None of the tested substances could completely eliminate Ef from the root canal space. Microsc. Res. Tech. 76:658–662, 2013. © 2013 Wiley Periodicals, Inc.

## INTRODUCTION

The endodontic treatment aims to eliminate bacteria from the infected root canal and to prevent reinfection. However, it has been reported that bacteria may survive inside the root canal even after careful chemomechanical preparation (Bystr6m and Sundqvist, 1985).

Mature biofilms might develop their own localized environments that dictate the metabolic activities of cells and protect them to some extent against changes in the environment (Shen, Stojicic and Haapasalo, 2011). Enterococcus faecalis is a facultatively anaerobic, Gram-positive coccus that has been implicated in persistent root canal infections. Its ability to form biofilms may provide it an ecologic advantage, making it difficult to eliminate from root canal (Molander et al., 1998; Sedgley et al., 2006).

Different substances have been used to reduce the microbial load inside the root canal system (Peters et al., 2002). It is well documented that NaOCl, besides being capable to kill endodontic pathogens, has a solvent action over organic tissues (Vianna et al., 2006). It is also documented that, beyond having a broad antimicrobial spectrum (Ferraz et al., 2007), CHX also presents substantivity (Rosenthal et al., 2004). Finally, it is known that EDTA has the ability to remove smear layer (Zehnder et al. 2005, 2006). Nevertheless, these are individual characteristics of each substance. One of the possible ways to enhance root canal disinfection and, to take together the advantageous properties from each substance, would be its association, without interferences on dentin structure (Vianna and Gomes, 2009). In this regard, Zehnder (2006) reviewed the current literature and suggested an irrigation regimen in which NaOCl would be used throughout instrumentation, followed by EDTA, and CHX would be used as a final irrigant. This regimen would be specially indicated in those cases of endodontic treatment failure, where higher proportions of Gram-positive bacteria are expected.

Therefore, the aim of the present study was to evaluate the effect of different endodontic auxiliary chemical substances over Enterococcus faecalis biofilm through confocal laser scanning microscopy.

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## MATERIALS AND METHODS

This study was approved by the Research Committee of the Dental School, and by the Ethics in Research of the Federal University of Rio Grande do Sul (protocol number: 20169).

### Sample Preparation

Forty five sound bovine incisors, obtained from disposable carcasses from a local slaughterhouse, were selected and stored in 0.2% thymol solution. The teeth were removed from storage and immersed in 5% NaOCl (Biodinâmica, Ibiporã, PR, Brazil) for 30 min. The organic tissues were removed from the outer root surface with a scalpel blade (Becton Dickinson Industries Surgical Ltda. Juiz de Fora, MG, Brazil). They were cleaned with pumice and water and then stored in distilled water at 4ºC.

Crowns were removed through a horizontal cut with a carborundum disk (KGSorensen, Rio de Janeiro, RJ, Brazil). Root length was standardized at 15 mm (from the apex to the cervical board).

A #20 K-Flexofile (Dentsply Maillefer, Ballaigues, Switzerland) was used to remove the pulp tissue. It was introduced further into the root canal until its tip could be seen at the apical foramen. The root was copiously flushed with 2.5% NaOCl solution to improve pulp tissue removal. The working length was set at 14 mm.

A buccal and a palatal straight line were drawn on the external root surface with Indian ink.

A metallic disc covered with diamond was mounted on a precision cutting machine (Extec Labcut 1010, Enfield, CT). Orientation sulci in both buccal and palatal external root surfaces were done in each sample, following the previously drawn lines. The root was split vertically in two fragments.

The segments were placed in an apparatus in the same position before cutting. This apparatus consists on a 1.5-mL plastic tube (Eppendorf, CRAL, São Paulo, SP, Brazil) without its tip. The repositioned fragments were inserted in the tube. The root outer surface was covered with two layers of nail varnish (Impala, Laboratório Avamiller de Cosméticos, Guarulhos, SP, Brazil), except the 3 mm that surround the apical area. If a space was observed in the edge between the root fragments and the plastic tube, an additional layer of nail varnish was applied to avoid marginal leakage. The root canal was irrigated with 2.5% NaOCl and dried with sterile paper points (Tanari, Tanariman, Manacaru, AM, Brazil) to enhance debris removal and to determine if there was additional leakage in the interface between the tube and the samples. The apex was then sealed with two layers of nail varnish. The apparatus containing the teeth attached to microcentrifuge tubes were sterilized through hydrogen peroxide plasma exposure.

### Bacterial Strains and Culture Conditions

E. faecalis ATCC 8750 was cultured overnight at 37ºC in Tryptic Soy Broth (TSB, Merck KGaA, Darmstadt, Germany) in an atmosphere with 5% $CO_2$. The colonies were diluted into fresh TSB and incubated to match the turbidity similar to 0.5 McFarland standard, corresponding to an optical density of 0.036 absorbance, at 550 nm.

Each sample was inoculated with 1mL of TSB containing the microorganism for 21 days to allow biofilm formation (Zapata et al., 2008). The culture media was replaced each week. The samples were kept in previously sterilized 10-mL glass bijoux bottles (Wheaton SA of Brazil, São Bernardo do Campo, SP, Brazil).

### Disinfection Protocols

All the samples were prepared by the same researcher. The mechanical preparation was performed with stainless steel files (Flexofile, Dentsply, Maillefer, Ballaigues, Switzerland). The initial file was the first file that fit in the apical area, reaching all the working length. Four additional files were used to enlarge the apical region and to shape the root canal of each sample. A 10-mL disposable plastic syringe (BD Brasil, São Paulo, SP, Brazil) with a needle (Ultradent, Ultradent Products, South Jordan, UT) was used to perform irrigation. The eppendorf lid and the 5 mm that surrounded it were removed from the apparatus to allow the root canal preparation.

Teeth were randomly divided into control and test groups. The negative control group (NCG, n 5 5) had no root canal preparation and all the samples were sterilized. In the positive control group (PCG, n 5 5), samples were irrigated with sterile saline solution followed by 17% EDTA for 3 min. The five test groups were:

Group 1. Irrigation with 2 mL of 2.5% NaOCl between each instrument throughout the preparation, followed by a final rinse with 17% EDTA during 3 min.

Group 2. Irrigation with 2% CHX gel. The root canal was filled with 2% CHX gel. It remained inside the root canal for 1 min, during instrumentation with each file. The 2% CHX gel was removed with sterile saline, before each file was changed. A final rinse with 17% EDTA was performed for 3 min.

Group 3. Irrigation with 2 mL of 2% CHX liquid between each instrument throughout the preparation, followed by a final rinse with 17% EDTA during 3 min.

Group 4. Irrigation with 2.5% NaOCl during root canal preparation, followed by a rinse with 17% EDTA during 3 min. This solution was removed with 2 mL of sterilized saline solution and 2% CHX gel (2 mL) was applied for 1 min as a final chemical agent. Finally, the gel was removed with saline.

Group 5. Irrigation with 2.5% NaOCl during root canal preparation, followed by a rinse with 17% EDTA during 3 min. This solution was removed with 2 mL of sterilized saline solution. The root canal was filled with 2% CHX liquid for 1 min, followed by a final rinse with sterile saline solution.

### CLSM Analysis

After instrumentation of the root canal, each sample was removed from the apparatus and the two segments were separated (mesial and distal). One slice of the tooth was placed over a glass coverslip (20 mm in diameter and 0.17 mm thickness). For determination of bacterial viability, "Live/Dead" BacLight™ Bacterial Viability kit L-13152 (Molecular Probes, Eugene, OR) was used. The SYTO9 probe (excitation at 480 nm

TABLE 1. Percentage of nonviable bacteria[a] out of the total bacterial load after different irrigation protocols

| NaOCl | CHX Gel | CHX Sol | NaOCl 1 CHX Gel | NaOCl 1 CHX Sol | Sterile saline |
|---|---|---|---|---|---|
| 30.53% | 23.12% | 8.64% | 37.37% | 32.66% | 22.82% |

[a]Red fluorescent voxels.

and emission at 500 nm) labels all bacteria in a population, while the propidium iodide probe (excitation at 490 nm and emission at 635 nm) penetrates only bacteria with damaged plasmatic membrane (Vitkov et al., 2005). The dyes were applied in the root canal for 5 min at a 1:1 ratio (total volume 5 50 mL).

Fluorescence from the stained cell was viewed by using CLSM (Olympus Europa Holding GmbH, Hamburg, Germany). Simultaneous dual-channel imaging was used to display green and red fluorescence. The mounted specimens were observed by using 603 lens with an additional zoom of 33. CLSM images were acquired through the software Olympus FluorView Version 1.7.

For imaging capture, the last millimeter of the apical third was excluded because the chemo-mechanical preparation did not cover this area. The apical third of the root was evaluated. Ten-micrometer-deep scans (0.5-mm step size, 20 slices/scan) were obtained were obtained from specimen.

### Quantitative Analysis

For the viability evaluation, quantitative analysis was performed with the software bio Image_L (Chávez de Paz, 2009). Briefly, the bioimage-L software produces information of the total biofilm population as well as the independent subpopulations represented by red and green fluorescent colors. The reduction of biofilm live cells, determined by the LIVE/DEAD technique, was obtained by calculating the percentage of the biovolume of the red subpopulation from the total biovolume. Each solution was considered an experimental group. The results were analyzed by using Kruskall–Wallis test and post hoc analysis (IBM SPSS v. 20.0, SPSS for Mac; SPSS, Chicago, IL), when necessary, at a significance level of $P < 0.05$.

### Qualitative Analysis

The sample analysis also comprised the assessment of the total bacterial load and the amount of remaining debris. The fifth slice after the dentin tubules appearance in the acquired image was selected and two blind and calibrated evaluators, with experience in confocal/biofilm research, determined specific scores for each CLSM image and classified the content of each image. Appropriate interobserver reproducibility was confirmed by using the Pearson correlation coefficient. The values of r ranged from 0.93–1.00, showing high correlation between the measurements. The total bacterial load corresponded to the total number of cells, regardless their viability. Therefore, score 1 represented :;25% of the entire surface covered with viable/nonviable bacterial cells, 2: > 25 :; 50% of the entire surface covered with viable/non viable bacterial cells; 3: > 50 to :;75% of the entire surface covered with viable/nonviable bacterial cells; and, 4: >75% of the entire surface covered with viable/nonviable bacterial cells. The presence of pulp tissue remnants and dentin chips attached to the root canal was considered remaining debris. Score 1 represented the absence of debris, while score 2 represented the presence of debris.

For this analysis, statistics was carried out in the SPSS v. 20.0 software (SPSS, Chicago, IL). The Kruskal–Wallis test was employed to compare groups considering the total bacterial load and the amount of debris. The Fischer Exact Test was used to evaluate the association between the criteria despite the irrigant solution. The significance level was set at 5%.

## RESULTS

The results showed the absence of differences between the total bacterial load, the presence of debris among groups and the bacterial viability ($P > 0.05$). Table 1 presents the mean percent reduction of total bacterial load after the irrigation protocols. There was a positive association between the total bacterial load and the presence of debris (P 5 0.019). Representative pictures of the scores are shown in Figures 1 and 2.

## DISCUSSION

The control of microbial viability, the removal of debris and microbial cells should be achieved with the chemomechanical preparation, and seem to strongly contribute to a favorable outcome in endodontic treatments. In the present study, all the tested protocols seemed to possess limited chemical effect over E. faecalis biofilm structure during the instrumentation procedures. Also, the enhanced properties expected by the

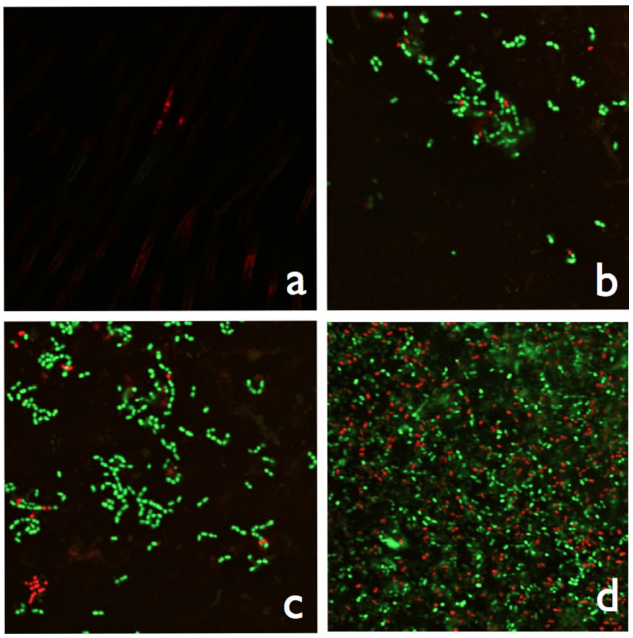


Fig. 1. Representative images for the assessment of the remnant bacterial load. (a) Score 1: :;25% of the entire surface covered with viable/nonviable bacterial cells. (b) Score 2: > 25 :; 50% of the entire surface covered with viable/nonviable bacterial cells. (c) Score 3: > 50 to :;75% of the entire surface covered with viable/nonviable bacterial cells. (d) Score 4: > 75% of the entire surface covered with viable/nonviable bacterial cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

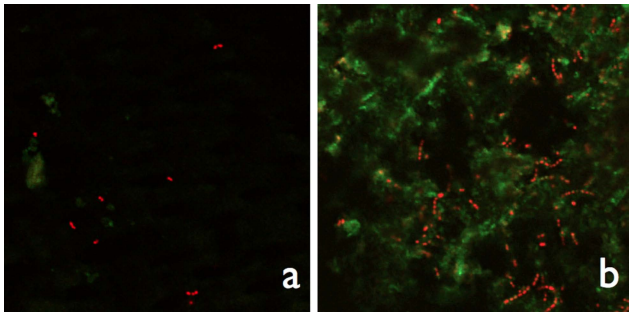


Fig. 2. Representative images for the assessment of the presence of debris. (a) Score 1: absence of debris. (b) Score 2: presence of debris. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

use of the 2% CHX final flush after the root canal preparation with NaOCl were not achieved.

The total bacterial load was assessed by the analysis of the root canal wall surface through the CLSM. It was represented by the surface area that was covered with E. faecalis biofilm, despite the cell viability. This analysis aimed on depicting the biofilm disruption ability that would be exerted by mechanical preparation associated with the auxiliary chemical substances. As reported by del Carpio-Perochena et al. (2011), disruption/dissolution of biofilms by irrigant solutions is crucial because such variables as the thickness and the age of the biofilm have an important impact on bacterial survival. In the present study, there was no statistical difference between groups, despite the protocol that was adopted. Possibly, the use of ultrasonic activation would be an alternative to supplement the antimicrobial effect of chemomechanical debridement.

Many root canal disinfectants present excellent antimicrobial activity in vitro whereas in vivo they often fail to completely kill all microbes. The antibacterial effect of sodium hypochlorite is well established. However, several factors can weaken the effectiveness of the disinfectants in the in vivo conditions, such as localization of microbes in the root canal system, poor penetration of the substance, low concentration, short exposure time, low overall volume of the irrigant, and the concentrations of bovine serum albumin (Pappen et al., 2010). Probably, if higher concentrated NaOCl solutions were used, they could physically disrupt and remove biofilms (Sena et al., 2006; Ma et al., 2011; Wang et al., 2012).

A number of laboratory studies have evaluated the efficacy of antimicrobial agents used in root canal treatment against E. faecalis biofilms at different stages of growth and on planktonic culture (Shen et al., 2009; Shen Stojicic and Haapasalo, 2011; Wang et al., 2012). According to Stuart (2003), the diffusion of substances inside the biofilm might be limited because fluid flow is reduced and diffusion distance is increased due to microbial growth. The maturity of the biofilm is known to influence its resistance to being killed by antibacterial agents (Shen, 2011). Bacteria in mature biofilm can resist the action of antibacterial irrigants and are remarkably difficult to eradicate, even though the reason for this resistance is not completely understood (Shen et al., 2011; Wang et al. 2012). To ensure that the tested substances would act against mature biofilms, we decided to use a 21-days biofilm.

The confocal laser scanning microscopy technique (CLSM) is a highly valuable approach to study the biofilm structure (Shen et al., 2009). It allows evaluating not only the surface analysis of the root canal, providing data regarding cells and debris distribution, but also determining the bacterial viability through imaging and live/dead staining protocols (Zapata et al., 2008). Another advantage of this technique is the wide evaluation of the intact biofilm, without the interference from the sample processing.

The use of dentin to grow biofilms can result in background difficulties, because the autofluorescence of dentin potentially obscures the bacterial count when the software-based analysis is performed (Bryce et al., 2011; Ma et al., 2011; Villette et al., 2008; Zapata et al., 2008). Although, Zapata et al. (2008) demonstrated that CLSM is able to show individual bacterial cells inside dentinal tubules and that the viability of bacteria in infected dentin can be determined in situ in an effective way. Our findings agree with Parmar et al. (2011) that the intensity of the background fluorescence was minimal within the tubules. It was also observed that compared with the control specimens treated with sterile water the low background fluorescence did not interfere with the fluorescent signal generated by the bacteria (Ma et al., 2011). Furthermore, the viable bacterial colonies have a unique round-shaped morphology, differing from both the green background and also from the debris.

Under the conditions of the present study, it may be concluded that none of the tested substances could completely eliminate Ef from the root canal space.

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## 4 CONSIDERAÇÕES FINAIS

A busca de uma solução irrigadora ideal na Endodontia ainda é um desafio. Apesar de o NaOCl ser o irrigante mais comumente utilizado para o preparo químico-mecânico dos canais radiculares, a CHX cada dia mais vem sendo uma alternativa, sendo inclusive, a solução irrigadora de primeira escolha em algumas escolas. Porém, devido a muitos resultados controversos encontrados na literatura, mais estudos sobre protocolos de irrigação se fazem necessários.

O controle da viabilidade microbiana, a remoção de debris e de células microbianas podem ser alcançados com o preparo químico-mecânico, e parece contribuir fortemente para um resultado favorável no tratamento de canais radiculares. No presente estudo, todos os protocolos testados parecem possuir efeito químico limitado sobre a estrutura do biofilme de *Enterococcus faecalis* durante os procedimentos de instrumentação. Também, os resultados esperados no uso do NaOCl 2,5% e irrigação final com a CHX 2% com o intuito de elevar suas propriedades antimicrobianas, não foram observados.

Em virtude disto, alternativas que possam contribuir na desinfecção e limpeza dos canais radiculares, como o uso de solução de NaOCl 5%, o uso de medicação intracanal entre as sessões e o uso de ultrassom durante o preparo químico-mecânico como coadjuvante podem ser avaliados em estudos futuros.

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