UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

JULIANA MARTINS SUMAN

O EFEITO DO ETANOL NA AÇÃO ANTIMICROBIANA DA CLOREXIDINA SOBRE *ENTEROCOCCUS FAECALIS*

Porto Alegre
2015

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Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Cirurgiã-Dentista.

Orientadores: Prof. Dr. Marcus Vinícius Reis Só e Prof. Dr. Francisco Montagner

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RESUMO

SUMAN, Juliana Martins. **O efeito do etanol na ação antimicrobiana da clorexidina sobre o *Enterococus faecalis*.** 2015. 27 f. Trabalho de Conclusão de Curso (Graduação em Odontologia) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2015.

Este estudo investigou o efeito do etanol 95% sobre as propriedades antibacterianas de clorexidina 2% (CHX) sobre biofilme de *Enterococcus faecalis* através de um método de cultura, e ao longo de múltiplas espécies biofilme através de microscopia confocal de varredura a laser (CLSM). Biofilme de *E. faecalis* foi induzido em 40 canais radiculares de dentes humanos extraídos. Os procedimentos de irrigação foram: S- solução salina; S/CHX- solução salina + CHX; E-etanol; e E/CHX- etanol + CHX. A coleta microbiana foi realizada em três períodos: antes (S1), imediatamente após (S2), e 72 h após a irrigação final (S3). Para o modelo de biofilme multiespécies induzido, 28 blocos de dentina bovina foram esterilizados e fixados em um dispositivo ortodôntico removível para permitir o desenvolvimento de biofilme intraoral. Sete amostras foram usadas em cada grupo. A análise estatística foi realizada por meio do teste KrusKal-Wallis e teste de Dunn para comparações múltiplas. Houve uma redução significativa na contagem de UFCs imediatamente após a irrigacao (S2) em todos os grupos experimentais ($P < 0,05$). No entanto, apenas S/CHX, E e E/CHX grupos tiveram contagem de UFCs perto de zero, sem diferenças entre elas ($P > 0,05$). Após 72h (S3), os grupos S/CHX e E/CHX tiveram contagens de UFCs perto de zero ($P > 0,05$). Houve um aumento de UFC no S3 para os grupos S e E ($P < 0,05$). A análise em microscopia confocal a laser mostrou que as percentagens de células vivas remanescentes foram semelhantes nos grupos S/CHX, E, e E/CHX ($P > 0,05$). O grupo S teve a maior porcentagem de células vivas ($P < 0,05$). O etanol de 95% não interferiu nas propriedades antibacterianas de CHX 2% sobre biofilmes de mono e multiespécies.

Palavras-chave: Biofilmes. Microscopia confocal. Irrigantes endodônticos. Clorexidina. Etanol. Paracloroanilina.

ABSTRACT

SUMAN, Juliana Martins. **The effect of ethanol on the antimicrobial activity of chlorhexidine on *Enterococcus faecalis*.** 2015. 27 p. Trabalho de Conclusão de Curso (Graduação em Odontologia) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2015.

This study investigated the effect of 95% ethanol on the antibacterial properties of 2% chlorexidine (CHX) over monospecies biofilm (Enterococcus faecalis) through a culture- based method, and over multispecies biofilm using confocal laser scanning microscopy (CLSM). For monospecies model, E. faecalis biofilm was induced in 40 root canals. The irrigation procedures were: S-saline solution; S/CHX—saline solution + CHX; E-ethanol; and E/CHX-ethanol + CHX. Microbial sampling was performed at three periods: before (S1), immediately after (S2), and 72 h after the final flush (S3). For multispecies biofilm model, 28 sterilized bovine dentin blocks were fixed on a removable orthodontic device to allow intraoral biofilm development. Seven samples were used in each group. Statistical analysis was carried out by using the Kruskal–Wallis test and Dunn's test for multiple comparisons. There was a significant reduction in CFUs count immediately after the final flush (S2) in all experimental groups ($P < 0.05$). However, only S/CHX, E and E/CHX groups had CFU counts close to zero, without differences among them ($P>0.05$). After 72h (S3), the S/CHX and E/CHX groups had CFU counts near zero ($P>0.05$). The CFU count increased in S3 for S and E groups ($P < 0.05$). CLSM showed that the percentages of remaining live cells were similar in S/CHX, E, and E/CHX groups ($P > 0.05$). The S group had the highest percentage of live cells ($P < 0.05$). The 95% ethanol did not interfere in the antibacterial properties of 2% CHX over mono and multispecies biofilms.

Keywords: Biofilms. Confocal microscopy. Root canal irrigants.

SUMÁRIO

1 INTRODUÇÃO

A irrigação endodôntica compreende uma das etapas do preparo químico e mecânico dos canais radiculares. Movimentação de partículas, ação antimicrobiana, solubilização de tecidos e detritos orgânicos e inorgânicos e lubrificação das paredes dentinárias são os seus objetivos fundamentais. É realizada antes, durante e após a conclusão da instrumentação endodôntica, empregando dispositivos e substâncias específicas para cada caso clínico (HAAPASALO et al., 2010).

O hipoclorito de sódio é universalmente aceito como a solução padrão para a irrigação dos canais radiculares. Entretanto, apresenta limitações diante de algumas condições microbianas endodônticas, elevado potencial alergênico e ação deletéria sobre a dentina radicular (ZHANG, 2010). Com a proposta de obter alternativas de irrigação, que sejam eficazes e desprovidas dos efeitos indesejáveis que o hipoclorito de sódio possui, tem sido recomendada a utilização do digluconato de clorexidina (ZEHNDER, 2006).

O digluconato de clorexidina é um composto bisguanida, com propriedade tensoativa catiônica, sendo utilizado no tratamento dos canais radiculares na concentração de 2% (ZEHNDER, 2006). Apresenta satisfatória ação antimicrobiana sobre *E. faecalis* e relativa baixa toxidade (LI; LIU; XU, 2012). Entretanto possui a desvantagem de praticamente não apresentar ação solvente de matéria orgânica nem ação neutralizante de produtos tóxicos de origem microbiana, tais como endotoxina (DE LA CASA et al., 2008; OLIVEIRA et al., 2007). Atualmente esse composto tem sido recomendado como agente de irrigação final, no intuito de contemplar as deficiências apresentar pelo hipoclorito de sódio (HAPASAALO et al., 2010).

Portanto, o protocolo de irrigação dos canais radiculares durante a fase de instrumentação poderá ser conduzida utilizando o hipoclorito de sódio. Por sua vez, após a conclusão desta fase, poder-se-ia realizar a toalete final com o digluconato de clorexidina (ZEHNDER, 2006). Entretanto, estas substâncias ao se combinarem, sejam simultaneamente ou em sequência, produzem um precipitado marrom-alaranjado (BUI; BAUMGARTNER; MITCHELL, 2008; TAY et al., 2006). Esse composto induz à descoloração dental e à obliteração dos túbulos dentinários, comprometendo a adesão do cimento obturador à dentina radicular (TAY et al.,

2006). Além disso, a paracloroanilina é citotóxica, sendo fundamental a sua eliminação do canal (BUI; BAUMGARTNER; MITCHELL, 2008).

Com o propósito de minimizar este inconveniente, tem sido proposta uma irrigação com o álcool absoluto entre cada uso da solução. Com esta conduta, não ocorre a precipitação de resíduos, contrário ao que ocorre quando utilizado a água destilada ou solução salina (MORTENSON et al., 2012). O ácido cítrico é outra opção que tem sido sugerida, porém, precipitando resíduo em menor intensidade (MORTENSON et al., 2012).

A formação do precipitado ocorre a partir da combinação de concentrações mínimas do hipoclorito de sódio, desde 0,023% a 6% (BASRANI et al., 2007). Diversas investigações foram realizadas para verificar a composição química deste precipitado (BASRANI et al*., 2007;* BUI; BAUMGARTNER; MITCHELL, 2008; BASRANI et al., 2010; THOMAS; SEM, 2010; MARCHESAN et al., 2007; BARBIN et al., 2008) sendo detectado a presença de Ca, Fe e Mg (NOWICKI; SEM, 2011). Inicialmente foi designado como sendo a para-cloroanilina (PCA) (NOWICKI; SEM, 2011), porém, atualmente, é sabido que o precipitado formado é composto de ao menos duas moléculas isoladas, menores e derivadas da clorexidina, designadas de para-clorofenil-uréia (PCU) e para-cloro-fenilguanidil- 1,6-diguanididil-hexano (PCGH) (KRISHNAMURTHY; SUDHAKARAN, 2010).

2 OBJETIVOS

OBJETIVO GERAL:

Avaliar o efeito da utilização do etanol na atividade antimicrobiana da clorexidina.

OBJETIVO ESPECÍFICO:

Verificar se o etanol prejudica a ação antimicrobiana da clorexidina sobre *Enterococcus faecalis*, através do grau de turbidez do meio de cultura e da contagem das unidades formadoras de colônias (UFCs).

3 ARTIGO CIENTÍFICO

O desenvolvimento do trabalho está apresentado na forma de artigo científico de periódico em inglês que foi publicado na revista Microscopy Research and Technique.

Title

Antibacterial Activity of Chlorhexidine After Final Irrigation With Ethanol: CLSM and Culture-Based Method Analysis

Introduction

In recent years, several substances have been investigated in endodontics for their use as final irrigants. Final irrigation attempts to optimize the irrigation provided by the main irrigant, and it is performed based on the associated use of two or more substances, after completing the root canal preparation (Alves et al., 2011; Baca et al., 2011; Paiva et al., 2012; Zehnder, 2006). These final irrigating substances aim, for example, to remove the smear layer (Zehnder, 2006), to dissolve remaining organic or inorganic tissue (Haapasalo et al., 2014), to reduce surface tension (Zehnder et al., 2005), and to enhance the antibacterial efficacy of the root canal treatment (Zehnder 2006). Alves et al. (2011) suggest that there may be a benefit of using passive ultrasonic irrigation (PUI) for the activation of sodium hypochlorite (NaOCl) followed by a final rinse with chlorhexidine (CHX) as supplementary steps in the treatment of infected oval-shaped root canals. Therefore, the combination of CHX, used as a final irrigant, with NaOCl, the main irrigating substance, provides benefits from both solutions.

On the other hand, authors have reported that when NaOCl and CHX come into contact, by-products might form (Prado et al., 2013; Rasimick et al., 2008). These by-products can be solid precipitates, in the form of a brownish-reddish mass (Zehnder, 2006), that are difficult to remove, and may occlude the dentinal tubules; they can also be toxic to the periapical tissues (Akisue et al., 2010; Prado et al., 2013; Vivacqua-Gomes et al., 2002).

To cope with this problem, some substances have been suggested as intermediate

irrigants to wash out the canal between the main and final irrigants (Prado et al., 2013). Although some studies have investigated the ability of intermediate irrigants to avoid precipitate formation, there is a lack of consistent evidence regarding the properties that might result from the combination of intermediate and final irrigating substances. One substance that may be used as intermediate irrigant is 95% ethanol (Krishnamurthy and Sudhakaran, 2010, Magro et al., 2015). Alcohol should be used as a flush between NaOCl and CHX, and it is indicated to remove residual NaOCl, preventing the formation of precipitates (Krishnamurthy and Sudhakaran, 2010). Nevertheless, many factors regarding the intracanal use of 95% ethanol are unknown, such as its influence on the antibacterial properties of CHX.

Therefore, the aim of this study was to investigate the effects of 95% ethanol on the antibacterial properties of CHX over monospecies biofilm (Enterococcus faecalis) using a culture-based method and over multispecies biofilm using confocal laser scanning microscopy (CLSM). The null hypothesis tested was that all irrigating procedures will promote similar antibacterial effect over mono and multispecies biofilm models.

Materials and methods

First, a monospecies biofilm model was used and extracted teeth were inoculated with Enterococcus faecalis (ATCC 29212). The second method inducted intraorally a multispecies biofilm. The data obtained over mono and multispecies biofilms were assessed by using colony forming units (CFUs) count and CLSM, respectively. The sample size was based on previous report from the literature [Magro et al. (2015) for the monospecies biofilm model; Del Carpio-Perochena et al. (2011), for the multispecies biofilm method], with a 95% level of confidence and power of the study equal to 80%. The study protocol was approved by the Ethics Committees in Research of the Federal University of Rio Grande do Sul (protocol no. 317.826).

Monospecies Biofilm Model and CFU Count

Forty human mandibular premolars were selected, cleaned, sterilized, and stored in 0.9% saline solution at 48ºC. All teeth were radiographed to confirm the presence of one canal, the absence of calcification, resorptions, immature foramen, and root canal filling. The crowns were removed and the working length was standardized at

12mm, 1mm shorter than the apical foramen as visually determined.

In order to standardize the width of the canals, they were instrumented under constant irrigation/aspiration with 2.5% NaOCl using the ProTaper UniversalVR system until F2 instrument. After preparation, the canals were irrigated with 17% EDTA (Biodinåmica, Ibiporã, PR, Brazil) and then irrigated with 5 mL of saline to wash out the EDTA. The apices were then sealed with composite resin (Magicfill Vigodent- Coltene, São Paulo, SP, Brazil) and the roots were sterilized using ethylene oxide. The efficacy of the sterilization was verified by inserting paper points into the canals, putting them into polypropylene tubes (Eppendorf do Brasil Ltda, São Paulo, SP, Brazil), and subsequently maintaining the tubes in an incubator at 37˚C for 7 days. The solution turbidity was evaluated every 24h.

The microbiological procedures were performed under aseptic conditions, in a laminar flow chamber. E. faecalis strain was used to infect the canals. A suspension was prepared by adding 1 mL of a pure culture of E. faecalis grown in brain–heart infusion broth (BHI; Difco, Detroit, MI) for 24 h at 37˚C in a bacteriological incubator. The concentration of the obtained microbial suspension was adjusted to 0.5 on the McFarland scale. Fifty microliters of the microbial broth was inoculated inside the canal and the roots were maintained for 7 days at 37˚C in the incubator.

Every two days, 25 µL of the microbial broth was removed from the root canals and replaced for fresh BHI broth q.s.p. to ensure cell nutrition and viability.

Microbial collection and counting were performed at three time points: before (S1), immediately after (S2), and 72 h after the final flush (S3). The initial sample (S1) was performed using three size 25 sterile paper points (Tanariman, Manacapuru, AM, Brazil) placed inside the root canal for 1 min and then transferred to a polypropylene tube (Eppendorf) containing 1 mL of sterile saline solution. The tubes were vortexed for 1 min (Vortex Ap 56; Phoenix, Araraquara, SP, Brazil). After decimal serial dilutions, aliquots were seeded into Petri dishes containing BHI agar (Difco) and incubated at 37˚C for 24 h to allow the counting of CFU. The culture purity was confirmed by the colony morphology, Gram staining, and the catalase test. No sample was discarded due to absence of culture purity.

After that, the irrigation procedures were performed as follows: each irrigant (5 mL)

was inserted into the root canal using a plastic syringe equipped with a NaviTipVR needle (Ultradent Products, Inc., South Jor- dan, UT). Once the irrigation was complete, the substance was then aspirated with an endodontic cannula. The four treatments used were: S—saline (5min); S/ CHX—saline (5 min) + CHX (5 min); E—ethanol (5 min); and E/CHX—ethanol (5 min) 1 CHX (5 min). After the irrigation procedures, S2 and decimal serial dilutions were performed identically to S1.

All canals were filled with 50 µL of BHI broth after S2 and the roots were stored at 37C. After 72 h, S3 was performed as described before. One evaluator blinded to the experimental groups performed the microbial counting always 24 h after the sample collections.

The intergroup analysis was assessed using Kruskal–Wallis and Dunn's tests to compare the CFUs' count after each microbial collection. Intragroup assessment was performed using Friedman's test to compare each protocol at different time points. The significance level was set at 5% for all statistical tests.

Multispecies Biofilm Method and CLSM Analysis

Twenty eight sterile bovine dentin blocks (3mm X 3mm X 2mm) were used. The samples were treated with 17% EDTA for 3 min to eliminate the smear layer produced during the sectioning process.

All dentin blocks were fixed on a removable orthodontic device to allow intraoral biofilm development (Del Carpio-Perochena et al., 2011). After signing an informed consent form, two healthy volunteers (aged 26 and 29) who had similar eating habits used the intraoral device continuously for 72h. Both volunteers only removed the intraoral device when consuming food and drink and when practicing oral hygiene. At the end of the infection period, the dentin blocks were removed from the orthodontic device and inserted into a polypropylene tube with 1 mL of BHI (Difco). The tubes were then stored in an incubator at 37˚C (Ordi- nola-Zapata et al., 2013).

After 24h, the dentin blocks were removed from the tubes and then rinsed with 1 mL of distilled water to remove the BHI medium and any non-adherent cells. Next, they were randomly divided in four groups (n=7): S—saline (5 min); S/CHX—saline (5 min) + CHX (5 min); E—ethanol (5 min); and E/CHX— ethanol (5 min) + CHX (5

minutes). Each specimen was immersed in 5 mL of the irrigant.

The analysis of biofilm viability was performed by using the SYTO 9/propidium iodide technique (Live/ Dead, Bacligth, Invitrogen, Eugene, OR). SYTO 9 is a green fluorescent stain that labels both live and dead microorganisms. Propidium iodide is a red fluorescent nucleic acid stain that only penetrates cells with damaged membranes (dead cells). First, the samples were cleaned with 2 mL of saline solution and then 0.25 µL of dye was placed over the biofilm. A CLSM (Olympus Fluoview 1000, Olympus Corporation, Tokyo, Japan) was used to visualize the samples (Ordinola-Zapata et al., 2012). The respective absorption and emission wavelengths were 494/518 nm for SYTO 9 and 536/ 617 nm for propidium iodide. The biofilm was randomly assessed at X60 magnification. Next, three confocal stacks from different random areas were obtained from each sample using a X40 oil lens, a 1- mm step size, and a format of 386 X 386 pixels. A total number of 21 stacks (3 operative fields X 7 specimens per group) were obtained for each group. The evaluator was blinded to the experimental groups. All images were analyzed using BioImage_L software (http://bio- image.com) for the total biovolume (mm^3), the total number of live cells (green), and the percentage of live cells (Chavez de Paz, 2009). The percentage of live cells (green) was assessed by using the Kruskal–Wallis test and Dunn's test for multiple comparisons (å = 5%).

Results

In the monospecies biofilm model, there was a significant reduction in CFUs count immediately after the final flush (S2) for all experimental groups ($P<0.05$) (Table 1). However, only the S/CHX, E and E/CHX groups presented counts near zero with no differences between them ($P>0.05$). After 72 h (S3), the S/CHX and E/CHX groups maintained CFUs' count near zero ($P > 0.05$) (Table 2). The CFUs' count increased significantly in S3 for S and E groups ($P < 0.05$).

CLSM showed that the percentages of remaining live cells were similar in the S/CHX, E, and E/CHX groups ($P>0.05$). The S group presented the highest percentage of live cells ($P < 0.05$) (Table 3). Table 3 also presents the total biovolume (μm^3 X 10^6), the biovolume of live cells, and the percentage of live cells (multispecies biofilm). Figure 1 presents CLSM representative images of multispecies biofilms after the four

irrigation procedures.

TABLE 1. Total percentage of remaining live cells after the microbial collection from the root canals in each group according to the experimental period: before final flush (S1), immediately after final flush (S2), and after 72 h (S3)

Irrigants	Experimental periods		
	S1	S2	S3
Saline	$100\%^{b}$	$9.5\%^{Ba}$	$33.9\%^{Bb}$
Saline + CHX	$100\%^{b}$	$0\%^{Aa}$	$0\%^{Aa}$
Ethanol	$100\%^{b}$	$0\%^{Aa}$	$34.3\%^{Bb}$
Ethanol + CHX	$100\%^{b}$	$0.1\%^{Aa}$	$0\%^{Aa}$

CHX, chlorhexidine.
Different superscript capital letters in each column represent statistical significance ($P < 0.05$), Kruskall–Wallis test and Dunn test ($\alpha = 5\%$).
Different superscript lower case letters in each row represent statistical significance ($P < 0.05$), Friedman's test ($\alpha = 5\%$).

TABLE 2. Comparison of CFUs between groups in each experimental period: before final flush (S1), immediately after final flush (S2), and after 72 h (S3)

Irrigants	S1	S2	S3
Saline	160^{B}	16.4^{Ab}	21.2^{ABb}
Saline + CHX	306^{B}	0.0125^{Aa}	0.0104^{Aa}
Ethanol	40^{B}	0.0009^{Aa}	22^{Bb}
Ethanol + CHX	128^{B}	0.8^{Aa}	0.01^{Aa}

CHX, chlorhexidine.
Different superscript capital letters in each row represent statistical significance ($P < 0.05$), Friedman's test ($\alpha = 5\%$).
Different superscript lowercase letters in each column represent differences ($P < 0.05$) after Kruskal–Wallis and Dunn post hoc tests ($\alpha = 5\%$).

TABLE 3. Median and percentiles P(25) and P(75) of cell biovolume and percentage of live cells on bovine dentine samples in each group

		Saline ($n = 7$)	Saline + CHX ($n = 7$)	Ethanol ($n = 7$)	Ethanol + CHX ($n = 7$)
Median, P(25), P(75)	Total biovolume ($\mu m^3 \times 10^6$)	16 (8.4/17.2)	21.2 (16.9/28.2)	21.4 (15.4/29.8)	15.9 (15.1/21.6)
	Biovolume of live cells ($\mu m^3 \times 10^6$)	15 (7.3/16.7)	13.7 (12.5/15.8)	13.8 (8.6/20.3)	13.8 (12.7/15.7)
	Live cells[a] (%)	93.4 (92.7/96.5)	79.6 (65.9/81.0)[b]	64.6 (53.3/75.7)[b]	76.3 (68.9/83.5)[b]

CHX, chlorhexidine.
[a](Biovolume of live cells/Total biovolume) × 100.
[b]Statistical significant difference in relation to saline group, Kruskall–Wallis test and Dunn test ($\alpha = 5\%$).

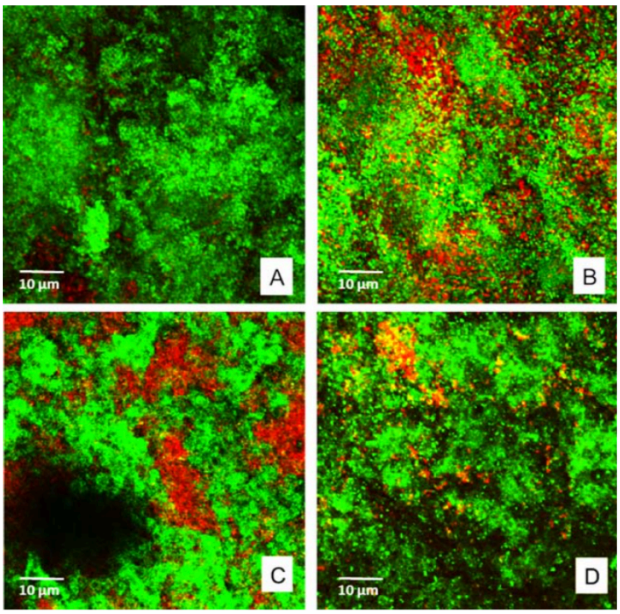

Fig. 1. CLSM representative images of multispecies biofilm model after the four irrigation procedures: (**A**) saline; (**B**) saline/chlorhexidine; (**C**) ethanol; and (**D**) ethanol/chlorhexidine. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

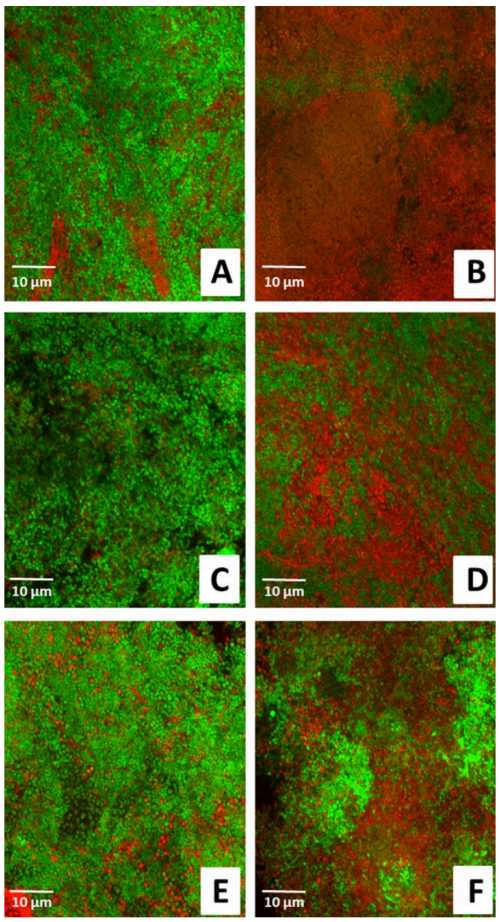

Fig. 2. Images from CLSM indicating predominance of live cells in deeper layers of biofilms (**A**, **C**, and **E**). In contrast, in biofilm superficial layers the irrigants were more effective in killing microorganisms (**B**, **D**, and **F**). A, B—Saline/CHX; C, D—ethanol; and E/F—ethanol/CHX. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Discussion

Alcohol has been indicated for use as an intermediate flush between NaOCl (main irrigant) and CHX (final irrigant) because it can prevent the formation of precipitates that occur as a consequence of mixing the two substances (Krishnamurthy and Sudhakaran, 2010). As reported previously, isopropyl alcohol prevented the formation of the insoluble neutral salt that results from the interaction of NaOCl and CHX (Krish- namurthy and Sudhakaran, 2010). Short et al. (2003) showed that 96% ethyl alcohol and 70% isopropyl alcohol were efficient in removing NaOCl crystals on the gutta-percha cones after immersion in 5.25% and 2.5% NaOCl for 1 min (Short et al., 2003). Thus, despite a recent study showed opposite results (Magro et al., 2015), 95% alcohol still seems adequate to remove residual NaOCl to prevent the formation of the precipitate (Krishnamurthy and Sudhakaran, 2010; Short et al., 2003). It is probable that the dilution of absolute alcohol with distilled water may compromise

their ability to remove the precipitates formed from the interaction between the NaOCl and CHX, despite the known antimicrobial activity of 70% alcohol (Magro et al., 2015).

Therefore, this study evaluated the influence of 95% ethanol on the antibacterial properties of CHX. The present results showed that the use of 95% ethanol as an intermediate irrigating substance did not affect the antibacterial properties of CHX over mono and multi-species biofilms (Tables (1–3)). The groups S and E received half the total volume of irrigation when compared to the groups S/CHX and E/CHX. There was a significant decrease in the microbial load for all the groups from S1 to S2, which can be associated with the mechanical effect of the irrigation. The increase of the CFU values at S3 was observed for groups S and E. The presence of residual microorganisms inside the root canal system and the limited antimicrobial actions of S and E may explain this behavior.

In the current study, results of viability for multispecies biofilm can be compared with those obtained at S2 in monospecies biofilm model because both assessed the antibacterial effect of the experimental protocols immediately after the contact of the microorganisms with the irrigants. In this scenario, both methods showed similar results. UFCs' count and CLSM assessments indicated that E, CHX, and E/CHX groups presented similar antibacterial properties ($P>.05$). The antibacterial effect of CHX has been well established in the literature (Du et al., 2014; Stojicic et al., 2013). Figure 1 shows a number of viable cells incompatible with Table 2, especially in the Saline 1 CHX and E 1 CHX groups. It is important to understand that when working with CSLM some images are captured in a deeper region of the biofilm where the chemical was not effective (Fig. 2). It should be recognized that no in vitro method can accurately reflect the conditions under which the microorganisms grow and develop within the root canal system. The multispecies biofilm model take into account that the endodontic pathogens are less susceptible to the action of antimicrobial agents when organized in communities adhered to the walls of the root canal. It can be one of the main reasons for the persistence of the microorganisms after disinfection protocols.

Another relevant finding from the present study was that 95% ethanol, when used alone, had antibacterial effects against mono and multispecies biofilms. It is well-

known that alcohol promotes disinfection of gutta-percha cones (Short et al., 2003). One explanation for this finding is that alcohol (both ethyl alcohol and isopropyl alcohol) is a tensioactive agent; it is highly electronegative (Krishnamurthy and Sudha- karan, 2010). Due to the low surface tension and the physical action of the irrigation procedure (irrigation/ aspiration), 95% ethanol significantly reduced the microbial count in the root canal at S2 (monospecies biofilm) and reduced the percentage of live cells (multi- species biofilm). On the other hand, Prado et al. (2013) observed that the association E/CHX produced a salt precipitation after 5s. The authors attributed the precipitate formation to the reduced solubility of the CHX salt in ethanol.

Microorganisms organized in biofilms may become established on any organic or inorganic surface substrate, and planktonic microorganisms prevail in a water-based solution (Mohammadi and Abbott, 2009). The literature has proven that when microorganisms are organized in biofilms, they are more resistant than the corresponding planktonic form of the same microorganism (Mohammadi and Abbott, 2009; Svensater and Bergenholtz, 2004). In this regard, the present study used both forms of microorganism organization. Therefore, mono and multispecies biofilms were induced and assessed using two different methodologies. Culture-based methods are useful due to their relative practicality and reduced cost. In addition to these advantages, they permit the ability to visually count the microorganisms; i.e., under ideal conditions of plating and incubation, the cells (or small cell clusters) grow singly, resulting in colonies known as CFUs (Fonseca et al., 2008). The use of CLSM is valuable for studying the structure of biofilms because it allows investigation of the intact biofilm without the interference of sample processing (Hohscheidt et al., 2013; Shen et al., 2009; Zapata et al., 2008). The immersion protocol employed to remove the multispecies biofilm formed over the dentin blocks did not allow to reproduce the dynamics of the irrigants that occur inside the root canal system. However, the aim of the study was to evaluate the antimicrobial properties of the irrigants alone or in association, and its potential residual effect.

The multispecies biofilm formation was inducted in a manner that was very similar to real clinical conditions. On the other hand, although the use of a monospecies biofilm (E. faecalis inoculated in the root canals) was useful for investigating the current

hypothesis, the use of a planktonic polymicrobial infection is suggested for further studies. Regarding the microbial counting performed at S3, this study showed that there was inhibition of microbial growth in the two groups with CHX (S/CHX and E/CHX). This inhibition probably occurred due to the residual effectiveness of the CHX, which is in line with the work by Baca et al. (2011). These authors incubated E. faecalis in extracted teeth to evaluate the antimicrobial effects of different final irrigation procedures. They concluded that an effective final irrigation regimen would be a combination of 2% CHX + 0.2% Cetrimide (a cationic surfactant agent), which exerts antimicrobial action over time.

Despite this advantage, the biocompatibility of ethanol when used as an endodontic irrigant is not yet well established (Krishnamurthy and Sudhakaran, 2010) and its effects on the periapical tissues remain a concern. Therefore, the results of the present study may not be directly applied as a clinical protocol. According to the results of the present study, 95% ethanol did not interfere in the antibacterial properties of 2% CHX solution over mono- and multispecies biofilms.

Acknowledgements

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

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4 CONCLUSÃO

Considerando as limitações deste estudo, é possível concluir que a utilização do Etanol 95% como irrigante intracanal intermediário, juntamente com o NaOCl e a CHX 2%, não interfere na ação antimicrobiana da CHX 2% sobre biofilmes mono e multiespécies. Portanto, avaliado como alternativa positiva para protocolos de irrigação.

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ANEXO A – PARECER CONSUBSTANCIADO DO CEP

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL / PRÓ-REITORIA DE PESQUISA -

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: O EFEITO DO ETANOL NA AÇÃO ANTIMICROBIANA DA CLOREXIDINA SOBRE Enterococcus faecalis

Pesquisador: Marcus Vinicius Reis Só

Área Temática:

Versão: 3

CAAE: 16298613.7.0000.5347

Instituição Proponente: Universidade Federal do Rio Grande do Sul

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 317.826

Data da Relatoria: 27/06/2013

Apresentação do Projeto:

Mantidas as especificações anteriores.

Objetivo da Pesquisa:

Avaliar o efeito do etanol na ação antimicrobana da clorexidina sobre o Enterococcus faecalis

Avaliação dos Riscos e Benefícios:

Apresentados de forma completa e adequada.

Comentários e Considerações sobre a Pesquisa:

Todas as solicitações do colegiado do comitê de ética foram atendidas.

Considerações sobre os Termos de apresentação obrigatória:

Todas as solicitações do colegiado do comitê de ética foram atendidas.

Recomendações:

Não existem recomendações adicionais.

Conclusões ou Pendências e Lista de Inadequações:

Não existem pendências.

Situação do Parecer:

Aprovado

Endereço: Av. Paulo Gama, 110 - 2º andar do Prédio da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Encaminhe-se.

PORTO ALEGRE, 27 de Junho de 2013

Assinador por:
José Artur Bogo Chies
(Coordenador)

Endereço: Av. Paulo Gama, 110 - 2º andar do Prédio da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

ANEXO B – TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Prezado Sr.(a),

Como é de seu conhecimento, existe a indicação terapêutica para a extração do (s) dente(s) _____, com o propósito de melhorar sua saúde, conforme registro no prontuário. Estamos realizando uma pesquisa com dentes extraídos intitulada: "O efeito do etanol na ação antimicrobiana da clorexidina sobre *Enterococcus faecalis*". Tal pesquisa tem por objetivo Avaliar o efeito do etanol sobre a ação antimicrobiana da clorexidina frente à *Enterococcus faecalis*, através do grau de turbidez do meio de cultura e da contagem das unidades formadoras de colônias (UFCs). Com este trabalho espera-se auxiliar os cirurgiões-dentistas a definir se a utilização do etanol como irrigante influencia a atividade antimicrobiana da clorexidina.

Pelo presente instrumento que atende as exigências legais, o(a) Sr.(a) _____ _____ portador da célula de identidade n^0 _____ e residente na Rua/Avenida _____ telefone ( )_____, ciente dos procedimentos à que será submetido, não restando quaisquer dúvidas a respeito do lido e explicado, firma seu CONSENTIMENTO LIVRE E ESCLARECIDO concordando em doar o(s) referido(s) dentes à pesquisa informada. Informamos que este(s) será(ão) utilizado(s) exclusivamente na pesquisa laboratorial a ser conduzida na Faculdade de Odontologia da UFRGS somente após certificação do Comitê de Ética responsável.

Caso tiver novas perguntas sobre este estudo e/ou sobre o órgão doado, poderá solicitar informações ao Prof. Marcus ViníciusReis Só (pesquisador responsável) no telefone (51) 33085357 ou para o Comitê de Ética e Pesquisa em Seres Humanos da UFRGS no telefone (51) 3308-3738.

Finalmente, ressaltamos que caso o(a) Sr.(a) não concorde em doar o(s) dente(s) para a pesquisa, não haverá qualquer interferência em seu atendimento odontológico.

Desde já agradecemos a atenção.

_____, ___ de _____ de 201__.

_____
Assinatura do doador ou responsável

_____
Assinatura e número do CRO do CD responsável pelo atendimento