Received: 20 June 2017 | Revised: 7 November 2017 | Accepted: 28 December 2017

DOI: 10.1002/jemt.22992

RESEARCH ARTICLE

WILEY MICROSCOPY RESEARCH & TECHNIQUE

Effect of ethanol on the antimicrobial properties of chlorhexidine over oral biofilm

Pedro Henrique Marks Duarte[1] | Paula Barcellos da Silva[1] | Ricardo Abreu da Rosa[1] | Francisco Montagner[1] | Marco Antônio Húngaro Duarte[2] | Milton Carlos Kuga[3] | Marcus Vinícius Reis Só[1]

[1]Department of Conservative Dentistry, School of Dentistry, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil

[2]Department of Dentistry, Endodontics and Dental Materials, University of São Paulo, São Paulo, Brazil

[3]Department of Restorative Dentistry, Araraquara Dental School, São Paulo State University, Araraquara, São Paulo, Brazil

Correspondence
Marcus Vinícius Reis Só, Rua Ramiro Barcelos, 2492, 90035-003 Porto Alegre, RS, Brasil.
Email: endo-so@hotmail.com

Review Editor: Prof. Alberto Diaspro

Abstract

The aim of this study was to evaluate the effect of 95% ethanol irrigation, with 5 or 10 min of action, on the antibacterial properties of 2% chlorhexidine (CHX), on oral biofilm, evaluated with confocal laser scanning microscopy (CLSM). Oral biofilm development was induced in 80 sterilized bovine dentin blocks, distributed in two groups (5 or 10 min) and 4 subgroups, according to time and the solution used: Saline (SALINE5, SALINE10); Saline followed by CHX (SALINE/CHX5, SALINE/CHX10); Ethanol (ETHANOL5, ETHANOL10), Ethanol followed by CHX (ETHANOL/CHX5, ETHANOL/CHX10). The surface of the block was dyed with *Live/Dead® BacLight*. Images from different areas were analyzed by BioImage L program. The total biovolum (μm^3), biovolum of live cells (green), percentage of live cells of the thickness of the biofilm visualized in CLSM and on surface biofilm were evaluated. Total biovolum and biovolum of living cells showed similar results among the different groups ($p > .05$). The percentage of living cells in total thickness of the biofilm also was similar among the groups ($p > .05$), except ETHANOL5, SALINE/CHX10, ETHANOL10, and ETHANOL/CHX10 that showed lower percentage than SALINE5 ($p < .05$). The ETHANOL10 and ETHANOL/CHX10 also showed lower percentage of living cells than ETHANOL/CHX5 and SALINE10 ($p < .05$). In relation to biofilm surface, SALINE/CHX5, SALINE/CHX10, ETHANOL5, ETHANOL10, ETHANOL/CHX5, and ETHANOL/CHX10 showed a lower percentage of living cells percentage than SALINE5 and SALINE10 groups ($p < .05$). Therefore, ethanol has no effect on antimicrobial properties of 2% chlorhexidine, prior when used as endodontic irrigating solution.

KEYWORDS
confocal microscopy, endodontics, root canal irrigants

1 | INTRODUCTION

The main goals of endodontic irrigation protocols are particle movements, antimicrobial action, tissue dissolution, and lubrification of the dentinal walls. It must be done during and after root canal preparation, using specific devices and chemical substances for each clinical case (Haapasalo, Shen, Qian, & Gao, 2010).

Sodium hypochlorite (NaOCl) is universally accepted as standard solution for endodontic irrigation of root canals due to its capacity to dissolve organic tissues and its antimicrobial property (Estrela et al., 2002). However, NaOCl presents limitations on some microbial endodontic conditions, high allergenic potential and deleterious effects over root dentin (Zhang et al., 2010). Besides, when in high concentrations, NaOCl is toxic to the periapical tissues (Pashley, Birdsong, Bowman, & Pashley, 1985).

Conversely, 2% Chlorhexidine (CHX) has been used after NaOCl to complement the deficiencies of each other (Haapasalo et al., 2010). Chlorexidine has low toxicity (Zehnder, 2006) and satisfactory action over *Enterococcus faecalis* (Li, Liu, & Xu, 2012). However, it has the disadvantage of not presenting organic tissues solving action and/or the capacity to neutralize toxic products from bacteria, such as endotoxin (De la Casa, Salas, Lopez, & Raiden, 2008; De Oliveira, Jorge, Carvalho, Koga-Lto, & Valera, 2007).

Despite this synergistic effect between both solutions, chlorexidine in contact with sodium hypoclorite produces a brown-orange precipitate

(Bui, Baumgartner, & Mitchell, 2008; Tay et al., 2006). This compound induces alteration of tooth color and the obliteration os dentinary tubules, compromising the adhesion of the endodontic sealer to the root dentin (Tay et al., 2006). Additionally, the p-chloraniline is a final product of the chemical reaction between both solutions, which has citotoxic effects to periapical tissues (Bui et al., 2008; Nowicki & Sem, 2011).

To minimize the formation of the precipitate, solution as saline solution, distilled water, citric acid, EDTA and, more recently, ethanol, have been studied as intermediate flush (Krishnamurthy & Sudhakaran, 2010). Krishnamurthy and Sudhakaran (2010) observed that absolute ethanol was the only solution that avoided the formation of the precipitate when compared to saline and distilled water. Other solutions, such as citric acid or EDTA also were recommended to avoid the formation of the precipitate, but Mortenson et al. (2012) showed that solutions also are ineffective for this purpose.

Although ethanol is the recommended solution for use as an intermediate solution, its effects and the influence of the previous time of activity on the antimicrobial property of chlorhexidine are still unknown. Therefore, the aim of this study was to evaluate if 95% ethanol changes the antimicrobial action of 2% chlorhexidine over oral biofilm, evaluated by confocal laser scanning microscopy (CLSM), in 5 and 10 min of contact with the tested solutions. The null hypothesis is that ethanol does not change the antimicrobial action of 2% chlorhexidine.

2 | MATERIAL AND METHODS

This study was approved by the Ethics Committees in Research of Federal University of Rio Grande do Sul (protocol- 708.353). Eighty bovine dentin blocks, with approximately 3 mm × 3 mm × 2 mm, were used. The samples were treated with 17% EDTA for 3 min to eliminate the smear layer produced during the sectioning process. The samples were placed on test tubes containing 5 ml of distilled water sterilized.

The blocks were fixed on a removable oral orthodontic device to allow intraoral biofilm development (Del Carpio-Perochena et al., 2011). Two healthy volunteers (aged 23 and 24) who had similar eating habits used the device continuously for 72 hr, and removed the intraoral device to consume food and drink and practice oral hygiene. At the end of the infection period, the blocks were removed from the orthodontic device and placed in a microtube (Eppendorf do Brazil, São Paulo, SP, Brazil) containing 1 ml of brain heart infusion (BHI) and stored in an incubator at 37°C, for 24 hr (Ordinola-Zapata et al., 2013).

After 24 hr the blocks were removed from the BHI and rinsed with distilled water, to remove nonadherent cells and BHI. The samples were randomly divided in four groups, according to chemical protocol (Saline and Ethanol, only or proceeded by 2% chlorhexidine, each with two subgroups ($n = 10$), according to irrigation time: SALINE5, Saline (5 min); SALINE10, Saline (10 min); SALINE/CHX5, Saline (5 min) + 2% Chlorhexidine (5 min), SALINE/CHX10, Saline (10 min) + 2% Chlorhexidine (10 min); ETHANOL5, Ethanol (5 min), ETHANOL10, Ethanol (10 min); ETHANOL/CHX5, Ethanol (5 min) + 2% Chlorhexidine (5 min), and ETHANOL/CHX10, Ethanol (10 min) + 2% Chlorhexidine (10 min).

Each sample was immersed in 5 ml of each irrigation solution. After the contact with the solution, the surface of the samples was cleaned with 2 ml of saline solution and then 0.25 μl of Live/Dead® BacLight Bacterial Viability (Invitrogen, Eugene, OR) dye was placed over the biofilm and it was submitted to analysis with CLSM (Olympus Fluoview 1000, Olympus Corporation, Tokyo, Japan).

The analysis of the biofilm viability was performed by using the SYTO9/propidium iodide technique (Live/Dead® BacLight Bacterial Viability), in which the SYTO9 is a green fluorescent stain that labels both live and dead cells and the propidium iodide is a red fluorescent nucleic acid stain that only penetrates cells with damaged cell membranes (dead cells).

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 100× magnification. Next, three confocal stacks from different random areas were obtained from each sample, with a 1 mm step size, and a format of 386 × 386 pixels. The evaluator was blinded to the experimental groups. All images were analyzed using BioImage_L software (http://bioimage.com) for the total biovolum (μm^3), the total number of live cells (green), and the percentage of live cells (Chavez de Paz, 2009). This analysis was performed in the total thickness of biofilm visualized in CLSM and of the most superficial portion of the biofilm.

The data obtained with the multispecies biofilm were analyzed using Bioestat 5.0 software (Instituto Mamirauá, Tefé, AM, Brazil). The normal distribution of data was confirmed by Shapiro-Wilk test. The percentage of live cells (green) was assessed by using the ANOVA (one way) and Tukey tests ($p = .05$).

3 | RESULTS

Thought the CLSM analysis in the thickness of the biofilm visualized, total biovolum (μm^3), and biovolum of living cells (μm^3) showed similar results among the different groups ($p > .05$). Conversely, the percentage of living cells in total thickness of the biofilm also was similar among the groups ($p > .05$), except ETHANOL5, SALINE/CHX10, ETHANOL10, and ETHANOL/CHX10 that showed lower percentage than SALINE5 ($p < .05$). Additionally, the ETHANOL10 and ETHANOL/CHX10 also showed lower percentage of living cells than ETHANOL/CHX5 and SALINE10 ($p < .05$). The mean and standard deviation of the total biovolum, biovolum of the living cells and percentage of living cells in the thickness of the biofilm visualized are described in Table 1. Figure 1 present a representative images of the 5 min groups, in which there is higher concentration of living cells (green) in the SALINE5 group and higher concentration of dead cells (red) in the ETHANOL5 group.

In relation to biofilm surface, total biovolum (μm^3) and biovolum of living cells (μm^3) were similar among the groups ($p > .05$). However, SALINE/CHX5, SALINE/CHX10, ETHANOL5, ETHANOL10, ETHANOL/CHX5, and ETHANOL/CHX10 showed a lower percentage of living cell percentage than SALINE5 and SALINE10 groups ($p < .05$). Table mean and standard deviation of total biovolum, biovolum of

TABLE 1 Mean and standard deviation of total biovolum, biovolum of the living cells, and percentage of living cells in the thickness of the biofilm visualized

			Saline	SALINE/CHX	ETHANOL	ETHANOL/CHX
5 min	Mean, (standard deviation)	Total biovolum (μm^3)	309.765 (135.154)	209.340 (40.111)	335.627 (177.559)	185.425 (62.661)
		Biovolum of living cells (μm^3)	300.386 (132.136)	119.487 (70.695)	147.752 (110.095)	117.736 (81.141)
		Living cells (%)	96.78 (2.76)	57.77 (32.83)	52.51[a] (27.14)[a]	73.16 (30.63)[a]
10 min	Mean, (standard deviation)	Total biovolum (μm^3)	263.778 (188.199)	320.903 (198.248)	335.369 (141.403)	205.677 (95.246)
		Biovolum of living cells (μm^3)	247.002 (187.542)	170.264 (149.738)	73.034 (123.231)	66104 (66.340)
		Living cells (%)	91.56 (10.32)	53.02 (21.15)[a]	26.98 (20.12)[a,b]	30.43 (19.48)[a,b]

[a]Statistical significant difference in relation to SALINE5 group.
[b]Statistical significant difference in relation to SALINE10 and ETHANOL/CHX5 groups.

living cells and percentage of living cells in the surface of biofilm are described in Table 2. Figure 2 present a representative images of the 10 min groups, in which there is higher concentration of living cells (green) in the SALINE10 group and higher concentration of dead cells (red) in the ETHANOL10 group.

4 | DISCUSSION

The use of ethanol, acting by contact either for 5 or 10 min did not affect the antimicrobial property of 2% chlorhexidine. The irrigation protocol using ethanol afforded the reduction of viable microorganisms in conditions similar to SALINE/CHX group.

In this study, it was used a intraoral device to allow growth of multispecies biofilm to analyses the antimicrobial action of irrigant solutions. This methodology seems to be the most indicated for this type of study because it represents what happens clinically in terms of microbial colonization. The formation of mixed biofilm was induced by microorganisms that, not only *E. faecalis*, which is a microorganism resist to the root canal treatment (Chavez de Paz, Bergenholtz, & Svensäter, 2010).

Besides, in biofilm form, microrganisms are more resistant to the antimicrobial agents when compared to planctonic microorganisms (Donlan & Costerton, 2002). Despite this advantages, it is important to highlight that this methodology does not emulate what occurs inside the root canal, where the action of the irrigant solution is hampered by the complexity of the root canals system and by the difficulty of difusion in the apical third (Susin et al., 2010).

The use of CLSM is useful to study biofilm structure, for it allows to investigate intact biofilms, without the interference of sample processing (Hohscheidt, Böttcher, Parolo, Montagner, & Grecca, 2013; Ordinola-Zapata et al., 2008; Shen, Qian, Chung, Olsen, & Haapasalo, 2009). The immersion protocol used does not reproduce the clinical intracanal irrigation. However, the aim of the study was only to evaluate the interference of ethanol on the antimicrobial action of chlorhexidine.

Chlorhexidine, in different concentrations is not capable of dissolving biofilm. Studies that used CLSM showed that CHX 2% seems to not cause any visible effect over the structure of the biofilm, specially in the areas with difficult access to endodontic instruments (Del Carpio-Perochena et al., 2011; Ordinola-Zapata et al., 2012). Ordinola-Zapata et al. (2012) asserted that, the antimicrobial action of CHX is expected in situations where dentinary walls are clean, biofilm-free.

Therefore, it should be used in association with NaOCl, being essential the use of an intermediate flush that removes the residual NaOCl from the root canals system to avoid the formation of a brown-orange precipitate. Krishnamurthy and Sudhakaran (2010) suggested the use of absolute alcohol, as intermediate flush between NaOCl and CHX, due to its ability to penetrate deeply to remove the residual NaOCl present in the canals and prevent the formation of the precipitate (*p*-chloraniline).

The results of this study are in agreement with the findings of Chavez de Paz et al. (2010), since the groups SALINE/CHX5 and

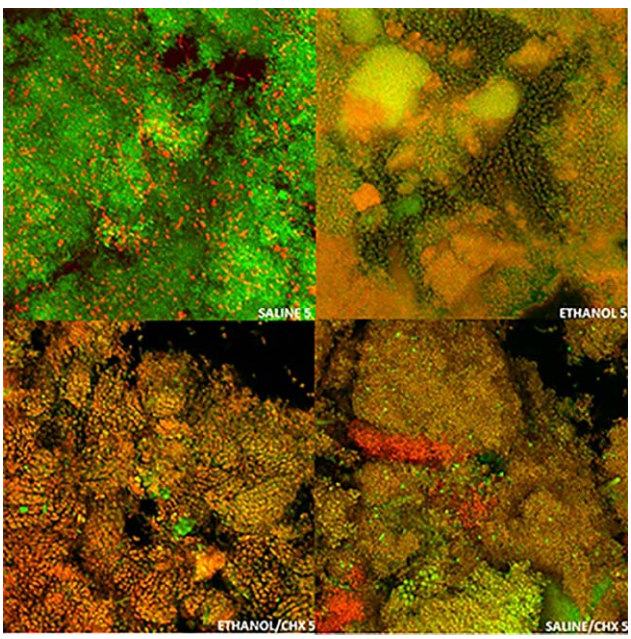

FIGURE 1 Representative images of the 5 min groups, in which there is higher concentration of living cells (green) in the SALINE5 group and higher concentration of dead cells (red) in the ETHANOL5 group [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Mean and standard deviation of total biovolum, biovolum of living cells, and percentage of living cells in the surface of biofilm

			Saline	SALINE/CHX	ETHANOL	ETHANOL/CHX
5 min	Mean, (standard deviation)	Total biovolum (μm^3)	20.701 (14.207)	10.399 (7.433)	8.864 (11.505)	10.819 (11.197)
		Biovolum of living cells (μm^3)	20.016 (13.625)	4.714 (4.708)	8.864 (11.505)	6.298 (8.934)
		Living cells (%)	97.10 (2.51)	42.68 (31.59)[a]	42.75 (24.89)[a]	50.45 (37.34)
10 min	Mean, (standard deviation)	Total biovolum (μm^3)	10.207 (12.300)	16.385 (15.288)	23.811 (19.113)	9.917 (13.823)
		Biovolum of living cells (μm^3)	9.158 (11.570)	2.876 (3.307)	3.574 (10.644)	1.474 (2.792)
		Living cells (%)	91.13 (14.57)	24.04 (10.48)[a]	23.70 (18.85)[a]	12.69 (14.65)[a]

[a]Statistical significant difference in relation to SALINE5 and SALINE10 groups.

SALINE/CHX10 presented percentage of living cells of 57.77% and 53.02%, respectively. Chavez de Paz et al. (2010) showed that the antimicrobial effects of CHX were not high in total biovolume. It was observed that the cells of the surface were more affected by CHX than the cells of deeper layers of biofilm. Zaura-Arite, Van Marle, and Ten Cate (2001) showed that with thicker biofilms, the effects of CHX decreases as it observes deeper layers, due to the lack of penetration in intact biofilm.

The present study sought to evaluate the effect of ethanol on the antimicrobial effect of chlorhexidine, in the thickness of the biofilm capable of being analyzed by confocal microscopy and in the most superficial layer of the biofilm, in 5 and 10 min of contact with the solutions. When it was evaluated only on the superficial layer of biofilm, the SALINE5 and SALINE10 presented higher percentage of living cells than the other experimental groups.

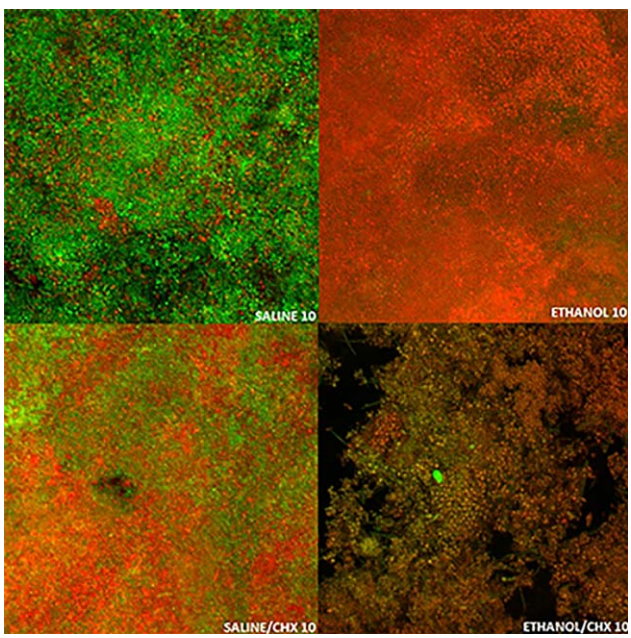

FIGURE 2 Representative images of the 10 min groups, in which there is higher concentration of living cells (green) in the SALINE10 group and higher concentration of dead cells (red) in the ETHANOL10 group [Color figure can be viewed at wileyonlinelibrary.com]

The results suggest a better antimicrobial action for ETHANOL10 and ETHANOL/CHX10 than SALINE10 and ETHANOL/CHX5 when evaluating on the thickness of the biofilm. SALINE/CHX10 group presented lower percentage of living cells than SALINE5 group. Therefore, ETHANOL, ETHANOL/CHX, and SALINE/CHX did not differ, in superficial and thickness analyses, in both experimental times, with the exception of ETHANOL/CHX5 group that differed from ETHANOL/CHX10 and ETHANOL10 groups.

The results of this study are in agreement with the finding of Suman et al. (2015), that evaluated the action of these combinations of irrigants with 5 min of contact. The groups analyzed were the same and the results were similar when it was analyzed the percentage of living cells, in the thickness of the biofilm (SALINE, 93.4%; SALINE/CHX, 69.6%; ETHANOL, 64.6%; ETHANOL/CHX, 76.3%).

Despite these results, the biocompatibility of ethanol, when used as irrigant, is not well established (Krishnamurthy & Sudhakaran, 2010) and its effects over the periapical tissues have not been studied sufficiently for ethanol to be used in a clinical protocol. Based on the methodology and results it is licit to conclude that, although it is not observed an influence of ethanol on the antimicrobial action of chlorhexidine, it is necessary caution to indicate ethanol as intermediate flush.

With these results it is possible to infere that ethanol does not interfere on the antimicrobial property of 2% CHX, both acting for 5 or 10 min prior when used as endodontic irrigating solution.

ORCID

Paula Barcellos da Silva http://orcid.org/0000-0001-9152-6849

REFERENCES

Bui, T. B., Baumgartner, J. C., & Mitchell, J. C. (2008). Evaluation of the interaction between sodium hypochlorite and chlorhexidine gluconate and its effect on root dentin. *Journal of Endodontics*, *34*(2), 181–185.

Chavez de Paz, L. E. (2009). Image analysis software based on color segmentation for characterization of viability and physiological activity of biofilms. *Applied and Environmental Microbiology*, *75*(6), 1734–1739.

Chavez de Paz, L. E., Bergenholtz, G., & Svensäter, G. (2010). The effects of antimicrobials on endodontic biofilm bacteria. *Journal of Endodontics*, *36*(1), 70–77.

De la Casa, M. L., Salas, M. M., Lopez, M. E., & Raiden, G. (2008). Protein content in irrigating solutions in contact with pulp tissue. *Acta Odontológica Latinoamericana*, *21*(1), 65–68.

De Oliveira, L. D., Jorge, A. O., Carvalho, C. A., Koga-Lto, C. Y., & Valera, M. C. (2007). In vitro effects of endodontic irrigants on endotoxins in root canals. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, *104*(1), 135–142.

Del Carpio-Perochena, A. E., Bramante, C. M., Duarte, M. A. H., Cavenago, B. C., Villas-Boas, M. H., Graeff, M. S., … Ordinola-Zapata, R. (2011). Biofilm dissolution and cleaning ability of different irrigant solutions on intraorally infected dentin. *Journal of Endodontics*, *37*(8), 1134–1138.

Donlan, R. M., & Costerton, J. W. (2002). Biofilms: Survival mechanisms of clinically relevant microorganims. *Clinical Microbiology Reviews*, *15* (2), 167–193.

Estrela, C., Estrela, C. R., Barbin, E. L., Spanó, J. C., Marchesan, M. A., & Pécora, J. D. (2002). Mechanism of action of sodium hypochlorite. *Brazilian Dental Journal*, *13*(2), 113–117.

Haapasalo, M., Shen, Y., Qian, W., & Gao, Y. (2010). Irrigation in endodontics. *Dental Clinics of North America*, *54*(2), 291–312.

Hohscheidt, G. L., Böttcher, D. E., Parolo, C. C. F., Montagner, F., & Grecca, F. S. (2013). Response of *E. Faecalis* biofilms to different associations of auxiliary substances during root canal preparation: A confocal laser microscopy analysis. *Microscopy Research and Technique*, *76*, 658–662.

Krishnamurthy, S., & Sudhakaran, S. (2010). Evaluation and prevention of the precipitate formed on interaction between sodium hypochlorite and chlorhexidine. *Journal of Endodontics*, *36*(7), 1154–1157.

Li, W., Liu, H., & Xu, Q. (2012). Extracellular dextran and DNA affect the formation of *Enterococcus faecalis* biofilms and their susceptibility to 2% chlorhexidine. *Journal of Endodontics*, *38*(7), 894–898.

Mortenson, D., Sadilek, M., Flake, N. M., Paranjpe, A., Heling, I., Johnson, J. D., & Cohenca, N. (2012). The effect of using an alternative irrigant between sodium hypochlorite and chlorhexidine to prevent the formation of para-chloroaniline within the root canal system. *International Endodontic Journal*, *45*(9), 878–882.

Nowicki, J. B., & Sem, D. S. (2011). An in vitro spectroscopic analysis to determine the chemical composition of the precipitate formed by mixing sodium hypochlorite and chlorhexidine. *Journal of Endodontics*, *37*(7), 983–988.

Ordinola-Zapata, R., Bramante, C. M., Cavenago, B., Graeff, M. S. Z., Gomes de Moraes, I., Marciano, M., & Duarte, M. A. H. (2012). Antimicrobial effect of endodontic solutions used as final irrigants on a dentine biofilm model. *International Endodontic Journal*, *45*, 162–168.

Ordinola-Zapata, R., Bramante, C. M., Minotti, P. G., Cavenago, B. C., Garcia, RB., Bernardineli, N., … Duarte, M. A. H. (2013). Antimicrobial activity of triantibiotic paste, 2% chlorhexidine gel, and calcium hydroxide on an intraoral-infected dentin biofilm model. *Journal of Endodontics*, *39*(1), 115–118.

Ordinola-Zapata, R., Bramante, C. M., Moraes, I. G., Bernardineli, N., Gasparoto, T. H., Graeff, M. S. Z., … Garcia, R. B. (2008). Confocal laser scanning microscopy is appropriate to detect viability of *Enterococcus faecalis* in infected dentin. *Journal of Endodontics*, *34*(10), 1198–1201.

Pashley, E. L., Birdsong, N. L., Bowman, K., & Pashley, D. H. (1985). Cytotoxic effects of NaOCl on vital tissue. *Journal of Endodontics*, *11* (12), 525–528.

Shen, Y., Qian, W., Chung, C., Olsen, I., & Haapasalo, M. (2009). Evaluation of the effect of two chlorhexidine preparations on biofilm bacteria in vitro: A three-dimensional quantitative analysis. *Journal of Endodontics*, *35*(7), 981–985.

Suman, J. M., Kuga, M. C., da Rosa, R. A., Santini, M. F., Grazziotin-Soares, R., Montagner, F., & Só, M. V. R. (2015). Antibacterial activity of chlorhexidine after final irrigation with ethanol: CLSM and cuture-based method analysis. *Microscopy Research and Technique*, *78*(8), 682–687.

Susin, L., Liu, Y., Yoon, J. C., Parente, J. M., Loushine, R. J., Ricucci, D., … Tay, F. R. (2010). Canal and isthmus debridement efficacies of two irrigant agitation techniques in a closed system. *International Endodontic Journal*, *43*(12), 1077–1090.

Tay, F. R., Pashley, D. H., Loushine, R. J., Doyle, M. D., Gillespie, W. T., Weller, R. N., & King, N. M. (2006). Ultrastructure of smear layer-covered intraradicular dentin after irrigation with BioPure MTAD. *Journal of Endodontics*, *32*(3), 218–221.

Zaura-Arite, E., Van Marle, J., & Ten Cate, J. M. (2001). Confocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm. *Journal of Dental Research*, *80*(5), 1436–1440.

Zhang, K., Tay, F. R., Kim, Y. K., Mitchell, J. K., Kim, J. R., Carrilho, M., … Ling, J. Q. (2010). The effect of initial irrigation with two different sodium hypochlorite concentrations on the erosion of instrumented radicular dentin. *Dental Materials*, *26*(6), 514–523.

Zehnder, M. (2006). Root canal irrigants. *Journal of Endodontics*, *32*(5), 389–398.

How to cite this article: Duarte PHM, da Silva PB, Rosa RA, et al. Effect of ethanol on the antimicrobial properties of chlorhexidine over oral biofilm. *Microsc Res Tech*. 2018;81:408–412. https://doi.org/10.1002/jemt.22992