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ORIGINAL RESEARCH

Antimicrobial Activity of Root Canal Irrigants associated with Cetrimide against Biofilm and Planktonic *Enterococcus faecalis*

[1]Camila Almeida Nascimento, [2]Mario Tanomaru-Filho, [3]Norberto Batista Faria-Junior
[4]Gisele Faria, [5]Juliane Maria Guerreiro-Tanomaru

ABSTRACT

Aim: To evaluate the antibacterial activity of sodium hypochlorite (NaOCl) and chlorhexidine (CHX) alone or associated with cetrimide (CTR), and QMiX against biofilm and planktonic *Enterococcus faecalis (E. faecalis)* [American type culture collection (ATCC) 29212].

Materials and methods: The solutions 2.5% NaOCl, 2.5% NaOCl + 0.2% CTR, 2% CHX, 2% CHX + 0.2% CTR, 0.2% CTR, and QMiX were evaluated. *E. faecalis* biofilms were induced for 14 days on bovine dentin blocks. The irrigants were evaluated after contact with *E. faecalis* suspension and biofilm for 1 and 3 minutes. After that, serial decimal dilutions were made and plated on tryptic soy agar (TSA) medium. Plates were incubated for 24 hours at 37°C and the colony-forming unit (CFU) 1 ml was determined. Data were subjected to ANOVA and Tukey's tests at 5% significance.

Results: All microorganisms were eliminated by direct contact of the irrigants with planktonic cells. Only NaOCl and NaOCl + CTR were able to completely eliminate the microorganisms by direct contact with *E. faecalis* biofilm. CHX presented effectiveness similar to CHX + CTR CTR, and QMiX after 1 minute of contact and similar to NaOCl and NaOCl + CTR after 3 minutes ($p > 0.05$), but was unable to completely eliminate the microorganisms. CTR and QMiX did not differ from each other.

Conclusion: CTR addition to CHX and NaOCl solutions did not improve the antimicrobial activity against biofilm. All evaluated irrigants and associations presented activity against planktonic *E. faecalis*. Only NaOCl and NaOCl + CTR eliminated biofilm after 1 and 3 minutes of direct contact.

Clinical relevance: Addition of CTR does not modify the antibiofilm effectiveness of CHX and NaOCl.

Keywords: Biofilm, Chlorhexidine, *Enterococcus faecalis,* Sodium hypochlorite, Root canal irrigant.

[1]MSc Student, [2,4,5]Professor, [3]PhD Student

[1-5]Department of Restorative Dentistry, Araraquara Dental School, São Paulo State University (UNESP), Araraquara São Paulo, Brazil

Corresponding Author: Mario Tanomaru-Filho, Professor Department of Restorative Dentistry, Araraquara Dental School, São Paulo State University (UNESP), Araraquara São Paulo, Brazil, Phone: 551633016390, e-mail: tanomaru@uol.com.br

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INTRODUCTION

Disinfection of the root canal system is essential for endodontic treatment success.[1,2] Irrigating solutions are used during root canal preparation in order to reduce the endodontic microbiota.[3,4] *E. faecalis,* a commonly detected microorganism in endodontic treatment failures[1,5] is able to survive under unfavorable conditions and organize in biofilm.

Sodium hypochlorite (NaOCl) in concentrations from 0.5 to 6% is the most widely used irrigant in endodontics due to its antimicrobial activity and ability to dissolve pulp tissue.[6,7] Chlorhexidine (CHX) is also recommended as a root canal irrigant, despite its lower antibacterial action against microorganisms in biofilm.[6]

Cetrimide (CTR) is a cationic surfactant used to decrease the cohesion between the extracellular matrix polymers and the bacterial cell wall.[8,9] Moreover, it can eliminate *E. faecalis* in biofilm after 1 minute of direct contact at a concentration of 0.2%,[10] and after only 30 seconds of contact at 0.5%.[11] CTR at 0.2%, associated with ethylenedia minetetracetic acid (EDTA) or citric acid at 15% eliminates biofilm after brief direct contact.[12] When associated with other solutions, CTR lowers the surface tension, conferring the irrigant greater ability to penetrate into the dentin tubules and anatomical irregularities of the root canal system.[9,13]

QMiX is an irrigating solution composed of CHX at 2%, EDTA and CTR.[14] This irrigant has demonstrated antibacterial activity comparable to NaOCl at 6% in dentin infected by *E. faecalis.*[9,13] QMiX showed stronger antibacterial activity against biofilm and planktonic *E. faecalis* in comparison with conventional CHX.[15] However, QMiX was unable to eliminate and remove microorganisms in biofilm from oral microbiota.[14]

The aim of the present study was to evaluate the antibacterial action of NaOCl at 2.5% and CHX at 2% associated

with CTR at 0.2%, and QMiX against biofilm and planktonic *E. faecalis*. The null hypothesis is that these solutions present similar effectiveness against the two forms of microbial organization.

MATERIALS AND METHODS

Direct Contact of the Bacterial Suspension with the Irrigating Solutions

The bacterial suspension was adjusted to 1.0×10^7 colony-forming units per milliliter (CFU ml^{-1}) using a spectrophotometer (600 Plus, Femto, São Paulo, SP, Brazil). Samples (1.45 ml) of each irrigant were placed in Eppendorf test tubes and 50 µl aliquots of *E. faecalis* suspension were added to each tube.[16] The direct contact periods of the irrigating solutions with the bacterial suspension ranged from 1 to 3 minutes. The decimal serial dilutions were performed using in the first three tubes a solution-specific neutralizing agent (Table 1). The fourth, fifth and sixth tubes contained sterile saline.

At the end of the process, three aliquots of 20 µl from each dilution were seeded on tryptic soy agar (TSA) plates and incubated at 37ºC under microaerophilic conditions. After 48 hours of incubation, the counting CFU were performed in order to determine the mean of the three areas of bacterial growth. The mean CFU counts were log 10 transformed and subjected to ANOVA and Tukey's tests at a significance level of 5%.

Direct Contact of the Biofilm with the Irrigating Solutions

Root segments from bovine central incisors were sectioned (Isomet—Buehler, Lake Bluff, IL, USA) to obtain blocks measuring 5 × 5 × 0.7 mm (width × length × thickness) and were sterilized by autoclaving.[17,18]

Enterococcus faecalis (ATCC- 29212) biofilms were allowed to grow on the dentin blocks in a shaking incubator for 14 days at 37ºC, under microaerophilic conditions.[18] The culture medium (BHI) of each specimen was replaced every 48 hours. The purity of the *E. faecalis* strain was tested by Gram stain and colony morphology.

For the direct contact test, the dentin blocks containing biofilm were immersed in 1 ml of each irrigating solution and combinations (Table 1) for 1 or 3 minutes. After the experimental period, each dentin block was rinsed under saline and transferred to test tubes containing glass beads (3 mm in diameter) and 1 ml of neutralizing agent.[15,16,19] Each tube was shaken for 60 seconds (Vortex AP 56, Phoenix, Araraquara, SP, Brazil) in order to resuspend the microorganisms attached to the dentin blocks. Then, serial decimal dilutions were prepared and plated on TSA medium.

RESULTS

Figures 1 and 2 show the mean $\log_{10}$ CFU ml^{-1} of *E. faecalis* after direct contact of the irrigants and associations with the bacterial suspension and biofilm for 1 and 3 minutes.

According to the results, all the irrigating solutions and associations were able to eliminate the planktonic microorganisms after 1 and 3 minutes of direct contact (Graph 1). NaOCl and NaOCl + CTR were able to eliminate the microorganisms in biofilm. CHX, CHX + CTR, CTR, and QMiX presented similar results after 1 minute of direct contact with

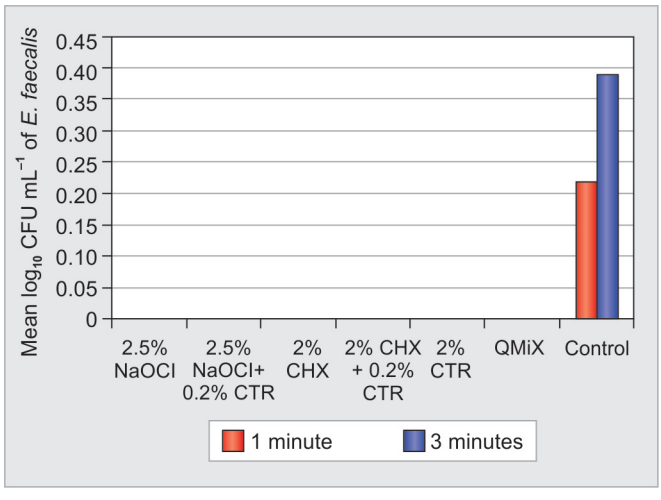

Fig. 1: Mean CFU ml^{-1} of *E. faecalis* after direct contact of the bacterial suspension with the irrigating solutions for 1 or 3 minutes

Table 1: Irrigating solutions and combinations, with their respective neutralizing agents (used after direct contact with bacterial suspension or biofilm)

Groups	*Irrigating solutions*	*Neutralizing solutions*
I	2.5% NaOCl*	1% sodium thiosulfate**
II	2.5% NaOCl + 0.2% CTR**	1% sodium thiosulfate + 3% tween 80 ** + 0.7% α-lecithin 0.7%**
III	2% CHX*	3% Tween 80 + 0.7% α-lecithin
IV	2% CHX + 0.2% CTR	3% Tween 80 + 0.7% α-lecithin
V	0.2% CTR	3% Tween 80 + α-lecithin at 0.7%
VI	QMiX***	3% Tween 80 + 0.7% α-lecithin
VII	0.85% Saline solution	0.85% Saline solution

*Compounding Pharmacy at the School of Pharmacy of Araraquara, Araraquara, SP, BR; **Sigma-Aldrich Brasil Ltda. São Paulo, SP, BR; ***Dentsply Tulsa Dental Specialties, Tulsa, OK, USA

biofilm ($p > 0.05$). CHX alone and CHX combined with CTR showed antibacterial activity similar to NaOCl and NaOCl + CTR ($p > 0.05$) after direct contact for 3 minutes, but were unable to eliminate *E. faecalis*. CTR and QMiX did not present significant difference from each other ($p > 0.05$). After 3 minutes of contact, QMiX had lower antibiofilm action than NaOCl and CHX, alone or associated with CTR (Graph 2).

DISCUSSION

Enterococcus faecalis has been used for evaluation of antimicrobial agents due to its association with endodontic treatment failures,[1,20] and ability to organize in biofilm. Hydroxyapatite,[16,18] human dentin,[21,22] bovine dentin[6,14] and polystyrene membrane[21] have been used as substrates for biofilm formation. *E. faecalis* biofilm shows organization after growing on bovine dentin blocks for 14 days.[18] Bovine dentin has been widely used[14,17,18] due to its similarities with human dentin. Biofilm maturity may influence its response to antimicrobial agents.[21,23,24]

The direct contact test quantifies viable microorganisms after different periods of contact with irrigating solutions.[10,25] The use of neutralizing agents prevents the occurrence of false negative results.[16,19,26] Tween 80 and lecithin are chlorhexidine-neutralizing.[4] Sodium thiosulfate inactivates sodium hypochlorite.[3,4,15,27]

In the present study, all the irrigating solutions and associations evaluated promoted elimination of planktonic microorganisms. Abdullah et al[26] observed that *E. faecalis* is eliminated after 1 minute of contact with NaOCl at 3%, and Gomes et al[28] verified elimination of planktonic microorganisms after less than 30 seconds of contact with CHX at 2%. However, Bidar et al[29] observed that 2.5% did not completely eliminate planktonic cells of *E. faecalis* in 15 minutes. When CHX was combined with CTR in concentrations lower than those used in the present study, the resulting solution demonstrated ability to eliminate two *E. faecalis* strains in planktonic phase after only 10 seconds.[30] QMiX showed similar results were observed after 5 seconds of contact.[15]

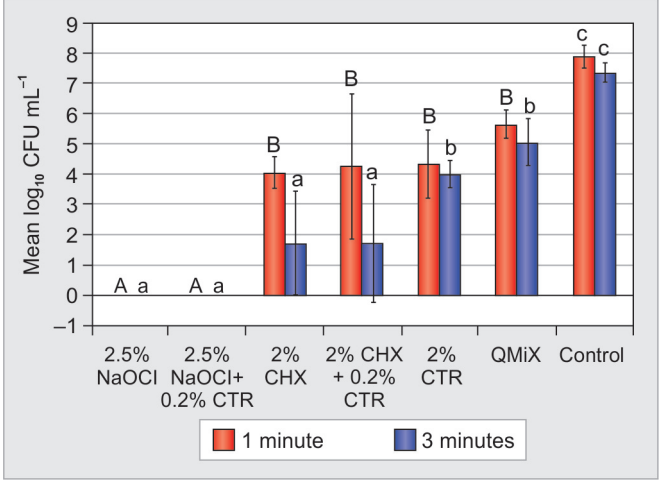

Fig. 2: Means and standard deviations for the CFU ml^{-1} of *E. faecalis* after direct contact of biofilm with the irrigants for 1 or 3 minutes. Upper case letters refer to the results for contact periods of 1 minute, and lower case letters indicate contact for 3 minutes. Different letters indicate statistically significant difference between the groups within the same experimental period ($p < 0.05$)

The results of the present study demonstrate that the irrigating solutions present lower activity against microorganisms in biofilm, confirming the greater resistance of bacteria in this form of bacteria in this form of organization.[15,26,30] NaOCl demonstrated effectiveness in eliminating *E. faecalis* biofilm after short contact periods, while CHX was unable to eliminate microorganisms after 1 minute. The limited ability of CHX to eliminate bacterial biofilm has been demonstrated by colony counting methodology,[26,31] scanning electron microscopy, and confocal laser scanning microscopy.[6,14,17,32] In the present study, despite its similarities with NaOCl, CHX was unable to eliminate the microorganisms after 3 minutes of biofilm contact. This observation is in agreement with Ariaz-Moliz et al,[11] who evaluated CHX in concentrations of up to 4% with contact periods of 2 minutes.

CTR has demonstrated antibiofilm activity. Baca et al[10] demonstrated the ability of CTR at 2% to eliminate *E. faecalis* biofilm after 1 minute of direct contact. These authors report antibiofilm action comparable to that of NaOCl at 1% and greater than CHX at 2%. CTR at 0.0078% eliminates microorganisms after 2 minutes of direct contact with *E. faecalis* biofilm, whereas CTR associated with CHX was capable of eliminate biofilm after 30 seconds of contact.[11] Addition of CTR enhances the antibiofilm activity of solutions such as EDTA, citric acid,[12] lactic acid,[25] and Biopure (MTAD).[16] The present study did not show increase in antibiofilm activity by the addition of CTR to CHX.

Stojicic et al[15] observed greater antibiofilm activity for QMiX compared with CHX after 1 and 3 minutes of contact with *E. faecalis* biofilm isolated from root canals (VP3-181 and Gel 31). In the present study, the contact of QMiX with biofilm for 1 minute did not show increased antimicrobial action, corroborating with Ordinola-Zapata et al,[14] who observed the poor cleaning ability when QMiX were applied for 5 minutes on multispecies biofilm.

Some dentin components may inactivate the action of endodontic antimicrobial agents.[30,33-35] Therefore, CHX, CTR and QMiX may have displayed lower antibacterial action due to the presence of bovine dentin used to substrate. Biofilm elimination has been observed in other studies, where polystyrene[11] and hydroxyapatite[15] were used as substrates.

Biofilm restricts penetration of antimicrobial agents into the bacterial cells due to the presence of a polymeric matrix

involving the microorganisms.[36] Accordingly, mature biofilms confer greater resistance against antimicrobial agents to bacterial cells, which are organized into overlapping layers deeply embedded in the matrix.[24,37] Thus, the use of mature biofilm (14 days old)[18] and dentin as the substrate may explain the difficulties for its complete biofilm elimination observed in the present study.

CONCLUSION

All evaluated irrigants and associations presented activity against planktonic *E. faecalis*. Only NaOCl and NaOCl + CTR eliminated biofilm after 1 and 3 minutes of direct contact. Addition of CTR does not modify the antibiofilm effectiveness of CHX and NaOCl.

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