In vitro study of the antimicrobial action of experimental intracanal medications on *Enterococcus faecalis* biofilm

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DOI: https://doi.org/10.14436/2358-2545.10.3.043-048.oar

ABSTRACT

Background: The elimination of Enterococcus faecalis of the root canals is fundamental for endodontic success, since these microorganisms are difficult to killed, especially when organized in biofilms. The search for drugs or their combinations that can eliminate these microorganisms is one of the main therapeutic aim. This study evaluated the antimicrobial action of experimental intracanal medications on Enterococcus faecalis biofilms. **Methods:** Forty uniradicular bovine teeth were used; their crowns were removed, and the roots were instrumented and sterilized. The roots were contaminated with suspension containing Enterococcus faecalis and kept in an oven at 37°C for 30 days. The roots were divided into 4 groups according to the intracanal medication: I- experimental medication 1 (0.2% CHX/metronidazole/doxycycline); II- experimental medication 2 (0.2% CHX/metronidazole/minocycline), III- 2% chlorhexidine (2% CHX), and IV- saline solution. The roots were sealed and kept in tubes containing TSB in an oven for 7 days. Dentin was collected and seeded for 24 h for perform of CFUs. The values obtained were compared using ANOVA and Tukeys tests ($p<0.05$). When comparing the results, there were no differences among groups I, II and III; however, they were significantly different from group IV. **Conclusion:** The experimental intracanal medications exerted an antimicrobial action on *Enterococcus faecalis* biofilms.

Keywords: Biofilms. Chlorhexidine. Doxycycline. Metronidazole. Minocycline.

How to cite: Machado NES, Mendes LO, Goldoni PR, Mori GG. In vitro study of the antimicrobial action of experimental intracanal medications on *Enterococcus faecalis* biofilm. Dental Press Endod. 2020 Sept-Dec;10(3):43-8. DOI: https://doi.org/10.14436/2358-2545.10.3.043-048.oar

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» The author Paulo Roberto Goldoni is responsible for formulating the medications used in this article, which he is interested in patenting in the future. The other authors report no commercial, proprietary or financial interest in the products or companies described in this article.

Submitted: August 22,2018. Revised and accepted: April 23, 2019

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Introduction

Enterococcus faecalis (E. faecalis) is a gram-positive coccus commonly associated with failure in endodontic treatments.[1,2] This microorganism has few requirements for growth and survival, can grow at temperatures between 10-45°C and survive with scarce nutrients, and can regulate pH, tolerating values higher than 11.5.[1] It also has the capacity to unite in biofilm form,[2,3] which is difficult to eliminate due to the presence of a mucopolysaccharide layer that protects the bacterial colonies from chemical agents.[4] Biofilms are commonly found in endodontic infections, especially in secondary or recurrent infections.[5]

Chlorhexidine is an antimicrobial agent of great importance in the endodontic clinic, and its efficacy is especially related to the elimination of *Enterococcus faecalis*.[6-8] This agent has been used as an irrigating solution and intracanal medication due to its antimicrobial capacity and its substantivity.[6-9] The chlorhecidine can be used in differents concentrations, however 2% is the concentration of the choice in endodontics.[11,12] The prolonged use of 2% chlorhexidine can generate such by-products as parachloroaniline and reactive oxygen species (ROS)[13-15] and, the search for products that can eliminate *Enterococcus faecalis* with decrease of side effects is benefit.

Intracanal medications that mix two or three antibiotics are becoming increasingly promising, especially for the decontamination of teeth with incomplete root formation.[16,17] Thus, in order to innovate and develop a medication effective against *Enterococcus faecalis* biofilms, this study proposed the association of two antibiotics with chlorhexidine at a lower concentration, culminating in the preparation of two experimental intracanal medications: experimental intracanal medication 1, composed of chlorhexidine at 0,2%, 2.5% metronidazole and 2% doxycycline, and experimental intracanal medication 2, consisting of 0.2% chlorhexidine, 2.5% metronidazole and 2% minocycline.

The use of the chlorhexidine in these medications is justified by its excellent ability to inactivate *Enterococcus faecalis*[6-8,11,12] and the fact that it potentiates its antimicrobial effect when used in association with other drugs. Clorhexidine's broad antimicrobial effect, spectrum of action, substantivity and ability to enter the dentinal tubules make it effective even in areas where mechanical preparation is not effective,[18-21] and the concentration of 0.2% was chosen to reduce the production of parachloroaniline and ROS.[13-15] Metronidazole has been suggested to have an antimicrobial action[15] on strains of Enterococcus faecalis.[21,22] The tetracycline derivatives used in the intracanal experimental medications were doxycycline (experimental intracanal medication 1) or minocycline (experimental intracanal medication 2). Khademi et al.[23] evaluated the action of doxycycline on *Enterococcus faecalis* in 2014 and verified that the substance has a significant antimicrobial action. Minocycline acts on bacteria of several species, including gram-positive, gram-negative, anaerobic, aerobic and others.[24]

Considering the factors discussed above, it is essential to determine the antimicrobial action of these experimental intracanal medications on *Enterococcus faecalis* biofilms present inside root canals. This study aimed to evaluate the antimicrobial actions of experimental intracanal medications on *Enterococcus faecalis* biofilms.

Materials and Methods

The methodology employed was adapted from the study of Elsaka and Elnaghy.[25]

Preparation of teeth

The study was conducted on 40 extracted single-rooted bovine teeth obtained from animals raised for human food and stored in saline solution (Darrow Laboratórios S.A., Rio de Janeiro/RJ, Brazil). The soft tissues and calculus adhered to the root surface were removed with periodontal curettes. Next, the teeth were stored in saline solution.

After that step, the tooth crowns were transversely sectioned with a carburundum disc (Dentorium, International, New York, USA) mounted in a low speed handpiece at a standardized length of 17 mm. The root length was confirmed by the introduction of a size 15 K-file (Dentsply Maillefer, Ballaigues, Switzerland) with a rubber stop; when the file tip passed the apical foramen, the stop was placed over the cervical edge of the root, and the length was noted. Roots with a total length different than 17 mm were discarded and replaced.

The biomechanical preparation was accomplished manually to 1 mm from the apex (16 mm) with the fabrication of an apical stop up to a #80 K-file and step-back up to a #120 K-file. Irrigation was performed at

each instrument change with 2.5% sodium hypochlorite using a 5 ml plastic syringe (Ultradent Products Inc., Salt Lake City, Utah, USA) and Navi Tip 30G (Ultradent Products Inc., Salt Lake City, Utah, USA). After the biomechanical preparation, a 15 K-file was introduced in the total length of the root canal (17 mm) to clean the apical foramen. Subsequently, the root canals were dried with absorbent paper points nº 80 (Tanariman Industrial Ltda., Manacapuru/AM, Brazil) and filled with 17% EDTA solution (Odahcam Dentsply Indústria e Comércio Ltda., Petrópolis/RJ, Brazil) for 3 minutes. After this period, the root canals were washed with saline solution. In sequence, the specimens were kept in an ultrasonic bath for 5 minutes, dried, wrapped in grade paper and autoclaved at 121°C for 20 minutes.

Contamination of specimens with Enterococcus faecalis

Twenty-five microlitres of 1.5 x 10^8 of *Enterococcus faecalis* suspension (ATCC®29212), according of McFarland' scale 1, was introduced into forty individual tubes containing 5 ml of tryptone soya agar (TSB) (Himedia Laboratories, Mumbai, India). After this step, one tooth was individually placed in one tube; these tubes were centrifuged and kept in an oven at 37°C for 30 days for the formation of a biofilm. At 15 and 30 days after the introduction of the teeth into the culture medium, one sample of TSB present in each of the tubes containing tooth and microorganism was collected and seeded in a petri dishes containing BHI agar (Himedia Laboratories, Mumbai, India); these petri dishes were stored in an oven at 37°C for 24 hours to check the growth or not of colony forming units. The results were positive to microbial growth in all specimens.

Antimicrobial test

After 30 days, the teeth were randomly divided using a random allocation software (http://www.openepi.com/Menu/Open EpiMenu.htm) in 4 groups according to the intracanal medication analysed. The following groups were defined:

» group I (n=10): experimental medication 1 (CHX 0,2%/metronidazole, doxacycline); this is a mixture of 0.2 CHX, 2.5% metronidazole and 2% doxycycline (Pharmacotécnica Fórmulas, Tupã/SP, Brazil).

» group II (n=10): experimental medication 2 (CHX 0,2%/metronidazole, minocycline); this is characterized by a mixture of 0.2 CHX, 2.5% metronidazole and 2% minocycline (Pharmacotécnica Fórmulas, Tupã/SP, Brazil).

» group III (n=10): 2% CHX (CHX 2%) (FGM Produtos Odontológicos, Joinville/SC, Brazil) (positive control).

» group IV (n=10): saline solution (negative control).

Initially, the apical foramen was sealed with glass ionomer (FGM Produtos Odontológicos, Joinville/SC, Brazil). Then, the root canal was irrigated with saline solution and dried with sterile absorbent paper points nº 80. Thereafter, the root canals were completely filled with intracanal medications according to the experimental groups, and the coronal surface of the root canals were sealed with glass ionomer. Subsequently, the teeth were placed in flasks containing 30 ml of TSB and kept in an oven at 37°C and 100% humidity for 7 days.

After that, the teeth had their coronary seal removed and the root canal irrigated with sterile saline. Afterwards, the root canals were dried with sterile absorbent paper points nº 80 and part of the dentin of these teeth was collected at 400 µm depth with #4 Peeso drills (Dentsply, Petrópolis/RJ, Brazil), which were introduced in the work length of the root canal (16 mm), and mounted in a rotary motor at a speed of 800 rpm and torque of 5.2 N/cm^2.The collected dentin was placed in a sterile tube containing TSB; these tubes were centrifuged and serially diluted up to 1:32 in saline solution. Thereafter, 5 µL of the 1:32 dilution were placed in a sterile petri dish containing BHI agar using an automatic micropipette, plated over the surface of sterile agar using a sterile bacteriological loop and then kept in an oven at 37°C for 24 hours to check the growth of colony forming units (CFUs).

An experienced blinded examiner determined the CFUs using a colony counter (Lupa Bender Indústria e Comércio S/A, Guarulhos/SP, Brazil). The amount of bacteria present in each CFU was determined from the concentration of the bacterial suspension used and the dilution after the collection of the contaminated dentin. Thus, each CFU contained approximately 2929 bacteria and it was expressed in $\log_{10}$ ($\log_{10}$ = 3.466).

The obtained values were recorded in a specific table, converted into $\log_{10}$ and statistically compared by one-way ANOVA and Tukey test at a significance level of 5% ($p<0.05$).

Results

The amount of the colony forming units (CFUs) are shown in table 1.

Group I (experimental medication 1 - 0.2% CHX/metronidazole/doxycycline) presented a small number of CFUs, since they were present in only 2 Petri dishes analysed, totalling 0.837 CFUs on average. Converting into log10, a value of 2.90 was determined, corresponding to 2450 bacteria. In group II (experimental medication 2 - 0.2% CHX/metronidazole/minocycline) and in group III (2% CHX), there were of 2,468 and 2,501 CFUs in the media, respectively. When converting to $\log_{10}$, one can determine a value of 8.554 and 8.668, corresponding to 7228 and 7325 bacteria in groups II and III, respectively. In group IV (saline solution), a large number of colony-forming units was observed, represented by 4,686 CFUs on average, and when converting into $\log_{10}$, a value of 16.24 was defined, corresponding to 13723 bacteria in this group.

Statistically comparing the results obtained in the experimental groups of this study, there was no difference between groups I, II and III; however, there were statistically significant differences in group IV (Table 1).

Discussion

Due to the anatomical complexity of root canals and the persistence of resistant bacteria inside the canal, irrigating solutions and intracanal medication are necessary to complement the mechanical action of endodontic instruments, favouring the repair of periapical tissues.[10]

In primary infections, where endodontic treatment has not been performed to date, Gram-negative anaerobic bacteria predominate, and in secondary and/or recurrent infections, there is a predominance of microorganisms resistant to endodontic treatment, and *Enterococcus faecalis* is found in 90% of these cases.[1,2,18]

These microbes can be found planktonically, in pairs, in chains and in biofilms.[1,2,18] The microbial biofilm is formed from the adhesion of bacteria, which proliferate and deposit an extracellular matrix, and acts as a protective layer against phagocytes, host defence factors and antibiotic action.[4] Studies have reported that endodontic treatment is capable of disrupting

Table 1. Number of colony forming units, expressed in log10, in experimental groups, as well as the mean and standard deviation.

Petri dish	Group I - experimental gel 1	Group II - experimental gel 2	Group III -2% chlorhexidine	Grupo IV - saline solution
1	0	4	5	4
2	0	6	11	64
3	0	12	5	8
4	2	0	0	42
5	0	0	3	14
6	14	0	0	8
7	0	9	0	33
8	0	0	4	12
9	0	3	5	83
10	0	1	0	5
mean ± standard deviation	0.837 ± 1.777a	2.468 ± 2.144a	2.501 ± 2.157a	4.686 ± 0.467b

p = 0.00035599

a statistically significant difference b

a large part of the biofilm present in the root canal. However, *Enterococcus faecalis* is persistent[7,8,18] and may survive and form a new biofilm.[3,26] Therefore, studies suggest that this microorganism is associated with the failure of endodontic treatment and the persistence of periapical lesions.[1,2]

Among the intracanal medications, calcium hydroxide is the most commonly used because it has a broad antimicrobial action, which promotes clinical success.[19] However, studies have shown that despite its excellent biological characteristics, the use of calcium hydroxide as an intracanal medication is not sufficient to eliminate *Enterococcus faecalis*[2,3,6,18] and may favour the permanence of this microorganism in the biofilm.[2]

Currently, the intracanal medication of choice to eliminate *Enterococcus faecalis* is 2% chlorhexidine.[6-8,11,12] Different studies have evaluated its effectiveness, considering its antimicrobial action, substantivity, large spectrum of action [6,11,18-20] and ability to penetrate dentinal tubules and apical delta.[15] According to studies by Lima et al.[20] in 2012, and Gomes et al.[8] in 2013, 0.12% and 2% chlorhexidine gels were effective on *Enterococcus faecalis*, justifying its use as a positive control. The data obtained in our study confirm the data found in the literature,[11,12] since there was no formation of CFUs at the experimental time points in this group (Table 1).

However, Barbin et al.[13] reported that the constant use of 2% chlorhexidine generate parachloroaniline and ROS and that the release of these products is proportional to the concentration of chlorhexidine.[13-15] Thus, to reduce clorhexidine's toxicity and lower the release of this product, a lower concentration of chlorhexidine can be used.[11,20] Therefore, in the preparation of the experimental gels, 0.2% chlorhexidine was used.

Other antimicrobial components were added for the composition of the experimental intracanal medications, aiming to increase the antimicrobial action on *Enterococcus faecalis*. The other components of the experimental intracanal medications were metronidazole and a tetracycline derivative with doxycycline in experimental intracanal medication 1 and minocycline in experimental intracanal medication 2. Metronidazole has a large spectrum of action on microorganisms[17], being effective on *Enterococcus faecalis*.[21,22] This drug alters the permeability of the bacterial cell membrane, binding to its DNA and promoting damage to its structure, leading to cell death. The drug is biocompatible with the periapical tissues, soluble in water and is gradually released, which provides substantivity.[27] In 2013, Bhangdia et al.[27] presented positive antimicrobial results when concentrations of metronidazole between 0.5 and 3% were used on *Enterococcus faecalis*. Thus, in our experimental medications, 2.5% metronidazole was used.

The tetracycline derivatives analysed were doxycycline and minocycline. Doxycycline exerts its effect on several bacteria, acting on protein synthesis and inactivating collagenase and metalloproteinase-9 to degrade the extracellular matrix.[18,28] Somayagi et al.[28] compared the action of doxycycline on biofilms of *Enterococcus faecalis*, and they observed that this drug has the capacity to significantly decrease levels of the microorganism. Khademi et al.[23] compared the substantivities of chlorhexidine and doxycycline and concluded that doxycycline showed a greater substantivity in the first three weeks within the root canal. These data justify the use of doxycycline as an intracanal medication. Minocycline has a large spectrum of action, inhibits protein synthesis, and has increased lipophilicity when compared to other antibiotics derived from tetracycline,[29] thereby justifying its greater effectiveness against several species of microorganisms.

According to our data, we verified the effectiveness of the mixture of the agents described above, since both experimental intracanal medications were acted against the biofilms of *Enterococcus faecalis*. Comparing the experimental intracanal medications with each other and with 2% CHX, there were no statistically significant differences between the groups ($p<0.05$) (Table 1). The effective action of the experimental intracanal medications can be justified by the joint action of their components.

Conclusion

Thus, through the results of this work, it was possible to conclude that the experimental intracanal medication presented antimicrobial action on the biofilm of *Enterococcus faecalis*. However, for the routine indication of these experimental intracanal medications in the endodontic clinic, studies involving the cytotoxicity and genotoxicity capacity of these intracanal medications are necessary.

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