EFFECTIVENESS OF DIFFERENT ENDODONTIC IRRIGANTS AGAINST *ENTEROCOCCUS FAECALIS*: AN *EX VIVO* STUDY.

Fabio Luis Miranda Pedro[1], Lenieser Fajardo Nunes[1], Thiago Machado Pereira[1], Kellin Pivatto[1], Cyntia Rodrigues de Araujo Estrela[1], Octavio Amezcua[2], Alvaro Henrique Borges[1]

1 Oral Sciences, University of Cuiaba, Brazil
2 Oral Sciences, University of Guadalajara, México

CORRESPONDING AUTHOR: fapedro@terra.com.br

ABSTRACT

Aim: To evaluate the effectiveness of different endodontic irrigants against *Enterococcus faecalis* (ATCC 29212).

Methods: Seventy bovine mandibular incisors were prepared, inoculated with a bacterial strain for 60 days and divided into the following groups: positive control; negative control; 2.5% NaOCl; 17% EDTA; 0.2% chitosan; 2.5% NaOCl + 0.2% chitosan; and 2.5% NaOCl + 17% EDTA. The irrigation protocol was performed using an experimental peristaltic pump device, with the irrigating solutions circulating within the apparatus at a constant flow for 10 min. Paper-point samples were then collected from the root canals and immersed in 7 mL of brain heart infusion broth, followed by incubation at 37°C for 48 h. Bacterial growth was assessed by turbidity of the culture medium.

Results: *E. faecalis* was present in all samples after the use of different irrigants.

Conclusion: The different irrigants tested were not effective in completely eliminating dentin bacterial contamination with *E. faecalis*.

KEYWORDS: Chitosan. *Enterococcus Faecalis*. Ethylenediaminetetraacetic Acid. Sodium Hypochlorite.
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INTRODUCTION

The ultimate goal of endodontic treatment is to eliminate bacteria from infected root canals by mechanical instrumentation combined with the use of irrigating solutions[1]. The root canal is an accessible environment for several microbial species, allowing attachment to the dentin surface and consequent formation of dense bacterial biofilms[2]. Among the microorganisms present in the root canal microflora, *Enterococcus faecalis* is a major pathogen associated with treatment failure in cases of persistent endodontic infections[3]. *E. faecalis* is a Gram-positive, facultative anaerobic microorganism with the ability to form biofilms, acquire several virulence factors, invade dentinal tubules, and grow in extreme conditions, such as at elevated pH[4]. Therefore, its present degree of pathogenicity[5] warrants further investigation.

Sodium hypochlorite (NaOCl) is a commonly used antimicrobial irrigant in endodontics, mainly because of properties such as the ability to dissolve organic matter, lubricate the instruments, and neutralize toxic agents[6,7]. Despite its antimicrobial activity and ability to remove the smear layer when used in combination with chelating agents[8], NaOCl is associated with cytotoxic effects[6]. Ethylenediaminetetraacetic acid (EDTA) was the first chelating agent to be used for the purpose of facilitating the instrumentation of atretic canals[8,9]. Therefore, a novel, accessible, biocompatible and low-cost irrigating solution that promotes disinfection has been the subject of research in several studies[10-12]. Chitosan is a natural polysaccharide obtained by deacetylation of chitin from crab and shrimp shells[12-14]. It has a chelating capacity and properties such as biocompatibility, biodegradability, bioadhesion, and absence of cell toxicity[14]. In endodontics, chitosan is considered an alternative to EDTA for root canal irrigation[14,15].

Because of the difficulty in controlling endodontic biofilm formation, investigation of biocompatible chemical substances, effective against resistant pathogens such as *E. faecalis*, is necessary. The objective of this *ex vivo* study was to evaluate the effectiveness of different endodontic irrigants against *E. faecalis*.

METHODS

BIOLOGICAL INDICATOR

The Gram-positive facultative anaerobic coccus *E. faecalis*, obtained from the American Type Culture Collection (ATCC 29212), was used as the biological indicator. The bacterial strain was inoculated into 7 mL of brain heart infusion (BHI) broth (Difco Laboratories, MI, USA) and incubated at 37°C for 24 h. The experimental suspensions were prepared by culturing the strain on the surface of BHI agar (Difco Laboratories), following the same incubation conditions. The bacterial cells were suspended in saline to give a final concentration of about $3x10^8$ cells/mL^{-1}, adjusted to No. 1 McFarland turbidity standard.

SOLUTION PREPARATION

The following solutions were tested in this experiment: 2.5% NaOCl (Longevitá, GO, Brazil); 17% EDTA (Longevitá); and 0.2% chitosan (Longevitá). For the preparation of chitosan, 0.2 g of chitosan were diluted in 100 mL of 1% acetic acid, and the mixture was stirred for 2 h using a magnetic stirrer until obtaining crystalline homogeneous solutions with 3.2 pH.

TOOTH PREPARATION

Seventy bovine mandibular incisors had their crowns removed with a diamond disc (American Burrs, SP, Brazil) at a 90° angle to the long axis of the tooth. Root lengths were standardized to 16 mm and the canals were emptied with a #15 K-Flex file (Dentsply Maillefer, Ballaigues, Switzerland) combined with conventional irrigation using 3 mL of 2.5% NaOCl (Longevitá). Root canals were dried and filled with 17% EDTA (Biodinâmica, PR, Brazil) for 5 min to remove the smear layer. All specimens were individually placed in 1.5-mL polypropylene Eppendorf tubes (Cral, SP, Brazil) containing BHI broth and autoclaved for 30 min at 120°C.

EXPERIMENTAL DESIGN

Five milliliters of BHI broth were mixed with 5 mL of the bacterial inoculum. The specimens were inoculated with *E. faecalis* for 60 days using sterile syringes of sufficient volume to fill the root canal. This procedure was repeated every 72 h, always using 24-h pure cultures prepared and adjusted to No. 1 McFarland turbidity standard. The teeth were maintained in a humid environment at 37°C. Ten uncontaminated specimens were used as a negative control group, serving as an aseptic control. Ten contaminated specimens served as a positive control. Positive and negative control groups were kept without irrigation and used to confirm contamination. After the contamination period, all specimens were filled with sterile distilled water. Sterile paper points (Tanari, Tanariman Indústria Ltda., AM, Brazil) were introduced into the root canal and maintained for 1 min. Three paper-point samples were collected from each root canal and immersed in 7 mL of Letheen Broth (Difco Laboratories), a medium containing or added with neutralizers [lecithin, Tween 80, and sodium thiosulfate (Art Laboratories, SP, Brazil)], followed by incubation at 37°C for 48 h. The presence or absence of bacteria was determined by the turbidity of the culture medium. The specimens were randomly divided into 7 groups (n=10) as described in Table 1.

EVALUATION OF THE ANTIMICROBIAL EFFECT OF IRRIGATING SOLUTIONS

To evaluate the antimicrobial efficacy of the irrigating solutions, a sterile urethane hose was connected to a polypropylene Eppendorf tube attached to the teeth and to the entrance of a peristaltic pump (Sarlo 90, SP, Brazil). The entrance of this apparatus was the urethane hose connected to the tube and its exit corresponded to the apical portion of the root canals. The irrigants circulated within the apparatus at a constant flow of 50 mL/min-1 for 10 min, as previously described by Estrela et al.[10]. After the 10-min period, each tooth was removed from the apparatus under aseptic conditions and irrigation was performed with 5 mL of sterile distilled water. Paper-point collection was performed as described earlier, followed by incubation at 37°C for 48 h. After evaluation of the culture medium, a 0.1-mL inoculum was transferred to 7 mL of BHI broth and subsequently incubated at 37°C for 48 h. After 48 h, bacterial growth was examined by visual analysis of the turbidity of the culture medium.

Table 1. Distribution of groups according to the experimental solutions.

Groups	Experimental solutions	Dilution concentration
C-*		
C+**		
G1	NaOCl	2.5%
G2	EDTA	17.0%
G3	Chitosan	0.2%
G4	NaOCl + chitosan	2.5% + 0.2%
G5	NaOCl + EDTA	2.5% + 17.0%

* C- (negative control): uncontaminated sample without the use of experimental solutions.
** C+ (positive control): contaminated sample without the use of experimental solutions.

Table 2. Antibacterial efficacy of irrigating solutions in root canals infected with *E. faecalis*.

Groups	Before	After
Negative control	- - -	- - -
Positive control	+ + +	+ + +
NaOCl	+ + +	+ + +
EDTA	+ + +	+ + +
Chitosan	+ + +	+ + +
NaOCl + chitosan	+ + +	+ + +
NaOCl + EDTA	+ + +	+ + +

+ + +: presence of bacteria.
- - -: absence of bacteria.

RESULTS

The antibacterial efficacy of the chemicals studied is described in Table 2. None of the endodontic irrigating solutions and experimental substances tested effectively eliminated dentin bacterial contamination with *E. faecalis.*

DISCUSSION

Infection control during endodontic treatment remains a challenge, and new auxiliary chemical substances to help in the disinfection process have been investigated[15-19]. In the present study, the evaluation of the turbidity of the culture medium and the qualitative evaluation of root dentin surface contamination using SEM showed that the different endodontic irrigants and substances tested were not effective in completely eliminating dentin bacterial contamination with *E. faecalis.* The limitations of the methodology used in this study should be considered when interpreting the results, since statistical analyses were not possible. Although some authors have reported limited efficacy of NaOCl combined with both EDTA and chitosan[16-19], there is still no report of a fully effective action of any of these solutions against *E. faecalis* in endodontics.

Several studies have investigated the antimicrobial efficacy of irrigants using different experimental models[2,7,10,14]. The choice of bovine dentin for infection and disinfection tests has been well accepted[14,20]. Bovine dentin appears to be a suitable substitute for human dentin, and factors such as ease of obtaining and handling and the use of bovine dentin in similar tests involving the removal of smear layer further support its indication[20]. In addition, its topography is an important factor in the survival of biofilms, since irregular surfaces increase bacterial adhesion and retention[14]. Our methodology included the use of a peristaltic pump device connected to the entrance of the root canals in order to simulate the root canal irrigation process[2,10]. Subsequently, the evaluation of the turbidity of the culture medium of microorganisms collected from the root canals allowed us to determine the microorganism growth rate. Studies investigating the antimicrobial efficacy of irrigating

solutions have used different methods, such as the agar well diffusion method[15], fluorescence assays[21], and SEM analysis[17]. However, methodological differences may lead to results that cannot be extrapolated to the clinical setting or at least should be done with caution.

Active chlorine generated by the dissociation of NaOCl in aqueous medium is a powerful oxidizing agent that exerts an antimicrobial effect by irreversible oxidation of bacterial cells[1]. Although lower concentration solutions have demonstrated antimicrobial efficacy, higher concentrations of NaOCI have a faster and greater bactericidal effect, but with increased cytotoxic effects[11]. The intensification of the antimicrobial effect of NaOCI when used in combination with EDTA may be related to the demineralizing action of EDTA, which prevents smear layer formation during instrumentation, resulting in increased penetration of NaOCI in the dentinal tubules[22]. However, this combination may cause erosion of the dentin, compromising its structural integrity[23].

Considering the lack of consensus on the efficacy, protocol of use and cytotoxicity of auxiliary chemical substances, new irrigants with improved antimicrobial and biological activity have been proposed. In this respect, the chelating capacity of chitosan in the dentin24 has prompted the study of its antimicrobial efficacy[14,15]. Chitosan is biocompatible, biodegradable, bioadhesive, and non-toxic to human cells[24]. Del Carpo-Perocheno et al.[14] attribute the antibacterial efficacy of chitosan to its polycationic nature, which reacts with the negatively charged membrane of bacteria, altering cell permeability[14]. Therefore, this substance may be a viable alternative to the irrigating solutions currently used in endodontics, given its effective action against microorganisms and less cytotoxic effects.

E. faecalis was chosen as a biological marker because of its possible microbial role in previously root-filled teeth with persistent periapical lesions[15]. Owing to characteristics such as being found more frequently in asymptomatic secondary endodontic infections and being able to invade dentinal tubules and compete with other microorganisms, this Gram-positive, facultative anaerobic microorganism has gained an important role in endodontic research[1,2]. The resistance of *E. faecalis* biofilms can be attributed to a series of microbiological factors inherent in the complex root system anatomy and dentin structure[1-4]. This microorganism has attracted much attention due to its ability to survive with limited nutrients, to maintain its pH level (due to the ability to block the cytoplasm), and to bond to the dentinal tubules[18].

Ex vivo studies are important for the determination of clinical behavior parameters. Although the results cannot be extrapolated to the clinical setting, the inability of all the irrigants tested to sterilize contaminated dentin, demonstrated in the present study, raises concerns about the implications of current root canal disinfection protocols. Therefore, inconsistent results with regard to the effectiveness of several irrigants against *E. faecalis*[15-21,24] reinforce the need to consider whether or not performing paraendodontic surgery in cases of endodontic failure. Although the evolution of endodontic systems has reduced the preparation time[25], resulting in less contact of the irrigant with dentin walls, there is still a need for further research on the effectiveness of irrigants in this scenario.

CONCLUSION

Based on the methodology and irrigation protocol used in this study, it can be concluded that the different irrigants and substances tested were not effective in eliminating dentin bacterial contamination with *E. faecalis.*

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