ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS AND CHLOROFORM ALONE AND COMBINED WITH CETRIMIDE AGAINST *ENTEROCOCCUS FAECALIS* BIOFILM

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The *Enterococcus faecalis* bacteria have been identified as the most commonly recovered species from teeth with persistent endodontic infections. The antimicrobial activity of essential oils and chloroform (CHL), alone and in association with various concentrations of cetrimide (CTR), against biofilm of *Enterococcus faecalis* was investigated. Solutions of CHL, eucalyptus oil (EO) and orange oil (OO) associated with CTR at 0.3%, 0.2%, 0.1%, and 0.05% were used to determine antimicrobial activity by exposing treated bovine dentine blocks to *E. faecalis*. Biofilms grown in the dentine blocks for 7 days were exposed to solutions for 2 and 5 min. Biofilm reduction between OO and EO at 2 min did not show any significant differences; however, OO had a higher kill percentage of biofilms than did the eucalyptus oil at 5 min ($p < 0.01$). Combinations with CTR at all concentrations achieved a 100% kill rate at 2 and 5 min. The association of CTR with solvent agents achieved the maximum antimicrobial activity against *E. faecalis* biofilms in dentine.

Keywords: essential oils, chloroform, biofilm, cetrimide, *Enterococcus faecalis*

Introduction

The elimination of microorganisms is one of the most important steps in endodontic therapy because bacteria and their metabolic products are considered to be the primary etiologic agents of pulpar and periradicular disease [1]. Anaerobic bacteria, especially Gram-negative species of *Prevotella* and *Porphyromonas*, are frequently associated with signs and symptoms of endodontic origin [2–4], and facultative species such as *Enterococcus faecalis* have been isolated in high frequency from refractory cases [5]. *E. faecalis* is rarely observed in cases of primary infections, but has been found to be the major component of microflora in cases of retreatment, amounting to as much as 70% of the microbiota isolated in these cases [6].

The removal of the root canal filling in retreatment procedures is necessary to clean any remnants of contaminated tissue or bacteria that are responsible for endodontic failure. Essential oils are able to dissolve most of the endodontic sealers [7]. Some of them, such as orange oil

(OO), eucalyptus oil (EO) and pine oil, have been reported as safe and useful for this purpose [8–11]. Furthermore, essential oils are known to possess antimicrobial effects against a wide variety of oral bacteria [12, 13]. For example, cinnamon oil exhibits a beneficial bactericidal activity when combined with chlorhexidine against both *Streptococcus mutans* and *Lactobacillus plantarum* biofilms [14], eucalyptus and mint oil when combined with methylparaben against *Pseudomonas aeruginosa* [15], or a combination of *Rosmarinus officinalis*/ciprofloxacin against *Klebsiella pneumoniae* [16].

Chloroform (CHL) is the most popular gutta percha solvent because it solubilizes gutta percha rapidly; it is moreover stable, and it exerts antimicrobial activity [17]. It is considered safe if used carefully, and its controlled use at appropriate doses poses no health risks to the patient [18, 19].

Cetrimide (CTR) is a cationic surfactant with the ability to increase the antibacterial effect of some antibiotics at concentrations between 0.01% and 0.04% [20]. It also has the capability of eradicating *E. faecalis* bio-

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films *in vitro* [21]. CTR has shown beneficial bactericidal activity in combination with EDTA, citric acid, and chlorhexidine against biofilm cultures [22, 23]. Recently, Pappen et al. [24] demonstrated that CTR improves the antimicrobial properties of Tetraclean and MTAD against *E. faecalis*.

To our knowledge, no studies have evaluated the combination of CTR with solvent agents. The purpose of this study was to evaluate, *in vitro*, the efficiency of orange oil, eucalyptus oil, and chloroform alone and in combination with cetrimide for the eradication of *E. faecalis* biofilm in dentine.

Materials and methods

Bacterial strains and test agents

The bacterial strain used in this study was *E. faecalis* from the American Type Culture Collection 29212, taken from a 4 °C stock culture and streaked out twice on Brain Heart Infusion (BHI) agar plates (Scharlau Chemie S.A., Barcelona, Spain) for 24 h at 37 °C. From the subculture of *E. faecalis*, a 1 McFarland standard suspension was prepared in BHI broth and then diluted 30-fold to obtain an initial bacterial suspension of approximately 1×10^7 colony-forming units per ml (CFU/mL).

The test agents were OO (Formula & Ação, São Paulo, Brazil), EO (Dentaflux, Madrid, Spain), CHL (Acofarma,

Barcelona, Spain) and their combinations with 0.3%, 0.2%, 0.1%, and 0.05% CTR (Sigma-Aldrich Chemie, Steinheim, Germany). To achieve a homogenization of combinations of solvents with CTR, they were mixed vigorously by vortexing with maximum speed of 3000 rpm and stirring radius of 3.5 mm in glass-covered tubes for 5 s immediately before their use.

Preparation of dentine blocks

Fifteen freshly extracted bovine mature teeth were stored in 0.1% thymol solution at 4 °C. The teeth were sectioned at the cementum-enamel junction using an Accuton-50 machine (Struers, Copenhagen, Denmark) at a high force of 3200 rpm with abundant water cooling. The apical and coronal thirds of the roots were discarded, leaving a section of 6 mm of the middle third. After removing the root cementum with a scalpel, the section was divided into three 2-mm slices (Fig. 1). Each slice was cut into four equal sections that were then adjusted in size using a calibrator and polished with 150, 220, and 600 grit silicon carbide papers in ascending order. Twelve dentine blocks were obtained from each root, and the smear layer formed during preparation of the dentine blocks was eliminated by submerging them in 17% EDTA for 2 min and then in 2.5% NaOCl for 1 min to standardize the specimens before their sterilization and separation into the different groups for study [23]. After sterilization, the specimens were in-

Fig. 1. Detailed description of achieving the dentin cubites employed in the study demonstrating the way of obtaining the samples

cubated in BHI during 24 h at 37 °C to ensure no bacterial contamination. The dentine blocks were kept in sterile saline solution until use.

The fifteen roots were randomly divided into three groups, CHL, OO and EO. The twelve dentine blocks/roots were assigned as follows: five dentine blocks to the corresponding solvent alone and its 4 combinations with CTR at each contact time, 2 and 5 min ($n = 10$ dentine blocks), while two were used as the positive and steril-

ity controls. Five replicates for each test agent and contact time were tested.

Biofilm antimicrobial activity test

The wells of a 96-well microtiter plate (Nuncclon Delta Surface; Nunc, Roskilde, Denmark) were inoculated with 180 μ L of the initial bacterial suspension. The sterile dentine

Fig. 2. a) Preparation of the dentine blocks for the study. b) Dimension of the root sections. c) The wells of a 96-well microtiter plate were inoculated with 180 μ L of the initial bacterial suspension. d) Specimens in details on Calgary biofilm device (CBD). e) Antibiotic susceptibility testing. f) Biofilm recovery placed in Eppendorf tubes with 200 μ L of BHI broth. g) Disrupted biofilm cultures were diluted serially and plated for viable cell counting. h) Antimicrobial activity against *E. faecalis* biofilm by different solvent solutions was expressed as the kill percentage of biofilm

Table 1. Mean kill percentage of *E. faecalis* biofilms by solvent agents alone and in combination with various concentrations of cetrime at two different time periods

Solvent solutions	2 minutes	5 minutes
CHL	E	E
CHL + 0.3% CTR	E	E
CHL + 0.2% CTR	E	E
CHL + 0.1% CTR	E	E
CHL + 0.05% CTR	E	E
OO	86 ^{a,1}	89.87 ^{a,2}
OO + 0.3% CTR	E	E
OO + 0.2% CTR	E	E
OO + 0.1% CTR	E	E
OO + 0.05% CTR	E	E
EO	71.62 ^{b,1}	78.50 ^{c,3}
EO + 0.3% CTR	E	E
EO + 0.2% CTR	E	E
EO + 0.1% CTR	E	E
EO + 0.05% CTR	E	E

E: eradication (100% kill)

CHL, chloroform; CTR, cetrime; OO, orange oil; EO, eucalyptus oil.

Read horizontally, the same letters show that differences are not statistically significant

Read vertically, the same numbers show that differences are not statistically significant

blocks were submerged in the inoculated wells, and they were incubated on the rocking table for 7 days at 37 °C and with 95% relative humidity. The BHI was refreshed daily to ensure the growth of *E. faecalis* on the dentine specimens, and the purity of the cultures was checked at regular intervals. After the exposure time, the dentine blocks with the biofilms were rinsed with 180 µl of 0.9% saline solution for 2 min to eliminate planktonic bacteria.

The antimicrobial activity assay was performed in glass tubes with 180 µL of the solvent solutions introducing their corresponding dentine blocks during 2 or 5 min. One non-treated dentine block/root was inoculated in 180 µL of the initial bacterial suspension as the positive control, whereas one non-treated dentine block inoculated in 180 µL of BHI served as the sterility control. Sterile absorbent paper disks (IVD; Becton, Dickinson and Company, Sparks, MD) were used to eliminate any excess solution from the dentine blocks. Afterwards, they were placed in Eppendorf tubes with 200 µL of BHI broth, vortexed for 2 s, and then sonicated for 10 min to assure biofilm recovery. Disrupted biofilm cultures were diluted serially and plated for viable cell counting (Fig. 2).

The results of antimicrobial activity against *E. faecalis* biofilm by different solvent solutions were expressed as the kill percentage of biofilm and calculated for each group of teeth (CHL, OO and EO) as follows: $(1 - [\text{mean CFU}_{\text{solution}} / \text{mean CFU}_{\text{positive control}}]) \times 100$. The term “eradication” was used to denote the killing of 100% of the bacterial population. To compare the efficacies of the different solutions when the kill percentage varied from 100%, as well as the reduction percentage related to the control, the Student's *t*-test was used, previously subjecting data to the Anscombe transformation [25].

Results

All negative controls showed no bacterial growth. The results of the antimicrobial activity of the tested agents are given in Table 1. CHL showed no bacterial growth alone or in combination with different concentrations of CTR at either period of time (2 and 5 min).

With respect to OO, the kill percentage at 5 min was significantly higher (89.87%) than at 2 min (86%) ($p < 0.001$). Comparison between the contact times in the EO group showed no significant differences in biofilm eradication ($p = 0.54$). The comparison of biofilm eradication between OO and EO at 2 min gave no significant differences ($p = 0.15$); at 5 min, however, the OO presented a kill percentage greater than the EO ($p < 0.01$). All combinations with CTR at all concentrations achieved 100% kill percentage at 2 and 5 min.

Discussion

The aim of this study was to determine the antimicrobial activity of CHL and two essential oils alone and in combi-

nation with CTR against *E. faecalis* biofilm. This bacteria has been identified as the most commonly recovered species from teeth with persistent endodontic infections [5, 6], especially when the bacteria are organized in biofilms attached to the canal walls or located in isthmuses and ramifications where they are definitely more difficult to eliminate. Bovine dentine specimens were selected because they are a satisfactory substratum for bacterial adhesion and biofilm formation. Based on a previous model that uses human dentine specimens [23], we designed a root dentine-volumetric test as a carrier to test the antimicrobial efficacy of solvents against biofilms. The specimens were easily size-standardized and infected, and they were simple to manipulate. At least twelve specimens could be obtained from each incisor at the middle root third; this permitted their assignment to different groups and reduced the inherent variability of the sample. Two and 5 min were considered adequate for their application in the endodontic retreatment regimen.

Under the aforementioned conditions, this study showed that CHL in effect eradicates the biofilm of *E. faecalis*; and although the essential oils OO and EO did not eradicate *E. faecalis*, they achieved percentages of biofilm reduction of over 85% for OO and 70% for EO. With respect to OO, biofilm reduction was not dependent on the time of action – there were no significant differences between 2 and 5 min – unlike the eucalyptus group, which was more effective at 5 min. The effectiveness of both essential oils tested in this work was recently found to be similarly effective in dissolving different root canal cements at 2 and 5 min. When CHL was used to dissolve gutta percha, bacterial growth was observed less frequently. Our results show that CHL alone eradicates *E. faecalis* biofilm, however, Edgar et al. [17], in an *ex vivo* study, showed that the use of CHL during endodontic retreatment significantly reduces intracanal levels of culturable *E. faecalis* without eradicating it, probably due to the residue left by the CHL/gutta percha mixture.

CTR was selected because it disrupts the extracellular polymeric matrix of the biofilm and reduces the surface tension of the liquids, thereby encouraging the penetration of solvents into the biofilm and allowing them to exert greater antimicrobial activity. CTR also lends stability to the emulsion formed with the essential oils [15]. The combined use of CHL and the essential oils at all concentrations of CTR, including the lowest (0.1% and 0.05%), killed 100% of *E. faecalis* biofilm at two time-tested periods. These findings point to the associated use of antimicrobial substances in correct proportions to enhance the killing of microbial biofilms instead of using a single substance at a much higher concentrations. Filoche et al. [14] demonstrated bactericidal activity against *S. mutans* and *L. plantarum* biofilms when combining essential oils with chlorhexidine.

In this work, essential oils showed a high percentage of inhibition against *E. faecalis* (71–89%), and eradication of biofilms was achieved when associated with CTR. It is known that 0.2% CTR, used alone [21] or in combination

with chelating agents [22] or chlorhexidine [23], has the capacity to eradicate *E. faecalis* biofilm. When the essential oils were combined with lower concentrations of CTR (0.05% and 0.1%), they effectively eradicated the biofilms, probably due to an enhancement of the antimicrobial properties of the essential oils, mainly attributed to lipophilic compounds – terpenoids and phenolics [15]. Some investigations suggest partial damage in the cell walls that cause an increase in cell permeability. Others report that essential oils can disturb the integrity of the cytoplasmic membrane, with extravasations of intracellular elements and interfere with bacterial enzyme metabolism [15]. Such cellular damage would be responsible for the high percentage of inhibition obtained when used alone, whereas eradication is dependent on the addition of CTR.

Although CHL is the most tested solvent and has demonstrated the highest antimicrobial properties in this study, it is necessary to consider its adverse effects on the bond strength of root fillings after root canal reobturation and its toxicity when it is not used in a clinically controlled manner [18].

The data obtained in this study suggest that the antimicrobial capacity of the solvent agents combined with CTR may contribute to disinfection in root canal retreatment. Nevertheless, further studies are needed to assess the capacity of associated solvents and antimicrobial agents in order to determine optimal combinations, i.e. those possessing adequate solvent capacity with no loss of antimicrobial activity. Moreover, taking into account the specificities and differences between a volumetric test in dentine and the complexity of the root canal system, *ex vivo* studies are necessary to evaluate the antimicrobial efficacy of combined agents in filled root canals.

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