ANTIMICROBIAL ACTIVITY OF ANTIBIOTIC PASTES USED IN PULP THERAPY THROUGH DIRECT CONTACT WITH A MULTISPECIES BIOFILM: A PILOT STUDY

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Palavras-chave: Dente Decíduo. Tratamento de Canal radicular. Combinação do Medicamentos. Antibacterianos. Testes de sensibilidade microbiana.

RESUMO

Objetivo: Avaliar a atividade antimicrobiana de pastas antibióticas utilizadas na técnica *Lesion Sterilization and Tissue Repair* (LSTR), através de nova metodologia de contato direto com membrana contra um biofilme multiespécies e estabelecer diluições adequadas para avaliação. **Métodos**: CTZ (cloranfenicol, tetraciclina, óxido de zinco) e duas formulações de pastas 3Mix (Ciprofloxacina, Metronidazol e Minociclina), 3Mix1 e 3Mix3, foram avaliadas, além dos grupos controle, negativo (solução salina a 0,9%) e positivo (clorexidina 0,2%). Biofilmes de *Candida albicans* e *Enterococcus faecalis* cultivados sobre membranas de celulose (n=10) durante 24 h foram expostos diretamente em contato com quantidades padronizadas de pastas frescas e controles (n = 2) por 24 h. As membranas foram imersas em 900 µL de solução salina e sete diluições seriadas foram obtidas por amostra. O plaqueamento para cada diluição (n = 2) foi realizado em meios de cultura para microrganismos totais e seletivos para *Candida* spp. e *Enterococcus* spp. para contagem de unidades formadoras de colônias (UFC). Para comparação entre grupos, os dados da contagem de UFC foram convertidos em $\log_{10}$ UFC / mL e o teste Mann-Whitney foi aplicado (p<0,05). **Resultados**: Observou-se inibição de UFC para todas as pastas, maior para CTZ no meio seletivo para *Candida* (p<0,001) e 3Mix1 nos demais meios (p<0,004). **Conclusão**: Concluiu-se que as pastas apresentaram atividade antimicrobiana contra o biofilme multiespécies testado e que a nova metodologia de contato direto proposta foi eficiente. Além disso, as diluições utilizadas mostraram-se adequadas para essa metodologia.

Keywords: Tooth Deciduous. Root Canal Therapy. Drug Combinations. Anti-bacterial agents. Microbial sensitivity tests.

ABSTRACT

Objective: To evaluate the antimicrobial activity of antibiotic pastes used in lesion sterilization and tissue repair (LSTR) technique, through a novel membrane direct contact methodology against a multispecies biofilm and to establish appropriate dilutions for this method. **Methods**: CTZ (chloramphenicol, tetracycline, zinc oxide) and two formulations of 3Mix pastes (ciprofloxacin, metronidazole, and minocycline), 3Mix1 and 3Mix3, were evaluated with negative (0.9% saline) and positive (chlorhexidine 0.2%) control groups. *Candida albicans and Enterococcus faecalis* (24-hour) biofilms (n=10) grown on cellulose membranes were directly exposed to standardized amounts of fresh pastes and control solutions (n=2) for 24h. Membranes were immersed in 900 µl of saline solution, and seven serial dilutions were made for each sample. Plating for each dilution (n=2) was performed on culture media for microbial colony-forming unit (CFU) counting of total microorganisms, *Candida* spp. and *Enterococcus* spp. Aiming the comparison between groups, CFU quantification data were transformed into $\log_{10}$ CFU / mL and the Mann-Whitney test was applied (p<0.05). **Results**: Inhibition of CFU was observed for all pastes, with greatest effects for CTZ paste in medium selective for *Candida* spp. (p<0.001) and 3Mix1 in non-selective (p<0.000) and selective for *Enterococcus* spp. (p<0.004). **Conclusion**: The pastes showed antimicrobial activity against the tested multispecies biofilm, and the proposed direct contact methodology was efficient. Moreover, the dilutions used proved to be appropriate for this methodology.

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INTRODUCTION

Instrument-free endodontic therapy, based on the lesion sterilization and tissue repair (LSTR) technique, aims to eliminate bacteria from the root canals of irreversibly infected teeth by the use of bacteriostatic and bactericidal drugs. These disinfect the lesion and promote repair by the host's natural tissue response, thus contributing to the health of the tooth and its supporting tissues until physiological exfoliation.[1,2]

CTZ and 3Mix pastes are examples of antimicrobial drug combinations employed in the LSTR technique. CTZ paste is composed of two broad-spectrum antibiotics, chloramphenicol and tetracycline, and zinc oxide and eugenol, which also exhibit antimicrobial activity.[3] 3Mix paste is composed of three broad-spectrum antibiotics (ciprofloxacin, metronidazole, and minocycline) added to distinct vehicles, such as macrogol and propylene glycol (MP), [4,5] or saline solution.[6]

Several factors may influence the success of antimicrobial drugs, among them the minimum concentration, the type of infection, and the bacterial resistance.[7] *Enterococcus faecalis* and *Candida albicans* have been reported as resistant to antibiotics and are associated with failure of endodontic treatments.[8] Thus, the evaluation of antibiotic combinations against these microorganisms becomes relevant for the pulp therapy of primary teeth. Although there is evidence that the pastes used in the LSTR technique exhibit antimicrobial properties on isolated microorganisms,[9,10,11] to date, the potential antimicrobial activity of distinct formulations of such primary teeth-targeted antibiotic pastes has not been investigated against multispecies biofilms.

Therefore, this pilot study aimed to evaluate the antimicrobial activity of antibiotic pastes, CTZ, and 3Mix in two formulations, through a direct contact antimicrobial assay against a polymicrobial biofilm composed of *C. albicans* and *E. faecalis* and to establish the appropriate dilutions for this assessment in future studies.

MATERIALS AND METHODS

The experiment was performed at the Multidisciplinary Laboratory of the School of Dentistry of the Federal University of Rio de Janeiro. Two membranes (Microlab Scientific, Yueqing City, Zhejiang Province, China) were used per group: CTZ, 3 Mix1, 3 Mix3, positive control (0.2% Chlorhexidine gel [CHX], Perioxidin® Bioadhesive Gel, Gross, Lacer, Rio de Janeiro, Brazil) and negative control (0.9% saline solution, Eurofarma Laboratórios S.A., São Paulo, Brazil). The experiment was performed in duplicate.

Preparation of antibiotic pastes

The enteric coating was removed by scalpel from those drugs obtained commercially in tablet form: Cipro® (Bayer SA, Socorro, Brazil), Flagyl® (Sanofi-Aventis Pharmaceutical Ltda., São Paulo, Brazil), and minocycline hydrochloride (Ranbaxy Laboratories Limited, Dewas, India). The tablets were separately pulverized in mortar and pestle and sieved (Tamis mesh 70 sieve) to standardize the particle size of each antibiotic powder. These were stored individually in opaque colored vials to prevent light exposure.

The antibiotic pastes were prepared as in previous studies:

a) CTZ capsules were prepared by a local pharmacy (Barraderm, Rio de Janeiro, Brazil). Each capsule contained all components in powder form (62.5 mg chloramphenicol, 62.5 mg tetracycline, and 125 mg zinc oxide).[12] Four drops of eugenol (SSWhite Dental Articles Ltd., Rio de Janeiro, Brazil) were added to the content of each capsule at the time of use.

b) 3Mix1 paste, composed of 500 mg ciprofloxacin (Cipro®, Bayer SA, Socorro, Brazil), 400 mg metronidazole (Flagyl®, Sanofi-Aventis Pharmaceutical Ltda., São Paulo, Brazil) and 100 mg minocycline hydrochloride (Ranbaxy Laboratories Limited, Dewas, India) were combined in a 1:1:1 ratio in excipients macrogol and propylene glycol (in a 1:1 ratio) (adapted from Nakornchai et al.[4]).

c) 3Mix3 paste, consisting of 500 mg ciprofloxacin (Cipro®, Bayer SA, Socorro, Brazil), 400 mg metronidazole (Flagyl®, Sanofi-Aventis Pharmaceutical Ltda., São Paulo, Brazil) and 100 mg minocycline hydrochloride (Ranbaxy Laboratories Limited, Dewas, India) were combined in a 1:3:3 ratio with 0.9% saline excipient (adapted from Divya et al.[6]).

All pastes were manipulated on sterile glass plates using a stainless steel spatula immediately prior to the experiment, at room temperature (25° C) using aseptic conditions, to obtain similar ointment consistency.

For the positive control (CHX), 0.2% chlorhexidine gel (Perioxidin® Bioadhesive Gel, Gross, Lacer, Rio de Janeiro, Brazil) was used, while 0.9% saline solution (NaCl, Eurofarma Laboratórios S.A., São Paulo, Brazil) was used for the negative control.

Preparation of inoculum and media

To obtain the mixed inoculum, reference strains of *Enterococcus faecalis* (ATCC 29212) and *Candida albicans* (ATCC 10231) were reactivated from original cultures on BHI

medium (Difco, Sparks, USA) for 48h at 37ºC with 5% CO_2. Bacterial colonies were collected and suspended with the aid of a sterile loop into BHI broth (Difco, Sparks, USA). The inoculum of microorganisms was standardized (in a spectrophotometer at 625 nm) at a concentration of 1×10^7 CFU / mL, corresponding to a 0.1 absorbance for *E. faecalis* and 10 for *C. albicans*. Brain Heart Infusion Agar (BHI) (Difco, Sparks, USA) was used for the selection of total microorganisms, CHROMagar™ Candida (Difco, Sparks, USA) for *Candida* spp. and BBLTM Enterococcosel™ Agar (Difco, Sparks, USA) for *Enterococcus* spp. BHI is a nutrient medium used for the cultivation of various microorganisms such as Streptococcus spp., Enterobacterium, yeast, and fungi. CHROMagar™ Candida (Difco, Sparks, USA) is a selective medium for Candida spp and for presumptive identification of some species in which colonies produce different colors, such as *Candida albicans*, whose colonies appear light green to medium green. BBLTM Enterococcosel™ Agar (Difco, Sparks, USA) is a selective medium used for rapid detection of enterococci.[13]

The media were prepared following the manufacturer's instructions and distributed into sterile Petri dishes (5 mL per plate). After solidification and drying, they were incubated at 37°C for 24 hours.

Evaluation of antimicrobial activity by direct contact of fresh pastes with multispecies biofilms

Two 13-mm diameter cellulose membrane discs (Microlab Scientific, Yueqing City, Zhejiang Province, China) distributed into 5 groups (n=10) were placed on BHI agar. A standard mixed microbial suspension (20 L) was pipetted over the discs for mixed biofilm formation. The plates were incubated under microaerophilic conditions for 24 hours at 37°C, after which, biofilm growth was observed on all membrane discs.

The freshly made pastes and the positive control were placed directly onto the biofilm that had formed on the surface of the membrane discs. An explorer probe was used to detach the paste from a specimen used to obtain standardized discs (7mm in diameter by 1mm in height). For the negative control, 2 drops of 0.9% saline solution were dropped directly onto the biofilm. The samples were incubated at 37°C, and the contact time was 24 hours.

The membrane discs were then transferred to 1.5 mL microtubes (Ciencor Scientific Ltda, São Paulo, Brazil) containing 900 µl of 0.9% saline and vortexed for 2 minutes. After shaking, cultures were serially diluted (10^0 to 10^{-7}) to allow microbial counting and assess microbial viability (CFU / mL).

For plating, 50 µl of each dilution were dispensed on the surfaces of the culture media and spread using a Drigalski loop, exchanged every two plates of the same medium.

Determination of appropriate microbial concentration

We analyzed five microbial dilutions per culture medium to determine the appropriate dilutions for this assessment. For non-selective BHI media, concentrations from 10^{-3} to 10^{-7} were used. For the other media, concentrations from 10^{-2} to 10^{-6} were used. The plates were incubated in microaerophilic conditions for 24 h at 37ºC, and the CFU was counted and the results demonstrated by CFU / mL.

Statistical analyses

Counting colony-forming unit data were tabulated in Excel version 2013 (Microsoft©, São Paulo, Brazil) and analyzed descriptively by the mean and standard deviation. CFU quantification data were log-logically transformed into $\log_{10}$ CFU / mL, and the Mann-Whitney test was applied (significance assigned at $p<0.05$) using the software Statistical Package for the Social Science (SPSS) for Windows, version 21.0 (IBM Corp., Armonk, NY, USA).

RESULTS

All antibiotic pastes showed some degree of inhibition in CFU number against the multispecies biofilm formed by *C. albicans* and *E. faecalis* in BHI medium. 3Mix1 paste demonstrated higher CFU inhibition than the other groups ($p = 0.000$) (Figure 1).

All pastes demonstrated inhibition of CFU number in mixed biofilm formed by *C. albicans* and *E. faecalis* in CHROMagar™ medium ($p<0.05$). The CTZ paste showed a high inhibition ability, significantly different from that of the control and the other pastes ($p<0.05$) (Figure 2).

All pastes reduced the number of CFU in mixed biofilm formed by *C. albicans* and *E. faecalis* in Enterococcosel™ medium. The largest differences were observed between paste activities and the negative control ($p = 0.000$). The 3Mix1 paste showed the highest degree of inhibition ($p<0.004$), while CTZ and 3Mix3 exhibited similar inhibition potential ($p = 0.005$) (Figure 3).

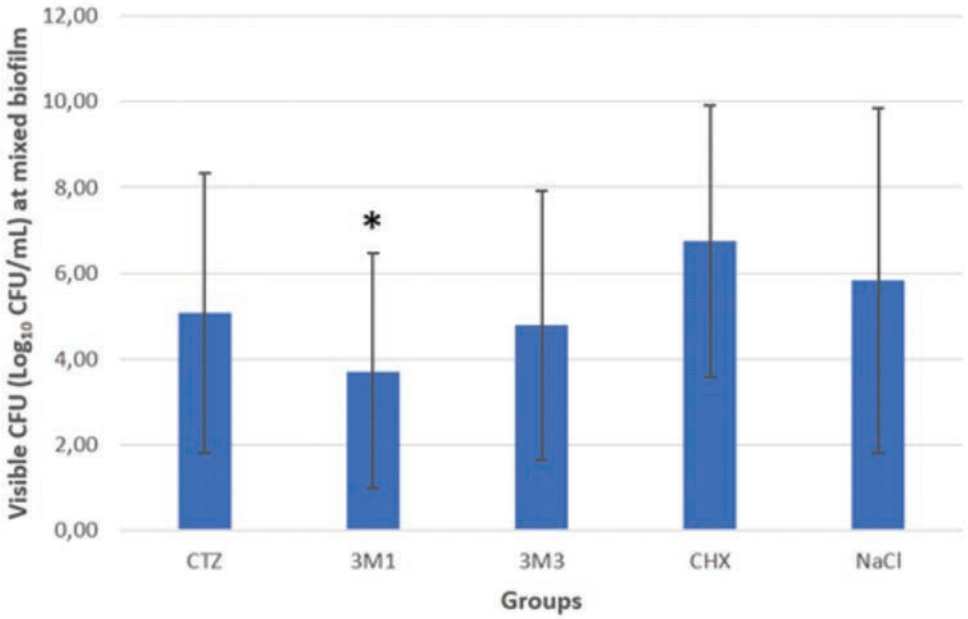


***Figure 1**: Average number of visible CFUs (Log10UFC / mL) in multispecies biofilm formed by* C. albicans *and* E. faecalis *in BHI medium per material. The vertical lines represent the standard deviation. * Significantly different compared to other materials.*

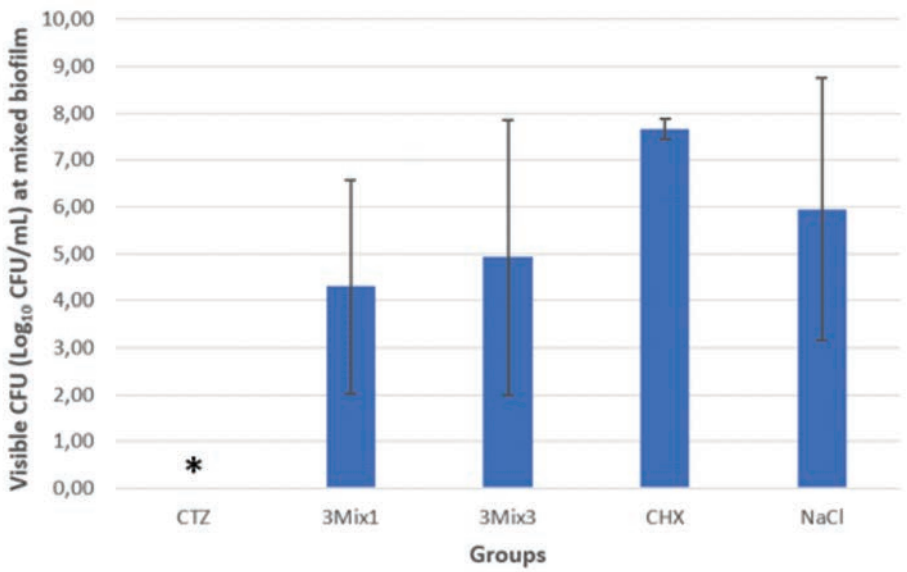


***Figure 2**: Average number of visible CFUs (Log10UFC / mL) in mixed biofilm formed by* C. albicans *and* E. faecalis *in CHROMagar™ medium per material. The vertical lines represent the standard deviation. * Significantly different compared to other materials.*

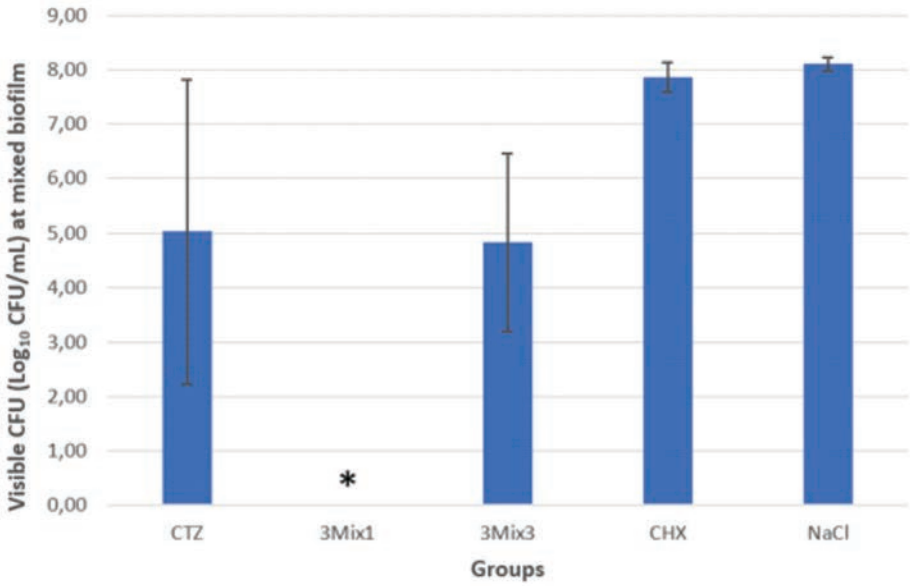


***Figure 3**: Average number of visible CFUs (Log$_{10}$UFC / mL) in mixed biofilm formed by* C. albicans *and* E. faecalis *in Enterococcosel™ medium per material. The vertical lines represent the standard deviation. * Significantly different compared to other materials.*

DISCUSSION

Pulpectomy success is directly related to bacterial reduction or elimination not only within the prepared root canals but also at the places not normally reached by chemo-mechanical preparation.[1] Tortuous root canals, the presence of multiple accessory canals and branches, large medullary bone spaces, the physiological resorption process, and, in some cases, inability to control infant behavior make the conventional treatment difficult and time-consuming.[1,15] Thus, non-instrumental endodontic treatment has been proposed as an alternative as it is a faster and easier treatment that employs antibiotic pastes to promote root canal disinfection and thus tissue repair.[16] For this reason, it is critical to understand the properties of the pastes used in this technique.

The microorganisms *E. faecalis* and *C. albicans* were chosen for this experiment since they are involved in cases of endodontic treatment failure, are antibiotic-resistant, and demonstrate virulence mechanisms that may hinder lesion management.[17]

The vehicles used in 3Mix pastes, whether saline or macrogol and propylene glycol, might have a direct influence effect on drug release, the onset of action, drug penetration into dentinal tubules, and drug dissociation.[18] The proportion of vehicles used in 3Mix pastes varies. In the studies by Hoshino et al.[11] and Lokade et al.[19], the ratio used was 1: 1 propylene glycol to macrogol, while in Takushige et al.[20], Nanda et al.[21], and Pinky et al.[22], the vehicle was propylene glycol with saline. Similarly, the proportion of antibiotics ranges from three equal parts each[4,11,19] to one-part ciprofloxacin and three parts metronidazole and minocycline.[6,20,21,22] We evaluated both antibiotic proportions and different vehicles.

Studies evaluating the antimicrobial activity of CTZ paste against *E. faecalis* demonstrated inhibition of bacterial growth.[23,24,25] Our results also showed decreased CFU count for CTZ in the selective medium for *E. faecalis*. Colony growth of *Candida spp.* on the selective medium was significantly inhibited by CTZ paste. This finding is consistent with previous results evaluated by tests such as agar diffusion.[3,22,25] In addition, the present study demonstrated, for the first time, the antimicrobial activity of CTZ on multispecies biofilms by direct contact with the paste.

The antimicrobial activity of 3Mix paste against *E. faecalis* was previously reported to be very satisfactory,[26,27,28] inhibiting all bacterial growth of single-microorganism cultures,[26] a finding corroborated by our results. In *E. faecalis* selective medium, 3Mix3 showed inhibition activity similar to CTZ, and 3Mix1 caused total inhibition of colony growth.

Most previous studies utilized a different methodological design than that in the present study, which may hinder comparison, especially in the use of multispecies biofilms, since the behavior of bacteria in their planktonic form differs greatly from their biofilm behavior.[29] This fact may explain the lower inhibition of CFU in BHI medium, a non-selective nutrient medium that promotes the growth of the two studied microorganisms. Moreover, we understand that the use of biofilm formed on a membrane does not exactly simulate the environment found inside the pulp canal

and that the number of samples used in this pilot study was small. In this sense, although the methodology was efficient for the initial screening of antimicrobial activity of pulp therapy pastes, future experiments using human or bovine teeth within multispecies biofilms on root canals are suggested.

Notably, the inhibition of CFU growth in the positive control group was not much higher than in the experimental groups. We believe this may be related to chlorhexidine concentration (0.2%) and / or gel presentation. Considering that no previous membrane methodology[30] study utilized a positive control group, a comparison is challenging. Since chlorhexidine at various concentrations is used as an irrigating solution on pulp therapy, it was chosen for this study.[31]

Chlorhexidine in higher concentrations and firmer consistencies should be evaluated in future studies. It is noteworthy that this did not impede the comparison of our results since we chose an inert negative control (0.9% saline), which allowed for the visualization of microorganism growth without antimicrobial agents. This emphasizes the importance of conducting pilot studies prior to laboratory experiments to detect methodological limitations to improve future study design.

The microbial dilutions enabled CFU counting in non-selective medium (10^{-3} to 10^{-7}) and *Enterococcus* spp. selective medium (10^{-2} to 10^{-6}). However, in the *Candida* spp. selective medium, it was not possible to quantify CFUs in 10^{-2} and 10^{-3} dilutions due to the large numbers of CFUs. Thus, we suggest that only larger dilutions be evaluated in future studies.

CONCLUSION

It was concluded that the pastes showed antimicrobial activity against the tested multispecies biofilm and that the new proposed direct contact methodology was efficient. Moreover, appropriate dilutions for this methodology were determined.

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