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Development of an Effective Triantibiotic formulation for *Enterococcus faecalis* in the Treatment of Endodontic Pathology

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ABSTRACT

Introduction: *The main objective of endodontic treatment is to minimize the number of microorganisms in the root canal system, to prevent or treat apical periodontitis [1]. The Enterococcus faecalis bacteria, most prevalent in persistent endodontic infections, resists antibacterial medications such as calcium hydroxide and multiconjugate pastes [2]. The increase in possible cases of refractory apical periodontitis due to said resistance has led to the search for alternatives, such as the use of broad-spectrum antibiotics alone or in combination as a possible solution to this problem [3].*

Objective*: To evaluate the effectiveness of a triantibiotic formulation on the inhibition of bacterial growth of E.feacalis by replacing Minocycline with Amoxicillin, to prevent dental pigmentation without losing the disinfection capacity of said paste.*

Materials and Methods*: Commercial presentations of the antibiotics and microdilution techniques were used to evaluate the antimicrobial activity against the bacterial strain of E. faecalis.*

Results*: In this study, Amoxicillin obtained the highest percentage of inhibition on E. faecalis*

Keywords

Antibiotics, Antimicrobial efficacy, *Enterococcus faecalis*, Periradicular diseases, Persistent endodontic infection.

Introduction

One of the most important objectives of endodontic treatment is to minimize the number of microorganisms and infectious remnants in the root canal system, to prevent or treat apical periodontitis [4-6]; This process is carried out through the chemical-mechanical disinfection of said canals, with the use of different irrigants such as sodium hypochlorite, EDTA (ethyldiaminetetraacetic acid), CHX (chlorhexidine), or with sonic, ultrasonic and, in some, activation methods. cases with the use of intracanal medications [7,8].

The anatomical variations that root canals can cause complications during access, conformation, disinfection, and three-dimensional sealing. These can lead to treatment failure due to the persistence of bacteria in said variations, which is why we look for strategies to maximize disinfection in these areas. A wide variety of microorganisms are found in pulp and periapical disease, with the predominant bacteria in primary endodontic infection being gram-negative anaerobes, while in secondary infections, gram-positive

anaerobic bacteria [9]; Within the latter, an important bacteria that has been shown to be more resistant to treatment and, detected with high frequency in isolates from endodontic retreatment and persistent periodontitis, is *Enterococcus faecalis (E. faecalis*) [2,4,10,11] and this is due to its high resistance to antibacterial agents and its ability to adapt to different environmental conditions; therefore, the use of intracanal medications is necessary to reduce the damage capacity of these bacteria. In cases of persistent infections and high contamination, different intracanal medications have been used, calcium hydroxide has been the most widely used because of its antibacterial properties and high pH [12]. Hoshino and Sato [11] have reported the use of a triple antibiotic paste (TAP) for the disinfection of the root canal system of deciduous teeth; This paste is composed of Ciprofloxacin, Minocycline and Metronidazole, medications that, when combined and applied topically in the canal, have great antimicrobial potential and are effective against pathogens found in the deep layers of dentin. TAP presents bacteriostatic (Minocycline) and bactericidal (Metronidazole and Ciprofloxacin) activity, but as a side effect the teeth suffer pigmentation due to the use of Minocycline, which is derived from Tetracycline [3,13]. These combined antibiotics form a paste frequently used empirically in endodontic treatments and its formulation has been described in different articles showing its preparation method, which consists of crushing the tablets and mixing them with sterile water or saline solution until the desired consistency is obtained [14]. The objective of this research is to evaluate the effectiveness of a triantibiotic formulation on the inhibition of bacterial growth of *E.faecalis* by replacing Minocycline with Amoxicillin to prevent dental pigmentation and without losing the disinfection capacity of said paste.

Materials and Methods

An *in vitro* study was developed to evaluate the effectiveness of triantibiotic paste on *Enterococcus faecalis*.

Strains used

The microorganism used in this study was the gram-positive coccus, a facultative anaerobic bacteria, *Enterococcus faecalis*. (ATCC 29212), the culture of the bacteria was maintained in BHI solid medium at 37°C with weekly renewal of culture medium. The initial concentration of bacteria used for the experiments was adjusted for turbidity to an OD of 0.08 equivalent to 1 x 10^8 CFU/ml.

Maintenance of the strains

The strains were preserved in Muller-Hinton solid medium at 4°C, making replicates every 7 days.

Inoculum preparation

The inoculum was prepared by making a direct suspension in Mueller-Hinton (MH) broth, of a colony isolated from one of the cultures on MH agar, incubating for 24 hours at 37°C. After the incubation time, the bacterial suspension was adjusted to an OD of 0.08 to achieve a turbidity equivalent to 0.5 on the McFarland scale, this is equivalent to a suspension containing 1 × 108 colony forming units (CFU/ml).

Preparation of the sample stock

Before preparing the stock of the antibiotics to be evaluated (Amoxicillin, Ciprofloxacin and Metronidazole), a solubility test was carried out with each of them. Concluding that Amoxicillin and Ciprofloxacin are soluble in water and that their solubilization rate must be increased by ultrasound for 1 min. The antibiotic Metronidazole required cosolvent to solubilize in water and acceleration of solubilization by ultrasound for 2 min. All antibiotics were prepared at a concentration of 1000 µg/ml with the presence of 30% cosolvent.

The formulation of each medication was made at 50 µg/ml of each antibiotic to maintain the 1:1:1 ratio proposed in the empirical triantibiotic paste. The excipients chosen were: cellosize (hydroxyethyl cellulose), PVP (Polyvinyl pyrrolidone), citric acid and dibasic sodium phosphate. Various concentrations were tested with each one until a fluid gel with a pH between 5-7 was achieved.

Determination of the inhibitory concentration IC50 and IC80 of each antibiotic on the strain

The determination of the inhibitory concentration was carried out by the microdilution method with Müller-Hinton broth. Making

Estéril	Control	Control	Anti 1 10 µg/ml	Anti 1 10 µg/ml	Anti 1 10 µg/ml	Anti 2 10 µg/ml	Anti 2 10 µg/ml	Anti 2 10 µg/ml	Anti 3 10 µg/ml	Anti 3 10 µg/ml	Anti 3 10 µg/ml
Estéril	Control	Control	Anti 1 5 µg/ml	Anti 1 5 µg/ml	Anti 1 5 µg/ml	Anti 2 5 µg/ml	Anti 2 5 µg/ml	Anti 2 5 µg/ml	Anti 3 5 µg/ml	Anti 3 5 µg/ml	Anti 3 5 µg/ml
Estéril	Control	Control	Anti 1 2,5 µg/ml	Anti 1 2,5 µg/ml	Anti 1 2,5 µg/ml	Anti 2 2,5 µg/ml	Anti 2 2,5 µg/ml	Anti 2 2,5 µg/ml	Anti 3 2,5 µg/ml	Anti 3 2,5 µg/ml	Anti 3 2,5 µg/ml
Estéril	Control	Control	Anti 1 1,25 µg/ml	Anti 1 1,25 µg/ml	Anti 1 1,25 µg/ml	Anti 2 1,25 µg/ml	Anti 2 1,25 µg/ml	Anti 2 1,25 µg/ml	Anti 3 1,25 µg/ml	Anti 3 1,25 µg/ml	Anti 3 1,25 µg/ml
Estéril	Control	Control	Anti 1 0,61 µg/ml	Anti 1 0,61 µg/ml	Anti 1 0,61 µg/ml	Anti 2 0,61 µg/ml	Anti 2 0,61 µg/ml	Anti 2 0,61 µg/ml	Anti 3 0,61 µg/ml	Anti 3 0,61 µg/ml	Anti 3 0,61 µg/ml
Estéril	Control	Control	Anti 1 0,3 µg/ml	Anti 1 0,3 µg/ml	Anti 1 0,3 µg/ml	Anti 2 0,3 µg/ml	Anti 2 0,3 µg/ml	Anti 2 0,3 µg/ml	Anti 3 0,3 µg/ml	Anti 3 0,3 µg/ml	Anti 3 0,3 µg/ml
Estéril	Control	Control	Anti 1 0,15µg/ml	Anti 1 0,15µg/ml	Anti 1 0,15µg/ml	Anti 2 0,15µg/ml	Anti 2 0,15µg/ml	Anti 2 0,15µg/ml	Anti 3 0,15µg/ml	Anti 3 0,15µg/ml	Anti 3 0,15µg/ml
Estéril	Control	Control	Anti 1 0,07µg/ml	Anti 1 0,07µg/ml	Anti 1 0,07µg/ml	Anti 2 0,07µg/ml	Anti 2 0,07µg/ml	Anti 2 0,07µg/ml	Anti 3 0,07µg/ml	Anti 3 0,07µg/ml	Anti 3 0,07µg/ml

Figure 1

serial dilutions of the compounds in a 96-well polystyrene microplate, from a concentration of 10 µg/ml to 0.07 µg/ml (Figure 1). The final volume after dilution was 100 µl.

Once the dilution of the antibiotics was prepared, 100 µl of inoculum prepared in MH medium and adjusted to an OD of 0.08 was added. The OD of each well was read at 625 nm at time 0H. Then, it was incubated for 18 H at 37°C. Again, the OD was read at 625 nm at time 18H.

Growth was calculated using the following formula:

% Growth = ((sample absorbance -control average absorbance) / control average abs orbance) × 100

Parallel to the determination of the IC50 concentration, the validation of the presence of the cosolvent in the medium was carried out, in order to verify that it did not have a biocidal effect on the bacteria. The final concentration of the cosolvent in the medium was 5%.

Determination of the inhibitory concentration IC50 and IC80 with three combined antibiotics

The determination of the inhibitory concentration IC50 and IC80 was carried out using the microdilution method with Müller-Hinton broth. Making serial dilutions of the compounds from a concentration of 10 µg/ml to 0.07 µg/ml, in the presence of a second antibiotic at a constant concentration. The concentration used in the second antibiotic was the MIC 50 found in the previous procedure. The MIC 50 was chosen to allow bacterial growth and to be able to observe the effect of the combination of two antibiotics.

Once the dilution of the antibiotics was prepared, 100 µl of inoculum prepared in MH medium and adjusted to an OD of 0.08 was added. The OD of each well was read at 625 nm at time 0H. Then, it was incubated for 18 H at 37°C. The OD was read again at 625 nm at time 18H. Growth calculations are carried out with the same formula as the previous procedure.

Preparation of the formulation

The solutions of citric acid and dibasic phosphate, each at a concentration of 1 mg/ml, were prepared by dissolving them. 30 ml of citric acid was taken to solubilize Ciprofloxacin with the help of sonication. Separately, 65 ml of the dibasic phosphate solution was taken and the Metronidazole and Amoxicillin were solubilized with magnetic stirring for 10 min. The resulting solutions of citric acid and dibasic phosphate were mixed. PVP and cellosize were added, and it was stirred and homogenized with the help of heating (55°C). Stirring was continued for approximately 10 min until gel formation was observed. It was packaged when the mixture was at a temperature below 40°C.

The formulation was developed using the compounds shown in Table 1.

Table 1: Compounds used in the preparation of the triantibiotic formulation.

COMPONENT	QUANTITY FOR 100 ML
Metronidazole	5 mg
Ciprofloxacin	5 mg
Amoxicillin	5 mg
Citric acid	(30 ml) 100 mg/100 ml
Dibasic phosphate	(65 ml) 100 mg/100 ml
Cellosize	1.5g
PVP	200 mg

Antibiotic drug samples

A formulation of the drug using only the excipients was used as a negative control for the experiment. As a positive control, the triantibiotic paste traditionally used in dentistry was used, which consists of (1) a 500 mg capsule of Amoxicillin, (1) a 500 mg tablet of Ciprofloxacin and (1) a 500 mg tablet of Metronidazole. macerated with 5 ml of sterile saline solution. The sample to be evaluated consists of a medication formulated with the excipients and the antibiotics Amoxicillin, Ciprofloxacin and Metronidazole at concentrations lower than 100 µg/ml. The composition of the formulations defined to develop the drug effectiveness test is shown in Table 2.

Table 2: Formulations defined for effectiveness testing.

Triantibiotic paste (PT)	Antibiotic formulation (FA)	Excipient formulations (EF)
Amoxicillin 500 mg capsule	Amoxicillin 5 mg	Without active ingredient
Ciprofloxacin 500 mg tablet	Metronidazole 5 mg	Without active ingredient
Saline solution 0.9%, 5 mL	Excipients q.s. 100 Ml	Without active ingredient

Evaluation of the formulations on the viability of the bacteria

For the comparison of the antimicrobial effect of the formulations, mature biofilms formed on a porous hydroxyapatite surface were used. The biofilms were formed for 168 hours in liquid MH medium at 37°C, with the addition of 200 µL of MH medium every 48 hours. The porous hydroxyapatite surface with the biofilm attached was rinsed with sterile saline to remove sessile bacteria. Subsequently, each treatment (PT, FA, FE) was added to the surface and left in contact with the biofilm for 15 min, rinsed with sterile saline solution and incubated again and then submerged in MH broth and at 37°C to verify the viability of the biofilm after treatment.

Results

Determination of the inhibitory concentration IC50 and IC80

The IC (Inhibitory Concentration) is defined as the concentration of antibiotic at which the growth of the bacteria is inhibited by 50 and 80%, using changes in turbidity as a measurement parameter. This turbidity is evaluated at 0 hours and 18 hours after putting the bacteria in contact with the antibiotic at 37°C. In these experiments it was observed that the wells that did not present turbidity were those that presented a growth \percentage of less than 20%, for this reason the MIC was calculated in terms of IC 80 (inhibitory concentration of 80% growth). The IC 50 (inhibitory concentration

of 50% growth) was calculated in order to find an experimental point that would allow us to observe the effects of the antibiotic combinations; at this midpoint, we were able to see the effects in the following experiments precisely, the growth indicator being the presence of turbidity.

The inhibitory concentration IC50 and IC80 of Amoxicillin, Metronidazole and Ciprofloxacin against *E. faecalis* is presented in Table 3. In the present study it was observed that Amoxicillin has greater bacterial inhibition (*E. faecalis*) compared to Metronidazole and Ciprofloxacin also showed the highest percentage of inhibition when used at its IC 50 dose, which indicates that a high concentration of the drug is not required to inhibit 50% of the population.

Table 3: IC50 and IC80 inhibitory concentration of antibiotics on E. faecalis. Inhibitory concentration test evaluated in liquid medium by micro dilution. n= 6 per concentration evaluated.

Inhibitory concentration against E. faecalis		
Amoxicillin µg/ml	**Metronidazole µg/ml**	**Ciprofloxacin µg/ml**
IC 50 IC 80	**IC 50 IC 80**	**IC 50 IC 80**
0.15 -0.078 031	**>10 >10**	**5 -2.5 >20**

Once the antibiotics were evaluated individually, the determination of the inhibitory concentration IC50 and IC80 was carried out again, but with pairs of antibiotics seeking to determine the synergistic, antagonistic or enhancing effects that were presented with the combinations of the antibiotics, the results are presented in Table 4.

The interaction of antibiotic mixtures and antibiotics as independent agents was evaluated, again resulting in Metronidazole reporting the lowest activities and Amoxicillin the highest, as well as the mixture with Ciprofloxacin. In this study, the mixture Amoxicillin, Ciprofloxacin, obtained higher percentages of inhibition, which could suggest future mixtures that also obtain low molecular weight for better penetration into the dentin.

Table 4: MIC E. faecalis. Inhibitory concentration test evaluated in liquid medium by microdilution with antibiotic combinations.

AMOXICILLIN	**IC 50 ug/ml**		**IC 80 ug/ml**	
Without combination	0.31-0.15	0.313	0.625	0.625
+Metronidazole 10 ug/mL	0.078-0.039	0.62-0.31	0.156	0.625
+Ciprofloxacin 2.5 ug/ml	<0.039	0.039	0.078	0.313
METRONIDAZOLE	IC 50 ug/ml		IC 80 ug/ml	
Without combination	>25	>25	>25	>25
+Amoxicillin 0.15 ug/mL	>25	12.5**	>25	>25
+Ciprofloxacin 2.5 ug/ml	<0.195*	>25	>25	>25
CIPROFLOXACIN	IC 50 ug/ml		IC 80 ug/ml	
Without combination	1,563	6.25-3.12	>50	>50
+Amoxicillin 0.15 ug/mL	0.781	3.125	1.56	6.25
+Metronidazole 10 ug/ml	1.563	6.25-3.12	>50	25*

Evaluation of the formulations on the viability of the bacteria

In the evaluation of the antibacterial effect of the different formulations, it was observed that the triantibiotic gel formulation was equally effective as the triantibiotic paste currently used, despite having a lot of A lower concentration of antibiotics it completely inhibits growth, this effect being evident in the absence of turbidity in the culture medium used in the incubation after contact with the formulation (FA). The positive growth in the formulation that only contains the excipients (EF) indicates that the chosen excipients and the pH of the formulation do not influence the antibacterial effect of the formulation since they allow the growth of the microorganism Table 5.

Table 5: Comparison of the antimicrobial effect of those formulated on mature biofilm of E. faecalis.

Treatment	**Viability prior to addition to treatment**	**Viability after addition of treatment**
Sterility control	Negative	Negative
Without treatment	Positive	Positive
Excipients (EF)	Positive	Positive
Excipients + antibiotics (FA)	Positive	Negative
Triantibiotic paste (PT)	Positive	Negative

Discussion

Most root canal infections occur as a consortium of microorganisms, in which bacteria such as *E. faecalis, Propionibacterium, Porphyromonas and Prevotella* predominate. For this research, E. faecalis was chosen, since it has been shown to be the most resistant bacteria against secondary infections in endodontic retreatments [14]. With the objective of verifying the sensitivity of *E. faecalis* against the antibiotics Amoxicillin, Ciprofloxacin and Metronidazole, a determination of the inhibitory concentration IC50 and IC80 of each antibiotic and in combination was carried out, seeking to determine the synergistic, antagonistic or enhancing effects. It was evident that at Amoxicillin concentrations between 5 - 0.625 µg/ml there were no alterations in the inhibition of bacterial growth, indicating that Metronidazole and Ciprofloxacin do not exert an antagonistic or enhancing effect with this antibiotic.

Metronidazole, despite the combinations with Amoxicillin and Ciprofloxacin, failed to inhibit more than 80% of growth at any of the concentrations evaluated. It was observed that with the combination with Ciprofloxacin, maximum inhibition was reached around 50% constantly at all concentrations evaluated, indicating that the biocidal effect that was presented there was only exerted by Ciprofloxacin at 2.5 µg/ml present in each well. This inhibition was confirmed considering that Ciprofloxacin alone at concentrations between 1.56 - 3.125 µg/ml presented growth inhibitions between 68-44%. A similar case occurred with Amoxicillin combined with the series of concentrations of Metronidazole, where there was a constant inhibition of around 20%, Therefore, it was determined that Metronidazole was not active against the pathogen evaluated and that it did not present an enhancing or antagonistic effect against the antibiotic amoxicillin.

The synergistic effect between Ciprofloxacin and Amoxicillin could be verified in the concentration range of Ciprofloxacin between 50 - 6.25 µg/ml, which went from inhibiting bacterial growth from 80% to 100% due to the combination with Amoxicillin. 0.15 µg/ml. The effect was considered synergistic because the two antibiotics caused growth inhibition of the *E. faecalis* bacteria.

Although Metronidazole did not inhibit the growth of the *E. faecalis* bacteria, it was included in the formulations of this study because, in clinical cases of root canal infection, it is common to find consortia between bacteria and fungi. Therefore, it is essential to have an antifungal active ingredient in the formulation.

Various studies have reported that the triple antibiotic paste, made up of Metronidazole, Ciprofloxacin and Minocycline, is a good alternative as an intracanal medication, complementing disinfection techniques in endodontic treatment [11,12], also that none of the antibiotic components of TAP could individually eliminate all the microorganisms responsible for dental infections and although Amoxicillin did not achieve this in this study, it did achieve a high percentage of inhibition even at the minimum concentrations evaluated.

In this study, it was chosen to replace Minocycline with Amoxicillin to avoid the pigmentation effect, in addition to being a commonly used antibiotic that has shown optimal results [12] and is within the group allowed by the pharmacological standard where the resistance and susceptibility parameters for each of the antibiotics reported in the M100-S23 supplement of the Clinical and Laboratory Standards Institute (CLSI) were followed for their use and combination with the other antibiotics evaluated, but which is usually classified as a medication that generates resistance when used freely and incorrectly. To date, there is not enough scientific evidence to support the combination described in this study: Metronidazole, Ciprofloxacin and Amoxicillin; and the lack of knowledge of the optimal concentration for their use.

Conclusions

The present study determined the effectiveness of a triple antibiotic paste composed of Amoxicillin, Ciprofloxacin and Metronidazole in inhibiting bacterial growth against *E. feacalis***.** The results indicated that the joint use of these three drugs is effective in inhibiting the growth of the bacteria *E. feacalis,* the effect was attributed to Amoxicillin and Ciprofloxacin. It would be pertinent to develop another study to verify the ex vivo effectiveness (natural teeth) and a subsequent implementation in humans. The development of triantibiotic pastes represents a promising future in the root canal disinfection process. Experimental and clinical implementation studies are lacking to evaluate the safety and clinical effectiveness of the new formulations contemplating the simultaneous use of the antibiotics Amoxicillin, Ciprofloxacin and Metronidazole.

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