Evaluation of the Antimicrobial Efficacy of Different Intracanal Medications on Enterococcus Faecalis Biofilm: A Comparative *In Vitro* Study

Eduardo Fernandes Marques* Alícia Costa Santos, Anna Clara Rodrigues Barbosa, Victória Arruda Gomes, Marcelo Faria da Silva and Carina Scolari Gosch

ITPAC University, Porto Nacional, Araguaína, Brazil

Corresponding Author

Eduardo Fernandes Marques
ITPAC University
Porto Nacional
Brazil.
E-mail: eduardo.marques@ceulp.edu.br

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Abstract

The total elimination of microorganisms during endodontic therapy, although it is desired, is difficult to achieve. Even after canal filling, microorganisms can be in the dentin mass and periapical region. This study aims to evaluate *in vitro* the antibacterial efficacy of different intracanal medications on an *Enterococcus faecalis* biofilm. It was used reference microorganisms, *Enterococcus faecalis* NEWP 0012, where the bacterial suspension was seeded uniformly on Mueller Hinton (MH) agar. The plates were incubated under aerobic conditions, at a constant temperature in the range of 35°C-37°C, for 24 hours. The intracanal medications tested were: G1 Calcium hydroxide associated with 2% Chlorhexidine, G2 Calcium hydroxide associated with ozone stratum, G3 Calcium hydroxide associated with paramonochlorophenol, G4 Calcium hydroxide associated with Otosporin, G5 Calcium hydroxide, G6 2% Chlorhexidine, G7 Ozone extract, G8 Paramonochlorophenol, G9 Otosporin, G10 Saline solution, G11 Calcium hydroxide associated with iodoform, G12 Iodoform, G13 Calcium hydroxide associated with NDP, G14 NDP. After handling the materials, they were impregnated on 5 mm diameter absorbent paper discs and distributed equidistantly on the plates seeded with the microorganism. The experimental data were obtained by measuring the zone of inhibition within 24h, 72h, and 7 days. In this way, the microorganism was classified as resistant or sensitive to the different products. The test was reproduced 3 times. After analysis, it was observed that within 24 hours, 72 hours and 7 days, only the groups G1 Calcium hydroxide associated with 2% chlorhexidine (18 mm of halo of inhibition), G5 Calcium hydroxide (13 mm of halo of inhibition), G6 2% chlorhexidine (16 mm of halo of inhibition) and G9 Otosporin (9 mm of halo of inhibition) showed a halo of inhibition in the time intervals. However, the groups did not show halo of inhibition, being ineffective against *E. faecalis*. According to the obtained data, chlorhexidine gel 2% showed larger halos of inhibition, however, calcium hydroxide was associated with chlorhexidine gel 2%, calcium hydroxide and Otosporin. Therefore, new researches with different methods should be used to verify the efficacy of the medications used, since they presented divergence compared to other researches already carried out.

Keywords: Functional independence measurement • Acute care • Sphincter • Social cognition • Multidisciplinary

Introduction

Endodontic treatment aims at the chemical-mechanical preparation of the root canal system, thus providing the removal of inflamed or necrotic pulp tissue, elimination of most microorganisms and subsequently the three-dimensional obturation [1]. However, some pathogens are resistant to the disinfection process such as *Streptococcus mutans, Lactobacillus, Candida, and Enterococcus faecalis* [2].

Enterococcus faecalis is a facultative anaerobic gram-positive pathogen that possesses the ability to adapt and tolerate adverse environmental conditions, resistant to phagocytosis, antibodies and antimicrobial agents in this way, making eradication difficult and relating to 22%-77% of persistent infection cases [3,4]. Thus, it is important to study adjunctive substances in endodontic therapy that assist in the eradication of resistant microorganisms, such as intracanal medications, especially in complex anatomical areas of root canal systems, such as apical deltas and isthmus regions [5]

To date, the ideal intracanal medication has not been found. However, calcium hydroxide ($CaOH_2$) is the most widely used intracanal medication in endodontics. Due to the release of hydroxyl ions that provides a highly alkaline environment with a pH of approximately 12, most microorganisms present in infected root canals are unable to survive. However, calcium hydroxide is not equally effective against all bacteria found in the root canal.

Currently, it is possible to observe studies with: calcium hydroxide associated with chlorhexidine gel 2%, calcium hydroxide associated with ozone extract, calcium hydroxide associated with iodoform, calcium hydroxide associated with paramonochlorophenol, calcium hydroxide associated with Otosporin, to enhance the effectiveness of calcium hydroxide in eliminating residual bacteria in the root canal system [6].

Other products have been studied to combat microorganisms in root canals, such as chlorhexidine gel 2% and NDP, to combat microorganisms in root canals. However, despite conflicting studies, no drug appears superior to another, and its usefulness has been questioned [7].

Given this, this work aimed to analyze different intracanal medications and associations with calcium hydroxide in order to potentiate them against microorganisms, being the *Enterococcus faecalis* of greater resistance, thus contributing to higher success rates in endodontic treatments.

Methods

Obtaining the microorganisms

A commercial strain of *Enterococcus faecalis* (NEWProv-0012), revitalized in BHI broth, incubated at 37°C ± 2°C in a bacteriological incubator until visible turbidity (18 h). Immediately after, the microorganisms were inoculated on a non-selective nutrient medium plate (Nutrient agar, KASVI) by the depletion technique and were again incubated at 37°C ± 2°C for 24 h. For the tests, the microbial concentration was adjusted to a concentration of 5x105 CFU/mL of BHI broth, controlled by turbidity reading in a spectrophotometer with a wavelength of 600 nm and subsequent counting of colonies on a plate by performing serial dilutions in 0.95% saline solution.

Drug sensitivity test

To determine the antibacterial action in plates, Mueller Hinton agar (MH, KASVI) was prepared, previously melted, sterilized, and cooled to 45°C-50°C, then distributed in 150 mm diameter Petri plates until a thickness of approximately 4 mm was reached. After obtaining solid consistency of the MH agar, sterile swabs were used to collect microorganisms in the bacterial suspension (≈ 5x105 CFU/mL) and the surface seeding technique was performed in the three directions on each plate, paying attention to a uniform distribution, avoiding the growth of isolated colonies.

Then, absorbent paper discs with a diameter of 6 mm were produced, followed by sterilization. These were impregnated with the different intracanal medications:

- G1- Calcium hydroxide associated with 2% Chlorhexidine
- G2- Calcium hydroxide associated with ozone extract

- G3- Calcium hydroxide associated with paramonochlorophenol (Calen with Pmcc)
- G4- Calcium hydroxide associated with Otosporin
- G5- Calcium hydroxide
- G6- Chlorhexidine 2%
- G7- Ozone
- G8- Paramonochlorophenol
- G9- Otosporin
- G10- Saline
- G11- Calcium Hydroxide associated with iodoform
- G12- Iodoform
- G13 Calcium hydroxide associated with NDP
- G14- NDP

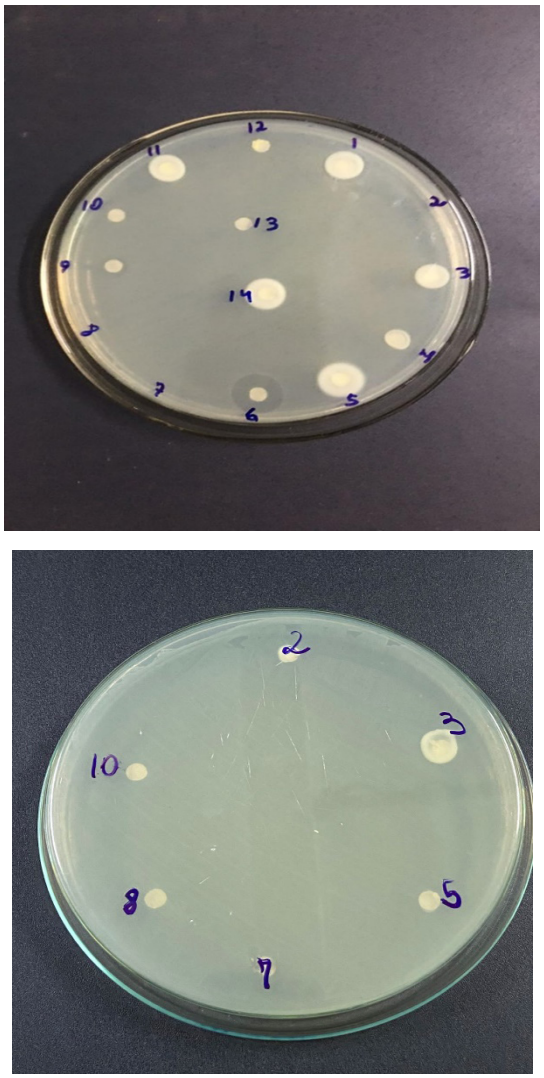

Figure 1. Halos of inhibition of the tested drugs.

Table 1. Inhibition halos of the materials used according to the time evaluated.

Experimental groups	24 h	72 h	7 days
Calcium hydroxide associated with 2% Chlorhexidine	Present	Present	Present
Calcium hydroxide associated with ozone extract	Absent	Absent	Absent
Calcium hydroxide associated with paramonochlorophenol	Absent	Absent	Absent
Calcium hydroxide associated with otosporin	Absent	Absent	Absent
Calcium Hydroxide	Present	Present	Present
Chlorhexidine gel 2%	Present	Present	Present
Ozone Extract	Absent	Absent	Absent
Paramonochlorophenol	Absent	Absent	Absent
Otosporin	Present	Present	Present
Saline solution	Absent	Absent	Absent
Calcium Hydroxide associated with iodoform	Absent	Absent	Absent
Iodoform	Absent	Absent	Absent
Calcium Hydroxide associated with NDP	Absent	Absent	Absent
NDP	Absent	Absent	Absent

The paper disks impregnated with the different medications were spaced out over the microbial plaque surface and the experiment was inoculated in a bacteriological oven at 37°C ± 2°C. The inhibition halos were measured using a pachymeter after 24 h, 72 h, and 7 days of incubation, and the results were expressed in millimetres.

All experiments were performed in triplicate. To determine the antimicrobial action of intracanal medication, the arithmetic mean of the inhibition halos obtained from triplicates of three consecutive experiments was considered as the result.

Results

In this study, an *in vitro* study was performed to analyze the efficacy of different groups of intracanal medication, through three experiments it was observed the low efficacy of some medications in inhibiting the growth of *Enterococcus faecalis* bacteria. After analysis, in the first experiment, it was revealed that within 24 hours, 72 hours and 7 days only the groups G1 Calcium hydroxide associated with 2% chlorhexidine (18 mm of halo of inhibition), G5 Calcium hydroxide (13 mm of halo of inhibition), G6 2% chlorhexidine (16 mm of halo of inhibition) and G9 Otosporin (9 mm of halo of inhibition), presented a halo of inhibition. The groups G2 Calcium hydroxide associated with ozone layer, G3 Calcium hydroxide associated with paramonochlorophenol, G4 Calcium hydroxide associated with Otosporin, G7 Ozone, G8 Paramonochlorophenol, G11 Calcium hydroxide associated with iodoform, G12 iodoform, G13 Calcium hydroxide associated with NDP and G14 NDP showed no halo of inhibition, being ineffective against *E. faecalis*. Figure 1 shows the culture medium and the halos formed.

Since the other tested drugs had no antimicrobial activity, two new disk diffusion tests were performed. It was then observed that in experiments 1, 2 and 3 the results were repeated, forming haloes only in: chlorhexidine, hydroxide, chlorhexidine associated with hydroxide and Otosporin. Among the results obtained, chlorhexidine without association was the medication that presented the highest efficacy against *Enterococcus faecalis* (Table 1).

Discussion

One of the reasons for failure in endodontic treatment is the presence of resistant microorganisms, which even after instrumentation remain in the root canal, in view of this, the use of irrigating agents and intracanal medications is indispensable [8].

The choice of medication is essential during the treatment because it aims to prevent the survival of microorganisms that may have remained even after the mechanical-chemical preparation, given that some medications are not as efficient for some microorganisms with *Enterococcus feacalis*, the present research aims to highlight which medications of best choice [9].

Otosporin is a combination of hydrocortisone, neomycin sulfate and polymyxin B and has anti-inflammatory, immunosuppressive, vasoconstrictor and antimicrobial properties, great tissue penetration ability. Neomycin sulfate is a broad spectrum antibiotic, being effective against aerobic and facultative anaerobic bacteria such as *E. faecalis, Staphylococcus aureus, Proteus vulgaris*. The present study corroborates with this work, as it was efficient in antimicrobial action to *Enterococcus faecalis*.

The camphorated paramonochlorophenol presents high antibacterial activity against strict anaerobic bacteria, low surface tension and has been associated with calcium hydroxide to combat root microorganisms (Lopes & Siqueira, 2015). However, in the present study, paramonochlorophenol and camphorated paramonochlorophenol associated with calcium hydroxide were not efficient in antimicrobial action to *Enterococcus faecalis* [10] evaluated *in vitro* the antimicrobial action of intracanal medications against bacteria present inside the root canal. Five medications were selected: NDP® and PRP®; Calcium Hydroxide+PMCC®; Otosporin®,

$Ca(OH)_2$+propylene glycol+iodoform. The drugs were submitted to antimicrobial activity evaluation by the agar diffusion technique using the disc method against the bacteria *E. faecalis* (ATCC 19433), *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923). The disks were soaked in the drugs and placed in the inoculated petri dishes, and then left at room temperature for two hours for pre-diffusion to occur then incubated in an oven at 370c for 48 hours.

For *E. faecalis,* the mean inhibition halos were $Ca(OH)_2$+PMCC® of 18.33 mm, Otosporin® of 15.66 mm, the association of calcium hydroxide, iodoform and propylene glycol of 8.33 mm, the NDP® of 10.66 and the PRP mean of 9 mm; for S. aureus were Otosporin 24.33 mm, the association of calcium hydroxide, iodoform and propylene glycol 23.66, $Ca(OH)_2$+PMCC® 18.33 mm, PRP® 11.66 mm and the NDP® 12 mm; for E. coli mean of 19 mm for Otosporin®, 18.66 mm for $Ca(OH)_2$+PMCC®, 12.33 mm for the association of calcium hydroxide®, iodoform and propylene glycol, 8.33 mm for NDP® and 6.66 mm for PRP®. Thus, all the drugs tested showed antimicrobial action on strains of *E. faecalis, S. aureus* and *E.coli.* The results found indicate that some medications that are quite usual in the clinical environment (Paramonochlorophenol, Cancoform and Iodoform), are not able to inhibit *Enterococcus faecalis*, which is a species frequently pointed out as one of the major causes of retreatment recurrence. Among the medications tested, those that obtained a greater halo of inhibition against *E. faecalis* were chlorhexidine, calcium hydroxide, chlorhexidine+calcium hydroxide and corticoid, where chlorhexidine had the greatest inhibition. These results corroborate the present study [11] evaluated *in vitro* the capacity of association of the antimicrobial effect of ozone to vehicles: olive oil, sunflower oil and propylene glycol; and indwelling dressings: Calen, Calen PMCC and propylene glycol plus calcium hydroxide in periods of one, seven, fifteen, thirty and one hundred and eighty days, after undergoing an ozonation process by bubbling, by means of the antimicrobial sensitivity technique (diffusion on agar and measurement of inhibition halo in bacterial cultures) using as indicator bacteria *P. aeruginosa* and *E. faecalis* as indicator bacteria.

The bacteria were seeded in 3 Petri dishes each, making a total of 6 cultured dishes per evaluation time. After 48 hours, for each evaluated time, we analyzed the inhibition halos formed. The authors concluded that Calen and Calen PMCC lagging dressings do not have synergistic association with ozone, but have their own antimicrobial activity and stability in time. Sunflower oil has a greater capacity of association with ozone than olive oil, and both have stability over time and greater antimicrobial action on *E. faecalis*. Propylene glycol plus calcium hydroxide has a lower capacity of association with ozone than propylene glycol and a higher capacity of association than sunflower oil, possessing antimicrobial action and stability in time. In this study, the association of calcium hydroxide with ozone and ozone extract were not effective against *Enterococcus faecalis*.

According to [12], clinical studies show that, on average, 20% to 30% of canals still have viable microorganisms after medication with calcium hydroxide in an inert vehicle. However, the present work showed inhibition of calcium hydroxide with inert vehicle against *Enterococcus faecalis* for 7 days. However, the study by [13] disagrees with this result, probably due to the different methodology employed.

Conclusion

According to the obtained data, chlorhexidine gel 2% showed greater halos of inhibition, however, calcium hydroxide was associated with chlorhexidine gel 2%, calcium hydroxide and Otosporin. Therefore, new studies with different methods should be used to verify the efficacy of the medications used, since they presented divergence in relation to other studies already carried out.

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