



Potentialiation of the action of calcium hydroxide on *Enterococcus faecalis* by proton pump inhibitor omeprazole

Deborah Meirelles Cogo^a, Sílvia Dias de Oliveira^b, Fernanda Camargo Antunes^b, Patrícia Maria Poli Kopper^c, Jéssica Stephanie Rodrigues Nasário^b, Fabiana Vieira Vier-Pelisser^a

Abstract

Purpose: Calcium hydroxide is not fully effective against *Enterococcus faecalis* (*E. faecalis*), a facultative anaerobic bacterium proven to be resistant to most conventional disinfection processes. The aim of this *in vitro* study was to evaluate the effect of calcium hydroxide, omeprazole and the association of these substances against *Enterococcus faecalis*, as well as to evaluate if the acid-catalysation of the omeprazole had any influence in the results.

Methods: The Minimum Inhibitory Concentration (MIC) of these drugs against *E. faecalis* (ATCC 29212) was determined using macrodilution test adapted from the CLSI (Clinical Laboratory and Standards Institute). Solutions with different concentrations of calcium hydroxide, associated or not to omeprazole, were tested. Data were statistically analyzed using ANOVA test with Tukey post-hoc, with a level of significance of 5%.

Results: The MIC to calcium hydroxide was 32 mg mL⁻¹ and, when associated with omeprazole, this was reduced to 16 mg mL⁻¹. The omeprazole and acidified omeprazole had similar activity.

Conclusions: Omeprazole potentiated the effect of calcium hydroxide, since the association of these drugs reduced the MIC for *E. faecalis*. The acidification of omeprazole, when associated with calcium hydroxide in different concentrations, did not influence its effect.

Key words: *In vitro*; Bacteria; *Enterococcus faecalis*; Calcium hydroxide; Omeprazole.

^a Department of Endodontics, Graduate Program, School of Dentistry, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, Brazil.

^b Laboratory of Immunology and Microbiology, Graduate Program, School of Biosciences, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, Brazil.

^c Department of Endodontics, Graduate Program, School of Dentistry, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

Potencialização da ação do hidróxido de cálcio sobre o *Enterococcus faecalis* pelo inibidor da bomba de prótons omeprazol

Resumo

Objetivo: O hidróxido de cálcio não representa uma estratégia totalmente eficaz contra *Enterococcus faecalis* (*E. faecalis*), uma bactéria anaeróbica facultativa resistente aos processos de desinfecção mais convencionais. O objetivo deste estudo *in vitro* foi avaliar o efeito do hidróxido de cálcio, do omeprazol e das associações destas substâncias contra *Enterococcus faecalis*, assim como avaliar se a catalização ácida do omeprazol pode ter influência sobre os resultados.

Métodos: A Concentração Inibitória Mínima (CIM) destes medicamentos contra *E. faecalis* (ATCC 29212) foi determinada pelo teste de macrodiluição adaptado do CLSI (*Clinical Laboratory Standards Institute*). Soluções com diferentes concentrações de hidróxido de cálcio, associadas ou não ao omeprazol, foram testadas. Os dados foram analisados estatisticamente pelo teste ANOVA com post-hoc de Tukey, com um nível de significância de 5%. **Resultados:** A CIM para o hidróxido de cálcio foi de 32 mg mL⁻¹ e, quando associada com omeprazol, foi reduzida para 16 mg mL⁻¹. O omeprazol e o omeprazol acidificado tiveram atividade semelhante.

Conclusões: O omeprazol foi capaz de potencializar o efeito do hidróxido de cálcio, uma vez que a associação dessas drogas reduziu a CIM para o *E. faecalis*. A acidificação do omeprazol, quando associado com o hidróxido de cálcio, em concentrações diferentes, não influenciou o seu efeito.

Palavras-chave: *In vitro*; Bactéria; *Enterococcus faecalis*; Hidróxido de cálcio; Omeprazol.

Correspondence:

Deborah Meirelles Cogo
deborahmeirellescogo@hotmail.com

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Introduction

The presence of the *Enterococcus faecalis* (*E. faecalis*) is highly associated to those cases considered refractory to the endodontic treatment [1,2] due to its resistance to conventional disinfection processes [2,3].

This facultative anaerobic bacterium has the ability to survive for many months in environments with limited nutrients in minimum metabolic condition [4]. Its elimination is difficult, especially when organized in biofilm [5]; this may be due to a phenotypic change in the biofilm, such as changes in the protein composition of the membranes or drug efflux pumps.

Several studies have shown that the calcium hydroxide is not very effective against *E. faecalis* [6,7]. Such bacterial tolerance may be attributed to the activation of the proton pump, of specific enzyme systems and/or buffering systems that assist in the maintenance of the bacterial internal pH practically constant [8,9].

Thus, in cases where the microbiota has been proved resistant, the association of other drugs with calcium hydroxide could reduce the infection [10,11]. However, in the presence of *E. faecalis*, the calcium hydroxide associated with the PMCC (Camphorated Paramonochloro-fenol) and the chlorhexidine has not shown promising results [3,10,11].

Currently, one of the proton pump inhibitors, used orally for the treatment of gastrointestinal disorders is omeprazole. Its mechanism of action takes place through the specific inhibition of the H⁺ + K⁺ -ATPase, a proton pump located in the secretory membrane of the parietal cells of the gastric mucosa, which is responsible for the final step of the acid secretion. Regarding the antimicrobial activity of the proton pump inhibitors, it has been demonstrated *in vitro* that omeprazole by itself has some effects against *Helicobacter pylori* [12]. Its association with metronidazole also made this microorganism more susceptible to antibiotics [13]. *In vitro* studies have investigated the MIC (minimum inhibitory concentration) of the calcium hydroxide and the omeprazole against different microorganisms, including *E. faecalis* [14,15].

Omeprazole is a prodrug which seems to require acid induction for its activation, as occurs in the compartment of the parietal cells. Therefore, *in vitro* studies previously dissolve the drug in acid substances [13,14]. However, to date, no studies have compared whether the acidification of this drug has a superior antimicrobial effect, particularly when associated with the calcium hydroxide, which has an alkaline pH.

Wagner, et al. [16] (2011) related that association of omeprazole with calcium hydroxide favored a superior repair of rat periapical lesions and seemed to display different selective activity over endodontic microbiota, in comparison with the conventional calcium hydroxide dressing. But in this study, the concentration of the substances was not evaluated, and the medications were not tested against a known microbiota of refractory periapical lesions.

Due to the high resistance of the *E. faecalis* and its presence in large numbers in cases of persistent infection, compounds capable of inhibiting the action of the proton pump of the bacterial membrane, which regulates the internal pH of the bacteria, could potentiate the effects of the calcium hydroxide, increasing the possibility of elimination of this bacterial species.

Therefore, the aim of this study was to evaluate *in vitro* the antimicrobial effect of the calcium hydroxide and the omeprazole, isolated and associated, against the *E. faecalis*, as well as to verify the need for acidification of omeprazole in this context.

Methods

The antimicrobial activity of the drugs was verified through determination of the MIC using macrodilution test adapted from the CLSI (Clinical Laboratory and Standards Institute) [17].

The inocula from *E. faecalis* (ATCC 29212) culture were prepared in 0.85% saline solution and adjusted at the 0.5 McFarland standard. The inocula were 10-fold diluted until to 10⁻⁵ and each dilution was cultured, in duplicate, in Plate Count Agar at 37°C for 24h to determine the bacterial concentration in colony-forming units (CFU) mL⁻¹.

To determine the maximum concentrations to be tested, were taken as reference previous studies, *in vitro*, which evaluated the MIC to calcium hydroxide [15] and for the omeprazole [14] against *E. faecalis*.

A standard solution of each drug was prepared by diluting the drugs in distilled water. The standard solutions were prepared to reach a concentration 10 times higher than the maximum concentration of each drug to be tested (Table 1). The experiment itself consisted of removing a certain aliquot (X) from the standard solution, in accordance with the desired concentration to be tested, and repeatedly inoculating them into tubes containing Y mL of sterile medium and 0.05mL of the inocula, up to X+Y=10 mL. The drug aliquot was reduced by 50% every time, until it reached zero (bacterial growth control), and the aliquot of medium was raised accordingly, up to 10 mL of the final volume.

In CHAO and AO groups, omeprazole was dissolved in 1mL of 2.85% acetic acid (Nuclear, Diadema, Brazil), 10 min before use, to assure acid-catalysation. A pilot study confirmed that there was no effect of the acetic acid on *E. faecalis* with the concentration used in this experiment.

The possible contamination of the drugs was evaluated by incubation of these with culture medium without inoculum under the same experimental conditions. In CHO and CHAO groups, the concentration of omeprazole was remained constant (0.512 mg mL⁻¹) and only the calcium hydroxide concentration varied. The tubes were mixed by vortexing for 30s and then incubated at 37°C for 24 hours.

The survival of the bacteria was assessed by 10-fold serial dilutions on agar plates. After incubation at 37°C for 48h, colonies on the plates were counted and CFU mL⁻¹ was determined. All experiments were made in triplicate.

Table 1. Groups and the drug concentrations statistically analyzed

Drug	CH/O Concentration (mg mL ⁻¹)	Group
calcium hydroxide (CH)*	32	CH32
	16	CH16
	8	CH8
calcium hydroxide* + omeprazole** (CHO)	32/0.512	CH32O
	16/0.512	CH16O
	8/0.512	CH8O
calcium hydroxide* + acidified omeprazole** (CHAO)	32/0.512	CH32AO
	16/0.512	CH16AO
	8/0.512	CH8AO
Omeprazole** (O)	0.512	O
Acidified Omeprazole (AO)**	0.512	AO

* Odontosul, Porto Alegre, Brazil.

** Cardila Healthcare TVT Ltda., Ahmedabad, Índia.

The MICs to drugs were determined and consisted of the lowest concentration of the drug at which bacterial growth could not be observed.

Data were statistically analyzed, with a level of significance of 5%. MIC required killing the microorganism were compared among the different groups. These data were then submitted to statistical analyses using the ANOVA test with Tukey post-hoc.

Results

The *E. faecalis* growth was not inhibited by omeprazole and acidified omeprazole in any tested concentration, similar to what has been observed in bacterial growth control (P=.319), although the acidified omeprazole group had better results comparing with the omeprazole group (P=.001).

MIC to calcium hydroxide was 32 mg mL⁻¹, while to calcium hydroxide + omeprazole and calcium hydroxide + acidified omeprazole it was 16 mg mL⁻¹. When calcium hydroxide, calcium hydroxide + omeprazole and calcium hydroxide + acidified omeprazole were tested at 16, 8 and 8 mg mL⁻¹, respectively, it was observed a reduction of the number of CFUs (Figure 1).

Discussion

The *E. faecalis* is one of the predominant species in cases of lesions refractory to treatment [6,18,22], since it has important characteristics that make it resistant to the therapeutic procedures [17,21,22]. Therefore, much of the current endodontic research seeks more efficient ways to combat this microorganism. The use of intracanal medication has been an alternative in an attempt to eliminate the maximum amount of the remaining bacteria after canal preparation.

The agar diffusion test is used extensively to assess the antimicrobial effect of these medications, despite its well-

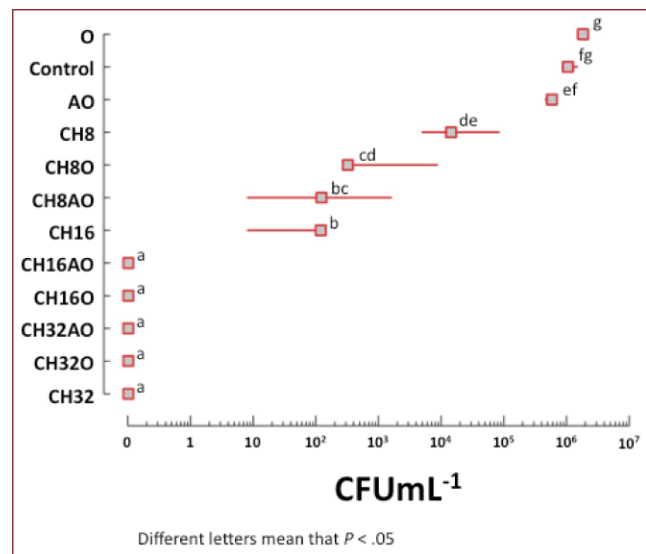


Figure 1. CFUs of *E. faecalis* after exposure to drug at various concentrations.

known limitations. Its results are influenced by the solubility and diffusibility of the material in the culture medium, not expressing their real antimicrobial effect [3]. Also, this test cannot distinguish the microbiostatic and microbicidal properties of the material [23]. On the other hand, the direct contact test does not have these disadvantages and it can be used to assess the antimicrobial effect of materials, providing quantitative and reproducible results [8,11,19].

The determination of the MIC to a drug is used by diagnostic laboratories to confirm what is the minimum concentration able to inhibit the bacterial growth, and often as a tool to determine the *in vitro* activity of new antimicrobials [16]. This study was conducted to determine the MIC to calcium hydroxide, to omeprazole, and to the association of these substances, and to check whether the

prior acidification of the omeprazole would have some influence in this context.

Considering that the omeprazole is a prodrug that requires acid induction, in this experiment we chose to add such variable, by acidifying it previously in some groups, as performed by Andersen et al. [2] and Jonkers et al. [9]. This investigation showed that the calcium hydroxide concentrations below 16 mg mL⁻¹ associated to the previously acidified omeprazole demonstrated better results than calcium hydroxide combined with the non-acidified omeprazole, which may be clinically relevant, requiring further investigations.

As the effects of the calcium hydroxide are closely related to its high pH, the addition of an acidic substance in this medication could have reduced its effectiveness. However, this effect was not observed because the results obtained showed no difference between calcium hydroxide + omeprazole and calcium hydroxide + acidified omeprazole when were used the concentrations of 32 and 16 mg mL⁻¹. Although omeprazole and acidified omeprazole have not shown significant difference compared to control, even at the highest concentration, it was found that the acidified omeprazole was able to reduce further the number of CFUs in comparison with the omeprazole. This finding supports the assertion that the omeprazole has its action enhanced when previously dissolved in an acid substance.

The acidified ethanol used by Andersen et al. [2] and Jonkers et al. [9] to promote de catalyzation of the omeprazole presented antimicrobial effect on *E. faecalis*, in a preliminary study. Therefore, it was not used in this investigation. The apple cider vinegar has already been proposed as an irrigating solution in endodontics by Estrela et al. [4], and the acetic acid is one of its main components [25]; that is why it was considered as a possible choice to acidify the omeprazole. Thus the omeprazole was previously acidified with that substance, which in the concentration used in the experiment did not cause inhibition of the *E. faecalis* growth.

The MIC to calcium hydroxide for the *E. faecalis* was 32 mg mL⁻¹, in contrast with another experiment, that used a similar methodology, in which it was 16 mg mL⁻¹ [16]. In our case, the drugs were diluted in distilled water and in their experiment it was used glycerin, this may have altered the results. Our results showed that calcium hydroxide has antimicrobial activity on this bacterium, which is enhanced when combined with omeprazole.

The findings of this research have shown that the omeprazole was not able to eliminate the *E. faecalis*, but when used in combination with the calcium hydroxide it was able to reduce its MIC in 50%. This fact may be related to the direct effect of the omeprazole on the proton pump present in the cytoplasmic membrane of *E. faecalis* [5], which would promote an increase in their internal pH, favoring the action of the calcium hydroxide. Thus, the calcium hydroxide and omeprazole could act synergistically, increasing its potential as an intracanal medication.

Conclusion

The results achieved in this study were obtained though *in vitro* analysis and cannot be directly related to the clinic, but they can be promising. Omeprazole potentiates the effect of calcium hydroxide, since the association of these drugs reduces the MIC for *E. faecalis*. The acidification of omeprazole, when associated with calcium hydroxide in different concentrations, did not influence its effect.

This study indicates that the association of calcium hydroxide with the proton pump inhibitor omeprazole improved the effects of the calcium hydroxide. Additional researches are still necessary to verify that these results will also occur in the complex root canal system, where direct contact with the microbiota of the medication is not always possible. Furthermore, it is necessary to verify the possibility of handling pastes with such concentrations of the drugs, since the calcium hydroxide paste usually used as temporary dressing should have adequate consistency which allows their introduction and residence in the root canal system.

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