Antibacterial activity of calcium hydroxide combined with triple antibiotic paste against *enterococcus faecalis*; an ın vitro study

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ABSTRACT
Objectives: The aim of the present in vitro study was to investigate the antibacterial efficacy of calcium hydroxide mixed with triple antibiotic paste at various rates.
Materials and Methods: Triple antibiotic paste (TAP; metronidizol, minocycline, ciprofloxacin) and calcium hydroxide powder was mixed in different proportions and tested for antibacterial activity against *E. faecalis* (ATCC 29212)using the agar diffusion method. The samples were divided into 10 different groups;Group 1: 100% $Ca(OH)_2$, Group 2: 99,5% $Ca(OH)_2$ + 0.5% TAP, Group 3: 99% $Ca(OH)_2$ + 1%TAP, Group 4: 97,5% $Ca(OH)_2$ + 2,5% TAP, Group 5: 95% $Ca(OH)_2$ + 5%TAP, Group 6: 90% $Ca(OH)_2$ + 10%TAP, Group 7: 75% $Ca(OH)_2$ + 25% TAP, Group 8: 50% $Ca(OH)_2$ + 50% TAP, Group 9: 25% $Ca(OH)_2$ + 75% TAP, Group 10: %100 TAP. Standard holes in the cultivated agar plates were filled with one of the samples and the zones of microbial inhibition were measured after incubation period (48 hours). The experiment was performed 8 times with each medicament.
Results: Group 1 had no effect against *E.faecalis*. Group 10 demonstrated high antibacterial activity than any other groups in this study. The antibacterial effects of the groups could be ranked from strongest to weakest as follows: Group 10, 9, 8, 7, 6, 5, 4, 3, 2, 1.
Conclusions: Adding the triple antibiotic paste increased he antibacterial efficacy of calcium hydroxide. In addition, the antibacterial effect of calcium hydroxide also increased with higher proportion of TAP.
Keywords: Antibacterial activity, calcium hydroxide, *enterecoccus faecalis***,** triple antibiotic paste.

INTRODUCTION

Elimination of bacteria from the root canal system is one of the major factors in successful endodontic treatment.[1] Although biomechanical preparation and root canal shaping effectively reduce microbiota, these procedures do not completely eliminate bacteria in the lateral and accessory root canals, isthmi, and apical deltas.[2] In addition, some microorganisms are more resistant than others. For example; obligate anaerobes are fairly easy to eliminate (or at least greatly reduce in number) by instrumentation and irrigation, in contrast to the facultative anaerobes that can survive such treatment.[3] *Enterococcus faecalis* is a gram positive anaerobe, and the prevalence of *E. faecalis* in failed endodontic cases ranges between 24 and 70 percent.[4] Like other facultative anaerobes, *E. faecalis* is very resistant to irrigation and biomechanical preparation. For this reason, intracanal medication between appointments is recommended to further reduce bacteria in the root canal system.[4]

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Calcium hydroxide ($Ca(OH)_2$) is one of the most commonly used intracanal substances in endodontics.[5] In vitro and in vivo studies have shown that most bacteria isolated from infected root canals are susceptible to calcium hydroxide.[6] Its antimicrobial activity stems from its alkalanity. But *E. faecalis* can survive in an alkaline environment.[7,8] Thus; a variety of antimicrobial agents have been tested for their ability to eradicate calcium hydroxide–resistant microorganisms (especially *E. faecalis*) from root canals and dentinal tubules, including camphorated paramonochlorophenol; camphorated phenol; combinations of steroids and antibacterial agents; and irrigants such as iodine potassium iodide, chlorhexidine, and sodium hypochlorite.[6,8,9]

Another intracanal medicament is triple antibiotic paste (TAP), a combination of the antibiotic drugs metranidozole, ciprofloxacin, and minocycline, described by Takushige et al.[10] The paste is used for oral infectious lesions such as dentinal, pulpal, and periradicular lesions with the aim of utilizing TAP is to eliminate target bacteria, which are possible sources of endodontic lesions.[10] Recently this paste has produced excellent clinical results.[11,12] The purpose of this study was to investigate the in vitro antimicrobial activity against *E. faecalis* of calcium hydroxide mixed with TAP in various concentrations.

MATERIALS AND METHODS

Triple antibiotic paste and $Ca(OH)_2$ powder (Sultan, Englewood, NJ, USA) were mixed in different proportions and tested for antibacterial activity against *E. faecalis* using the agar diffusion method. Each test material was mixed with sterile saline solution at 1.5:1 (wt/vol) ratio. Freshly prepared pastes were used for each test.

The samples were divided into the following groups: **Group 1**, 100% $Ca(OH)_2$; **Group 2**, + 2.5% TAP; **Group 5**, 95% $Ca(OH)_2$ + 5% TAP; **Group 6**, 90% $Ca(OH)_2$ + 10% TAP; **Group 7**, 75% $Ca(OH)_2$ + 25% TAP; **Group 8**, 50% $Ca(OH)_2$ + 50% TAP; **Group 9**, 25% $Ca(OH)_2$ + 75% TAP; **Group 10**, 100% TAP.

The *E. faecalis* (ATCC 29212) strain was used for this study was inoculated in Tryptic Soy Broth (Biomerieux, Marcy l'Étoile, France) and incubated at 37 °C for 24 hours. The bacteria were then resuspended in saline to standardize the final concentration of 1.5×10^8 cells/mL equivalent to the 0.5 standard of the McFarland scale. Next Petri plates with 20 ml of tryptic soy agar (TSA) (Biomerieux, Marcy l'Étoile, France) were inoculated with 0.1 ml of the microbial suspension using sterile swabs that were spread on the medium and four wells (5 mm deep and 5 mm in diameter) were made in the inoculated agar plates with a sterile stainless steel cylinder. The wells were completely filled with one of the test materials and each agar plate included only one test material. The experiment was performed eight times with each medicament (n=8). The agar plates were maintained for 1 h at environmental temperature and then incubated at 37 °C for 48 h. The diameter of the zone of bacterial inhibition around the wells containing the test materials was measured and recorded in millimeters.

RESULTS

The average values of inhibition zones created by calcium hydroxide and TAP combinations are presented in figure 1 and table 1. No statistical methods were applied owing to the possible error factors originating from the group that had zero value and/or zero standard deviation. Group 1 had no effect against *E. Faecalis* while Group 10 demonstrated the best antibacterial effect in this study. The antibacterial effects of the groups ranked from strongest to weakest as follows:

Group 10, 9, 8, 7, 6, 5, 4, 3, 2, 1. Increasing the TAP proportion resulted in an increase in antibacterial efficiency.

Table I. Average of the inhibition zones of all groups against *E. Faecalis.*

	100% $Ca(OH)_2$	0,5% TAP 99,5% $Ca(OH)_2$	1% TAP 99,% $Ca(OH)_2$	2,5% TAP 97,5% $Ca(OH)_2$	5% TAP 95% $Ca(OH)_2$	10% TAP 90% $Ca(OH)_2$	25% TAP 75% $Ca(OH)_2$	50% TAP 50% $Ca(OH)_2$	75% TAP 25% $Ca(OH)_2$	100% TAP
E. faecalis	0,0 (0,0)	23,0 (0,9)	26,85 (0,47)	30,50 (0,47)	30,80 (0,67)	31,70 (0,48)	32,30 (0,48)	32,60 (0,70)	32,90 (0,39)	39,0 (0,82)

Values are presented as mm
()SD. N: 8/group.

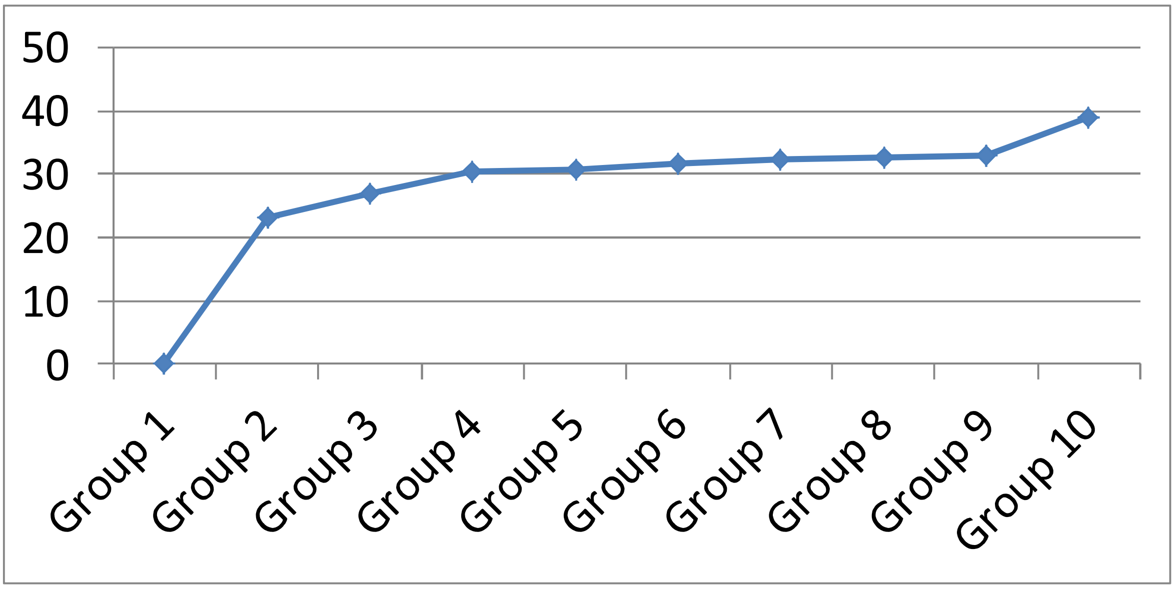

Figure 1. Average of the inhibition zones of all groups against *E. Faecalis* (mm).

DISCUSSION

The results of the present study show that additional antimicrobial benefits against *E. faecalis* can be achieved by combining calcium hydroxide with TAP.

In the study, the agar diffusion test, which is one of the most frequently used methods for assessment of the antimicrobial activity of endodontic materials, was used.[13] This test allows direct comparisons of the test materials against the microorganisms found in the local microenvironment of the root canal system.[14] Most of the research on calcium hydroxide pastes has used the agar diffusion method because of its simplicity, standardization, and reproducibility.[15,16]

E. faecalis was chosen as a test organism since it is considered the most resistant microorganism in endodontic infections and is implicated as a possible cause of root canal treatment failure.[17] In addition, it is a facultative organism that is non-fastidious and easy to grow.[18,19] The ATCC 29212 strain used in this study has been used in several in vitro studies to test the antimicrobial action of intracanal medication.[19]

Even though $Ca(OH)_2$ is routinely used as an intracanal medicament in dental clinics, the poor antibacterial effect of $Ca(OH)_2$ against *E. faecalis* has been well documented by earlier studies.[18,20] Our results accord with other findings showing *E. faecalis*'s resistance to $Ca(OH)_2$.[16,21] The antibacterial properties of $Ca(OH)_2$ are attributed to its alkalinity. However, *E. faecalis* can survive in an alkaline environment.[7,8] leading to the addition of some antimicrobial agents to $Ca(OH)_2$ to provide antimicrobial efficacy.

Antibiotics have been applied in endodontic treatment locally since 1951 when Grossman[22] first used poly antibiotic paste. While systemic antibiotics appear to be clinically effective, their administration has the potential risk of adverse systemic effects, such as allergic reactions, toxicity, and the development of resistant strains of microbes.[23] Local antibiotic usage prevents systemic consequences and complications. Moreover, antibiotics can be used in higher concentration locally.[24] Therefore, local application of antibiotics within the root canal system may be a more effective mode for delivering the drug.[23]

Many studies investigating the antimicrobial efficacy of different antibiotics against *E. faecalis* in local administration can be found in the published literature.[25-28] Kritthikadatta et al.[25]showed that metronidazole has an antibacterial effect on *E. faecalis*, although it is known to be more effective against obligate anaerobic bacteria than against aerobic and facultative anaerobic bacteria. The combination of calcium hydroxide and antibiotics also has been studied. One study argued that antimicrobial treatment with calcium hydroxide in combination with either erythromycin or tetracycline had a significant effect on enterococci. This study also showed that erythromycin had a superior effect compared with tetracycline.[26]

Different protocols using the local administration of a combination of antibiotic drugs (TAP) were described by Takushige et al.[10] The paste is used to eliminate target bacteria, which are possible sources of endodontic lesions, and is being employed successfully in current dental practise. In a recent case report on retreatment of a failed endodontic case, where the prevalence of *E. faecalis* ranged between 24 and 70 percent and was associated with a large periradicular lesion, TAP produced excellent clinical results.[4,12] There are also many case reports on the treatment of large periradicular lesions with TAP.[11,12] In spite of these results, no previous study has evaluated the antibacterial efficacy of TAP combined with Ca(OH)2 against *E. faecalis*. In this study, the combination of $Ca(OH)_2$ with TAP provided better antibacterial efficacy against *E. faecalis* even at low concentrations than only $Ca(OH)_2$ usage .

The results of the present study are similar to those of Pallotta et al.[28] They determined the minimum inhibitory concentrations of iodoform, calcium hydroxide, iodine potassium iodide, and CFC (ciprofloxacin, Flagyl [metronidazole], and calcium hydroxide) required to kill *S. aureus, P. aeruginosa, E. faecalis*, and *B. Fragilis*.[28] CFC produced the best results. The antibacterial action of CFC results from the presence of two antibiotics: ciprofloxacin, which is an antibiotic specific for enterobacteria such as *E. faecalis* and *P. aeruginosa*, and metronidazole, which can eliminate anaerobic bacteria such as *B. Fragilis*.[29]

CONCLUSION

The combination of $Ca(OH)_2$ with TAP provided the greatest antibacterial efficacy against *E. Faecalis* among the different groups in this study. In addition, the antibacterial effect increased with a higher proportion of TAP. However, in vitro results should be carefully analyzed before they are adapted to clinical use.

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