Assessment of the Antibacterial Activity of Calcium Hydroxide Combined with Chlorhexidine Paste and Other Intracanal Medications against Bacterial Pathogens

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ABSTRACT

Objectives: The purpose of this study was to assess the *in vitro* antibacterial activity of four formulations of calcium hydroxide [$Ca(OH)_2$] pastes against *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus mutans*.

Methods: A broth dilution test was performed, and the lengths of time for different pastes to kill the microbial cells were recorded and statistically analyzed. The following medications were assessed: Group I – $Ca(OH)_2$ + 2.0% chlorhexidine (CHX) gel; Group II – $Ca(OH)_2$ + camphorated paramonochlorophenol (CMCP) and propylene glycol; Group III – $Ca(OH)_2$ + propylene glycol; Group IV – $Ca(OH)_2$ + saline.

Results: The results showed that *E. faecalis* was the most resistant microorganism. Groups II and III eliminated all the microbial cells in 15 seconds. Group I took 45 seconds to eliminate *E. faecalis*.

Conclusions: Under the conditions of this study, it was concluded that all the intracanal medications tested showed antibacterial activity. However, the association of $Ca(OH)_2$ and PMCC or $Ca(OH)_2$ and propylene glycol showed a better performance, since Groups II and III took a shorter length of time than the other groups to eliminate *S. aureus* and *E. faecalis*. (Eur J Dent 2011;5:1-7)

Key words: Calcium hydroxide; Chlorhexidine gel; Intracanal medications; *Enterococcus faecalis*.

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INTRODUCTION

The success of root canal treatment depends on mechanical preparation, irrigation, microbial control and complete filling of the root canal system.[1,2] Bacteria and their products are considered the primary etiologic agents of pulp necrosis and perirradicular lesions. Microorganisms infecting the root canal may survive endodontic procedures,[2-4] due to anatomical complexities and consequent limitations of access by instruments and irrigants.[5]

Microorganisms may also infiltrate through a poor temporary seal during the period between appointments for endodontic treatment.[6] Therefore, the use of a root canal dressing is important for obtaining[7] and maintaining a disinfected canal after mechanical instrumentation and before root canal obturation.[2,8,9] Despite its viscous consistency, the paste made by mixing calcium hydroxide [$Ca(OH)_2$] and chlorhexidine (CHX) gel 2% was found not to influence the sealing ability of the obturation technique.[10]

Calcium hydroxide plays an important role in endodontics due to its ability to induce hard tissue formation,[11] its antibacterial effect[12] and its ability to act as a physical barrier to prevent root canal reinfection.[13] In an attempt to enhance the antimicrobial activity of $Ca(OH)_2$, different substances have been used as vehicles. The combination of $Ca(OH)_2$ and camphorated paramonochlorophenol (CMCP) was proposed by Frank[14] to extend the antibacterial spectrum of $Ca(OH)_2$, mainly against some facultative or anaerobic bacteria. However, previous studies have clearly shown the toxicity of CMCP *in vivo* and *in vitro*.[15] Therefore, the use of these phenolic compounds should be treated with caution in dentistry.[15]

Chlorhexidine gluconate has been widely used as an endodontic irrigant, because of its antimicrobial activity against Gram-positive and Gram-negative microorganisms.[16-19] Chlorhexidine gluconate may also present residual antimicrobial activity on the dentin surface after prolonged contact (at least one week) with the root canal.[20-22]

Recent studies have suggested that CHX could be used in combination with $Ca(OH)_2$ to improve antimicrobial efficacy against $Ca(OH)_2$–resistant microorganisms.[4,17,19,23-25]

The broth dilution test has been used to investigate the *in vitro* antimicrobial activity of CHX (in gel and liquid formulations) and sodium hypochlorite against several endodontic pathogens.[26-31] The agar diffusion method only indicates the medicament potential to eliminate bacteria within the root canal system,[30] but does not quantify its action timing. Unlike the agar diffusion method, the broth dilution test is not dependent on the solubility and diffusibility of the substance evaluated in the culture medium.

The purpose of this study was to assess the *in vitro* antimicrobial effect of $Ca(OH)_2$ when combined with saline, propylene glycol, CMCP plus propylene glycol and 2% CHX gel against *S. aureus*, *S. mutans*, *E. faecalis* and *P. aeruginosa*.

MATERIALS AND METHODS

The methodology of this study was adapted from Vianna et al[26] and Gomes et al,[29] who used a broth dilution test to quantify the time required for an antimicrobial agent to inhibit microbial growth.

$Ca(OH)_2$ pastes were prepared using $Ca(OH)_2$ P.A. (Biodinâmica, Ibiporã, PR, Brazil) combined with different vehicles, as follows: Group I – $Ca(OH)_2$ + 2.0% CHX (Endogel, Itapetininga, SP, Brazil); Group II – $Ca(OH)_2$ + CMCP (Biodinâmica, Ibiporã, PR, Brazil) + propylene glycol (SIAFARMA, Campinas, SP, Brazil); Group III – $Ca(OH)_2$ + propylene glycol; Group IV - $Ca(OH)_2$ + saline (Ecibra, Santo Amaro, SP, Brazil). The $Ca(OH)_2$ pastes were prepared in a proportion of 2:1 or 2:1:2 (group II) and the consistency was similar to that of a toothpaste. Saline was used as a negative control. Antibacterial activity was evaluated against the Gram-positive microorganisms *E. faecalis* (ATCC 29212), *S. aureus* (ATCC 25923), *S. mutans* (UA 159) and *P. aeruginosa* (ATCC 27853). The strains were inoculated into brain-heart infusion (BHI) broth and incubated in an aerobic atmosphere at 37°C. After 24 hours, the turbidity of the culture medium was assessed using a spectrophotometer (Ultrospec 1000; Amersham Pharmacia Biotech, Cambridge, UK).

For the broth dilution test, 10 µl of each tested substance, as well as sterile saline (control group), were placed in 24-well cell culture plates (ref. no. 3524, vol. 3.2 mL; Corning, NY, USA). Thus, 20 µl of the microbial suspension were added to each well containing the substances or the control solution. Six wells were used for each time period and medication (i.e. from each well, only one time

period and medication were tested). Overall, 1080 wells were used, comprising 864 for all the test groups and 216 for the control group.

The well cell culture plates were placed onto an upside down 250 mL stainless steel griffin beaker (BK 1122, MGL Scientific, Elko, NV, USA) inside an ultrasonic cleaner (Bransonic Ultrasonics Corporation, Danbury, CT, USA) that had been previously filled with 1400 mL of distilled water up to the operating level. These plates were ultrasonicated for 10 s and left to stand for different periods of time: 15, 30, 45, 60 seconds, 5, 15, 30 minutes, 1 and 24 hours. After each period of time, 10 µl from each well was transferred to a tube containing 2 mL of fresh broth media (BHI), containing neutralizers in order to prevent continued action of the substances.

The neutralizer for CHX was Tween 80 plus 0.07% lecithin, whereas citric acid was used for $Ca(OH)_2$.[26] All tubes were left at 37°C for 48 hours under appropriate gaseous conditions. After this period, 10 µl from each tube were inoculated onto agar plates to evaluate bacterial growth. The purity of the positive cultures was confirmed by Gram staining, biochemical tests and analysis of the colony morphology on blood agar plates. The time needed for each substance to achieve complete inhibition of microbial growth was recorded and transformed into seconds.

RESULTS

Figure 1 presents the period of contact required for the 4 groups to produce negative cultures of all tested microorganisms. Groups II and III eliminated all the microorganisms in 15 seconds. Only 15 seconds were needed for the $Ca(OH)_2$ + 2.0% CHX combination to produce negative cultures of *S. mutans* and *P. aeruginosa*. However, this group took 45 seconds to eliminate *S. aureus* and *E. faecalis*. The same time, 45 seconds, was needed for the substances of Group IV to produce negative cultures of *E. faecalis*.

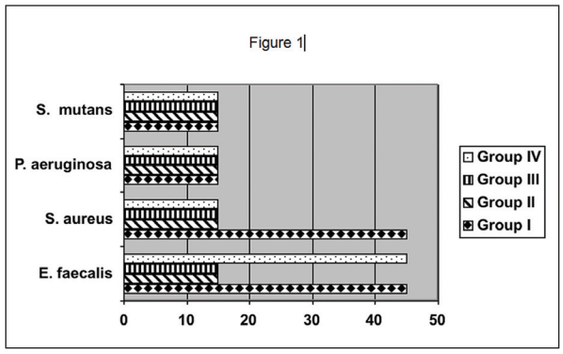

Figure 1. Maximum time (in seconds) needed for all the test substances to produce negative cultures of each microorganism.

According to the present study, the microbial resistance to all substances tested can be ranked from the strongest to the weakest as follows: *E faecalis* was the strongest, followed by *S. aureus*, *P. aeruginosa* and *S. mutans* (the latter two at the same level).

DISCUSSION

Establishing the spectrum of activity of any antimicrobial agent is essential for improving infection control.[27] The *in vitro* techniques that have been used for this purpose have advantages and disadvantages. The agar diffusion method may not express the full potential of the test substance, since the inhibition zones depend on its solubility and diffusibility in the culture medium.[9,28] In the present study, the broth dilution test was used, which relies on direct and close contact between the test microorganism and materials.[32] It is considered a more precise method to analyze medications than the agar diffusion, in which the medicaments dissociate and diffuse differently through the media.[28] In summary, the broth dilution test uses a liquid culture medium and allows liquid and paste formulations to have a more similar diffusion conditions than the agar diffusion method.

There are several reasons for a poorer in vivo performance of medicaments as compared with their *in vitro* results, including poor penetration of the medicament/irrigants, low concentration, short exposure time, small overall volume, poor exchange of irrigants in the apical portion of the root canal, and inactivation of the medicament in the root canal. Resistant, surviving microbes can be isolated even on the walls of the main root canal after cleaning and shaping procedures. The results of this study showed strong activity of the substances tested when in direct contact with microorganisms. However, $Ca(OH)_2$, in combination with CMCP and/or propylene glycol, mixed very well with the bacterial suspension, and immediately exerted its antimicrobial action, whereas the gel formulation, which did not mix as easily, delayed required direct contact between bacterial cells and CHX, thus requiring a longer time to act against *E. faecalis* and *S. aureus*.

Calcium hydroxide is the recommended intracanal medication for the treatment of apical periodontitis. Its antimicrobial action mechanism is influenced by the speed of its dissociation into calcium ions and hydroxyl ions[23] and by inactivating enzymes of the cytoplasmic membrane of microorganisms, thus causing toxic effects on the bacterial cells.[27] To be effective against the bacteria located inside dentinal tubules, the hydroxyl ions must diffuse at sufficient concentrations and exceed the dentin buffering ability, thus producing pH levels high enough to destroy or inactivate microorganisms.[33] Another mechanism of action of this medication is its ability to absorb carbon dioxide, thus leading to the death of CO_2–dependent bacteria, such as *Actinomyces*.[34]

Different vehicles have been added to $Ca(OH)_2$ in an attempt to enhance its antimicrobial activity, dissociation and biocompatibility.[21,23,25,27,33,35,36] The combination of $Ca(OH)_2$ with CMCP has previously been shown to be more capable of inhibiting the growth of bacteria than CHX and $Ca(OH)_2$ combined with sterile saline.[37] Chang et al[15] demonstrated that CMCP is cytotoxic to the target periodontal ligament cells by inhibiting cell viability and proliferation. The present study found that this association kills microorganisms in 15 seconds.

Propylene glycol is a clear, colorless, and odorless liquid with a characteristic taste that resembles that of glycerin. Its wide application in endodontics as a vehicle for intracanal medicaments is attributable to its strong antibacterial action against microorganisms commonly found in infected root canals.[38] Another advantage of this substance is its consistency, which improves the handling qualities of the paste.[12] According to Simon et al,[39] by the use of this vehicle it is possible to control the level of pH rise and Ca^{2+} release that can be sustained for a long period. The authors also stated that there is no justification to continue the use of CMCP as a vehicle for $Ca(OH)_2$. Instead propylene glycol that possesses many desirable qualities can be routinely used in preference to all the other vehicles. The effects of glycerine and propylene glycol mixing vehicles on the pH of $Ca(OH)_2$ preparations was investigated using conductivity testing by Safavi et al.[40] A range of 10–30 percent for a glycerine/water mixture and 10–40 percent for a propylene glycol/water mixture resulted in the greatest amount of conductivity. In our study, only 15 seconds were needed for $Ca(OH)_2$ combined with propylene glycol to kill all microorganisms.

Chlorhexidine gluconate is a broad-spectrum antibacterial agent whose positively charged molecules can be adsorbed onto dentin and prevent microbial colonization on the dentin surface.[2,41] Ferguson et al[42] showed that chlorhexidine diffuses through the root canal, and possibly into the dentinal tubules, thus being an effective anticandidal agent. Interestingly, $Ca(OH)_2$ has been shown to be inefficient in the killing of both facultative anaerobes and yeasts.[43] The aim of combining $Ca(OH)_2$ and 2% CHX gel (CG) is to enhance antimicrobial effectiveness, particularly against resistant microorganisms such as *E. faecalis* that are implicated in the failure of root canal treatment.[44,45] The association of CHX and $Ca(OH)_2$ has been tested against *E. faecalis* in infected bovine root dentine, demonstrating that the combined medicaments were effective.[46,47]

Enterococcus faecalis are enteric facultative anaerobic Gram-positive cocci that have been associated with persistent endodontic infections.[48] A distinguishing characteristic of *E. faecalis* is its ability to grow at an alkaline pH that normally inhibits other bacteria. Evans et al[49] found that a functioning intracellular proton pump was the primary factor triggering alkaline resistance of *E. faecalis*, because it can maintain pH homeostasis within a narrow physiological range, allowing enzymes to work normally.[50]

The direct contact method revealed that the combination of $Ca(OH)_2$ and PMCC was more effective than the combination of $Ca(OH)_2$ and CHX, since Group I took 45 seconds to produce negative cultures for *S. aureus* and *E. faecalis*. As previously mentioned, the results of this method can be justified by the high viscosity of the CHX gel, which may impair direct contact between the medication and microorganism. Estrela et al[27] demonstrated that the lower the viscosity of the vehicle, the higher the ionic dissociation of the $Ca(OH)_2$. Athanassiadis et al[51] reported that the reduced antibacterial activity of $Ca(OH)_2$ and CHX association may be because chlorhexidine is a cationic biguanide whose optimal antimicrobial activity is achieved within a pH range of 5.5–7.0. Therefore, it is likely that alkalinizing the pH by adding $Ca(OH)_2$ to CHX will lead to precipitation of the CHX mol-

ecules and thereby decreases its effectiveness. In a study using agar diffusion, Haenni et al[52] could not demonstrate any additive antibacterial effect by mixing $Ca(OH)_2$ powder with CHX (0.5 percent). In fact, they showed that the CHX had a reduced antibacterial action. However, $Ca(OH)_2$ did not lose its antibacterial properties in such a mixture. This may be due to the deprotonation of CHX at a pH greater than 10, which reduces its solubility and alters its interaction with bacterial surfaces as a result of the altered charge of the molecule. Mohammadi & Abbott[53] provided a systematic review regarding different aspects of CHX of relevance to endodontics, concluding that the usefulness of mixing $Ca(OH)_2$ with CHX still remains unclear and controversial.

CONCLUSIONS

Based on the results of this research, it can be concluded that all the intracanal medications tested showed antibacterial activity. However, the association of $Ca(OH)_2$ and PMCC or $Ca(OH)_2$ and propylene glycol showed a better performance, once Groups II and III took a shorter length of time than the other groups to eliminate *S. aureus* and *E. faecalis*.

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