Evaluation of different pH levels of calcium hydroxide on *Enterococcus faecalis*: an *in vitro* study

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Abstract

The aim of the study was to determine the effective pH of calcium hydroxide Ca(OH)_2 against *Enterococcus faecalis*, the most frequently isolated bacterial species in endodontic failure. Solutions of Ca(OH)_2 with different pH values of 9, 11 and 12.5 were prepared by dissolving 1 mg of Ca(OH)_2 in distilled water. The test solutions were grouped as follows: Group I, vials containing phosphate buffered saline pH of 7.2 as control; Group II, aqueous calcium hydroxide with pH of 9; Group III, aqueous calcium hydroxide with pH of 11; Group IV, aqueous calcium hydroxide of pH 12.5. Forty sterile paper points were transferred to Trypticase Soy Agar broth containing *Enterococcus faecalis* for a period of 10 min, then transferred to vials containing 1 mL of the test solutions and incubated for 1 h at 37°C. The paper points were then transferred to neutralizing broth with glass beads to resuspend the microorganisms. Ten-fold serial dilutions were performed using aliquots of 250 µL from the broth and the same amount was plated onto blood agar plates. The plates were incubated anaerobically at 37°C for 24 h. The number of colony forming units for each dilution was counted using the colony counter. The inhibitory effects of different pH groups on the bacteria were as follows: Group IV > Group III > Group II > Group I in an ascending order. This study revealed that Ca(OH)_2 at a pH 12.5 was effective in complete elimination of *Enterococcus faecalis*.

Introduction

The success of root canal treatment should aim to eliminate bacteria from the infected root canal and prevent re-infection. Cleaning, shaping and irrigating root canals has been shown to greatly reduce the number of bacteria in infected canals. However, obtaining complete disinfection of root canals has proved difficult in some cases.¹ Deep-seated microorganisms, in particular *Enterococcus faecalis*, may escape killing, remaining viable for multiplication under favorable conditions and thus cause endodontic treatment failure.² The use of intra-canal medications helps to eliminate bacteria that remain after mechanical debridement.¹

The most popular intra-canal medicament is calcium hydroxide, which has a profound antimicrobial effect against most of the microorganisms found in the root canals. The primary advantage of Ca(OH)_2 is that it is capable of killing microorganisms without direct contact by absorbing the CO_2 required for bacterial growth and thereby releasing hydroxyl ion (OH^-) in the environment. This results in a highly alkaline pH 12.5, which does not favor the growth of most endodontic pathogens.¹

Enterococcus faecalis is one of the most prevalent species found in persistent root canal infections refractory to endodontic treatment.³ It is commonly found in a higher percentage of root canal failures and it is able to survive in the root canal as a single organism or as a major component of flora.² It is well recognized that such microbial communities are noticeably resistant to antimicrobial agents and are difficult to eradicate. This study aimed to evaluate the optimal pH value at which calcium hydroxide is effective against *E. faecalis*.

Materials and Methods

Bacterial strains

Enterococcus faecalis (ATCC 29212) was collected from the Department of Microbiology and Immunology, CMC-Vellore, Tamilnadu, India, and preserved by lyophilization in an ampoule. Cells were reviewed by subculturing a loopful of the bacteria on blood agar and plates were incubated at 37°C for 24 h.

Preparation of test suspensions

Samples of Ca(OH)_2 with different pH (9, 11 and 12.5) were prepared by dissolving 1 mg of Ca(OH)_2 powder (Merck, Mumbai, India) in 100 mL of distilled water at room temperature to obtain a liquid phase and a solid phase. The

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Key words: calcium hydroxide, endodontic failure, *Enterococcus faecalis*, Trypticase Soy Agar broth, TSA broth.

Conflict of interests: the author reports no conflict of interests.

Received for publication: 21 March 2012.

Revision received: 22 May 2012.

Accepted for publication: 11 June 2012.

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Microbiology Research 2012; 3:e22

doi:10.4081/mr.2012.e22

clear supernatant liquid phase was pipetted into a separate beaker and the pH measured with a pH meter (Mark IV). The pH of this clear supernatant was 12.5. The pH values of aliquots of the supernatant were adjusted to 9.0 and 11.0, respectively. Phosphate buffered saline was used as a control in all experiments. The solutions with different pH values were assigned to groups as follows: Group I Control (pH=7.2); Group II (pH=9.0); Group III (pH=11.0) and Group IV (pH=12.5). Forty sterile paper points (Dentsply, USA) 5 mm long were placed in a sterile glass tube and sterilized at 160°C for 30 min.

Preparation of bacterial culture

Enterococcus faecalis was subcultured onto blood agar plates and then incubated at 37°C for 24 h. A well isolated colony was transferred into Trypticase-soy broth (TSA) and incubated anaerobically. Cell growth was assessed according to the presence of turbidity. The paper points were then transferred to TSA broth containing *Enterococcus faecalis* for 10 min. Each pH group consisted of 10 test samples. The paper points were removed from the broth and transferred to vials containing 1 mL of the test solutions, and vials were incubated at 37°C. After incubation, the paper points were removed from the test solution and transferred to bottles containing a neutralizing broth with glass beads. The contents in the bottles were vortexed for 30 s to suspend the microorganisms in the neutralizing broth. Aliquots of 250 µL from the broth was serially diluted 10-fold and 250 µL was then plated onto blood agar plates. The plates were incubated anaerobically at 37°C for 24 h. The number of

colony forming units (cfu/mL) from each dilution was counted using the colony counter. The results obtained were analyzed by Statistical software SPSS 15.0, MedCalc 9.2.1.0 and Systat 11.0. to determine the optimal pH value (12.5) at which $\text{Ca}(\text{OH})_2$ would effectively inhibit the growth of *Enterococcus faecalis*.

Results

Logarithmic non-linear regression analysis has been carried out to find the correlation of growth and the dilution levels for pH 7.2, pH 9, pH 11 and pH 12.5. The statistical softwares SPSS 15.0, MedCalc 9.2.1.0 and Systat 11.0 were used for data analysis.

Ten serial dilutions were made for each group (Table 1). Results were:

Group I: 262 colonies of bacteria were observed at 10^{-1} dilution, and bacterial growth was also observed at 10^{-10} dilutions, indicating that phosphate buffered saline was not efficient in inhibiting the bacterial growth;

Group II: 181 colonies were observed at 10^{-1} dilution and growth was observed only up till 10^{-6} dilutions;

Group III: 73 bacterial colonies were observed at 10^{-1} dilution and bacterial growth was observed up till 10^{-4} dilutions;

Group IV: no colony growth was observed at the initial or further dilutions, indicating complete inhibition of bacteria (Figure 1).

To summarize, inhibitory effects of different calcium hydroxide pH groups was as follows: GROUP IV > GROUP III > GROUP II > GROUP I in an ascending order.

Discussion

Mechanical instrumentation and irrigation are the main methods of removing bacteria from the root canal space. In particular, irrigation with antibacterial agents and placement of intra-canal medications are essential to kill bacteria in infected dentinal tubules and root-canal ramifications which cannot be eliminated mechanically.⁴ Calcium hydroxide was chosen for this study because it plays an important role in endodontics through its ability to induce hard tissue formation, moderate antibacterial activity, and its tissue dissolving properties.⁵ Its antimicrobial mechanism of action is influenced by its speed of dissociation into calcium ions and hydroxyl ions in a high pH environment which inhibits enzymatic activities that are essential to microbial life, i.e. metabolism, growth and cellular division.⁶ Athanassiadis *et al.* reviewed several $\text{Ca}(\text{OH})_2$ combinations and concluded that the choice of

which intra-canal medication to use during endodontic treatment depends on having an accurate diagnosis of the condition to be treated.⁷ Studies have demonstrated that water is an effective carrier for calcium hydroxide, producing a rapid dissociation of OH^- and Ca^{2+} ions. Esberrad *et al.* found that a mixture of $\text{Ca}(\text{OH})_2$ and sterile water produced a rapid increase in pH.⁸ Calcium hydroxide alters the biological properties of bacterial lipopolysaccharides in the cell walls of gram negative species and inactivates membrane transportation mechanisms, resulting in cell toxicity. The lethal effects of hydroxyl ions are probably due to:

- damage to the cytoplasmic membrane
- protein denaturation
- damage to DNA.⁹

A recognized pathogen in post-treatment endodontic infections, *E. faecalis* is frequently isolated both in mixed flora and in monocultures. *E. faecalis* is probably the species that

can best adapt to and tolerate the ecologically demanding conditions in the filled root canal. Eradication of *E. faecalis* from the root canal with chemo-mechanical preparation, and the use of disinfecting irrigants and antibacterial dressings is problematic.¹⁰ It can occupy ecological niches created by the removal of other microbes and grow in a low-oxygen environment.¹¹ *E. faecalis* has certain virulence characteristics including lytic enzymes, cytotoxins, aggregation substances, pheromones and lipoteichoic acid. It has been shown to adhere to host cells, express proteins that allow it to compete with other bacterial cells, and alter host responses. Evans *et al.* reported that *E. faecalis* was resistant to $\text{Ca}(\text{OH})_2$ at a pH of 11.1, but unable to survive at a pH higher than 11.5.¹² which is in accordance with the present study. A dentine block model may have been used to assess the antimicrobial effectiveness of calcium hydroxide, as this may be more clinically relevant to the known inhibitory action

Table 1. Colony forming units at different serial dilutions of experimental groups.

pH levels of calcium hydroxide	1 (10^{-1})	2 (10^{-2})	3 (10^{-3})	4 (10^{-4})	5 (10^{-5})	6 (10^{-6})	7 (10^{-7})	8 (10^{-8})	9 (10^{-9})	10 (10^{-10})
Group 1 (control)	262	197	135	80	42	21	9	3	1	1
Group 2 (pH=9)	181	130	84	47	23	10	0	0	0	0
Group 3 (pH=11)	73	42	25	10	0	0	0	0	0	0
Group 4 (pH=12.5)	0	0	0	0	0	0	0	0	0	0

Figure 1. Growth of bacteria at different dilutions in different experimental groups.

of dentine on root canal medications.¹³ The dilution susceptibility test, which is an effective method to evaluate the antibacterial properties of antimicrobial agents, was used. This method allows the solution to have a direct contact between the bacterial cells and the agent to be tested. Such a method proves critical when evaluating the antimicrobial activity of calcium hydroxide, which has a low solubility and diffusibility.¹⁴

Conclusions

Under the conditions of this study, we can conclude that aqueous calcium hydroxide at pH 12.5 is very effective in killing 100% of the bacteria (*E. faecalis*). The ability of aqueous Ca (OH)₂ solution to kill the bacteria increased with a subsequent increase in pH of the solution.

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