Comparison of Antibacterial Effect of Calcium Hydroxide Combined With Chlorhexidine and Povidone-Iodine Against Enterococcus faecalis in Dentinal Tubules of Human Incisors: An In Vitro Comparative Study

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Background and Objectives: It has been found that the microorganism behind the failure of root-filled teeth is Enterococcus faecalis, which shows resistance to most of the intra-canal medicaments. Therefore, the purpose of this study was to evaluate the antibacterial efficacy of three intra-canal medications—calcium hydroxide with saline, combinations of calcium hydroxide with 2% chlorhexidine (CHX), and calcium hydroxide with 5% povidone-iodine—against E. faecalis in dentinal tubules of human incisors. Materials and Methods: Forty permanent maxillary central incisors were made into standardized segments and infected with E. faecalis. They were treated with a paste made of calcium hydroxide and 2% CHX, calcium hydroxide and 5% povidone-iodine, and calcium hydroxide and saline for 1 week. Dentinal shavings collected from the canal were suspended in thioglycollate broth solution and spread on brain heart infusion agar. Colony-forming units (CFUs) were enumerated and the CFU per milligram of dentin was calculated. The pH of the medicaments used was measured with the help of pH meter. Results: The results showed that the paste made from calcium hydroxide and 2% CHX was significantly more effective than that made from calcium hydroxide and povidone-iodine, and calcium hydroxide and saline. The addition of CHX or povidone-iodine did not affect the alkalinity of calcium hydroxide. Conclusion: This study concludes that Ca(OH)2 + 2% CHX are effective against E. faecalis. Combinations of calcium hydroxide and 5% povidone-iodine showed better antibacterial effect than calcium hydroxide and saline. Ca(OH)2 + saline was ineffective against E. faecalis.

KEYWORDS: Calcium hydroxide, CFU, chlorhexidine, Enterococcus faecalis, intra-canal medicament

INTRODUCTION

Microorganisms play a pivotal role in the development and perpetuation of pulpal and periradicular diseases[1] and are the major causative factor associated with endodontic treatment failures.[2] Endodontic research assumes special importance in finding newer methods and materials to significantly eradicate root canal infection. An endodontic treatment can only be successful if we are able to completely eliminate the bacteria and their by-products. Intra-canal medicaments play a pivotal role in this context and calcium hydroxide is universally considered as the gold standard. The antibacterial action is mainly attributed because of its high pH. At the same time, Enterococcus faecalis can also be resistant to the bactericidal effect of...
Ca(OH)_2 in case a high pH is not maintained, which is attributed to its ability to passively maintain a pH. The efficacy of Ca(OH)_2 against *E. faecalis* can be increased by combining it with another medicament that could produce additive or synergic effect. Chlorhexidine (CHX), an intra-canal medicament that has recently emerged, has excellent antimicrobial properties. Its bacteriostatic action at low concentration and bactericidal action at high concentration give a better effect on the microbial flora. Povidone-iodine and CHX\(^2\) may be useful in killing calcium-hydroxide-resistant bacteria. Supplemetning the antibacterial activity of calcium hydroxide with CHX or povidone-iodine preparations may play an important role to improve the efficacy of intra-canal treatment. CHX and povidone-iodine have got antibacterial and antifungal effects when used in the treatment of pulp gangrene and in cases where conventional therapy is not working. This study was conducted to evaluate the antibacterial efficacy of an intra-canal medication composed of calcium hydroxide with CHX or povidone-iodine preparations against *E. faecalis* in dentinal tubules of human incisors. As alkalinity is considered as an important antimicrobial effect of calcium hydroxide, the influence of povidone and CHX on the pH in combination was also studied.

**Materials and Methods**

This study was conducted to determine the antibacterial efficacy of calcium hydroxide combinations on *E. faecalis*. The study was conducted in the Department of Pedodontics, Yenepoya Dental College, Mangalore, Karnataka, India, in collaboration with the Department of Microbiology, Yenepoya Medical College, Mangalore.

The organism selected in this investigation was *E. faecalis* (ATCC29212; Hi Media, Mumbai, India), as it has been the most prevalent bacteria in the failed root canal system.

**Preparation of 2% CHX**

Ten milliliters of 20% CHX solution was taken and 90 mL of sterile water was added to prepare a 100 mL solution of 2% CHX.

**Preparation of samples**

Forty freshly extracted non-carious permanent maxillary central incisors, which were extracted for periodontal reasons, were stored in saline till use for the study after disinfecting with 5.25% NaOCl to remove debris. The tooth was horizontally sectioned into coronal, middle, and apical section using a carbide disc in handpiece. The 5 mm middle segment was taken and the root canal of each specimen was enlarged with 10 sized round bur to standardize the internal diameter of canal.

**Smear layer removal from the samples**

The smear layer was removed by placing it in a 17% EDTA followed by 5.25% NaOCl for 5 min. Each segment was then placed in a sterile tray and then transferred to Department of Microbiology, Yenepoya Medical College, Mangalore.

**Sterilization of samples**

Samples were sterilized by autoclaving at 121°C for 30 min.

**Microorganism**

Standard strain of *E. faecalis* (ATCC29212) from Hi Media was used in this study.

**Preparation of the inoculum**

Six morphologically similar magenta-colored colonies of *E. faecalis* were picked up from MacConkey’s agar with a straight wire and transferred to a test tube containing 5 mL of sterile brain heart infusion (BHI) broth. The test tube was then incubated at 35°C for 6 h to produce a bacterial suspension.

**Inoculation of the segments**

The segments were placed in BHI broth containing the culture of *E. faecalis* and incubated for 5 days at 35°C to infect the dentinal tubules. After 5 days, the segments were removed from the broth with a sterile forceps, rinsed with sterile water, and blotted dry with sterile gauze. The 40 specimens were randomly divided into 4 groups of 10 specimens each and were kept upright in petri dishes that contained nutrient agar by pressing the segment into the media as follows:

Group 1: A paste made from Ca(OH)_2 + 2% CHX (2:1)
Group 2: Ca(OH)_2 + povidone-iodine (5%).
Group 3: Ca(OH)_2 + saline
Group 4: Control (no medicament)

The medicaments were mixed on a sterile glass slab. The glass slab was sterilized by passing over a Bunsen flame three times and the cement spatula by passing in a Bunsen flame for 1 min. The powder was dispensed with a cement spatula and the liquid medicament using a sterile syringe and needle. The powder and the liquid were mixed to a creamy consistency. The creamy medicament was then taken in a lentulo spiral and applied into canal space, and the entire space was filled. The pH of the medicaments immediately on mixing was measured with help of a pH meter. The
medicaments were placed in the canal space of each segment, and these were placed back in nutrient agar and kept for incubation at 35°C and 100% humidity for 1 week. At the end of 1 week, the segments were removed from the petri dishes and the paste was removed using 2 mL of sterile water irrigation and dried with gauze and paper points. To test for bacterial survival, dentinal shavings within the canal were collected using round burs of increasing diameter. They were collected on a piece of sterile aluminum foil and weighed. These were suspended in 1 cc solution of thioglycollate broth. Of this solution, 0.01 cc was taken with the help of standard wireloop (having a diameter of 0.4 mm, which delivers 0.01 cc of the fluid) and was streaked on to BHI agar. The sterile metal loop was used to spread the suspension evenly throughout the BHI agar plate. They were incubated for 24 h and the colony-forming units (CFUs) were enumerated using a digital colony counter. The number of CFU per milligram was determined according to Miles and Misra technique. The mean CFU per milligram and standard deviation values were calculated for groups 1, 2, 3, and 4.

The obtained results were compared using Kruskal–Wallis and Mann–Whitney U test to detect the statistical difference among and between groups.

**RESULTS**

The total number of CFU of *E. faecalis* per milligram of dentin is shown in Table 1. Control group 4 (mean = 4.440670 + 0.86570) was very significantly different from group 1 (mean = 0.076215 + 0.07685), group 2 (mean = 0.5344 + 0.3008), and group 3 (mean = 2.2304 + 0.77012), indicating that group 1 was effective against the species *E. faecalis*. Calculation of the Kruskal–Wallis test indicated that calcium hydroxide paste with 2% CHX was more effective at eliminating *E. faecalis* in the dentinal tubules than other calcium hydroxide combinations. The mean value among the groups showed a very highly significant difference (*P* < 0.001) as shown in Table 2. In our study, pH of calcium hydroxide mixed with saline was 12. Calcium hydroxide combined with 2% CHX achieved a pH of 13. There was no significant change in pH values of Ca(OH)₂ paste following the addition of povidone-iodine (pH > 12.5) as shown in Table 3.

**DISCUSSION**

Intra-canal medicaments between visits have already proved to have a definite and important role to play in endodontic therapy. They can penetrate into areas not reached by instruments or irrigants and thereby preventing regrowth of residual microorganism. *E. faecalis*, which is an opportunistic facultative

| Table 1: Comparison of total number of CFU of *E. faecalis* per milligram of dentin between four different groups |
| --- |
| **Group 1** | **Group 2** |
| Dentine (mg) | CFU | CFU/mg | Dentine (mg) | CFU | CFU/mg |
| 19 | 0 | 0 | 22 | 10 | 0.4545 |
| 22 | 3 | 0.1363 | 16 | 18 | 1.125 |
| 24 | 0 | 0 | 14 | 8 | 0.5714 |
| 21 | 2 | 0.0952 | 18 | 12 | 0.6666 |
| 17 | 4 | 0.2352 | 15 | 14 | 0.9333 |
| 18 | 1 | 0.0555 | 13 | 4 | 0.3076 |
| 14 | 2 | 0.1428 | 12 | 6 | 0.50 |
| 24 | 1 | 0.0416 | 14 | 4 | 0.2857 |
| 14 | 0 | 0 | 15 | 3 | 0.2 |
| 18 | 1 | 0.0555 | 20 | 6 | 0.3 |
| **Group 3** | **Group 4** |
| Dentine (mg) | CFU | CFU/mg | Dentine (mg) | CFU | CFU/mg |
| 15 | 20 | 1.3333 | 24 | 140 | 5.8333 |
| 13 | 34 | 2.6153 | 18 | 98 | 5.4444 |
| 20 | 62 | 3.1000 | 21 | 72 | 3.428 |
| 22 | 43 | 1.9545 | 22 | 84 | 3.818 |
| 18 | 31 | 1.7222 | 16 | 60 | 3.75 |
| 16 | 54 | 3.375 | 18 | 96 | 5.333 |
| 15 | 48 | 3.2 | 20 | 80 | 4.000 |
| 24 | 34 | 1.4166 | 14 | 70 | 5.000 |
| 21 | 38 | 1.8095 | 18 | 72 | 4.000 |
| 18 | 32 | 1.7777 | 20 | 76 | 3.8 |
Anaerobe, was chosen as the test organism because it is a well-recognized pathogen associated with persistent apical lesions in endodontically treated teeth and was found to be highly prevalent in root canal failure. Extracted human teeth were used in this study to simulate the clinical situation as accurately as possible. The middle segment of root was used because the diameter of dentinal tubules is larger in the middle segment.

Calcium hydroxide, which was discovered by Hermann in 1920, has been a proved intra-canal medicament since ages. Its antimicrobial properties can be explained based on its high pH (11–12.5). It can dissociate into highly interactive and lethal hydroxyl ions that can kill bacterial cells by damaging the cytoplasmic membrane, protein denaturation, and damaging the DNA.\(^3\) It has high ability to absorb carbon dioxide that deprives capnophilic bacteria, which mainly relies on it for nutrition from thriving.\(^3\) It has got a wider spectrum of antimicrobial action against a variety of organisms including the \textit{E. faecalis}.\(^11\) It has got a primary alkaline nature. Its optimum action is proposed when the root canal pH reaches 3.5. It is at this pH that CHX is di-cationic, which enables it to adsorb onto the tooth as well as to the bacteria. This can be attributed to the antimicrobial activity of CHX due to its interaction between positively charged CHX molecule and negatively charged groups on the bacteria cell wall. This interaction encourages the permeability of the bacterial cell wall and thus allows CHX to penetrate into the cytoplasm and results in death of the organism by precipitation of the cytoplasm. Results in our study have shown that calcium hydroxide and CHX have good efficacy against \textit{E. faecalis} corroborating previous studies.\(^{12-14}\)

In this study, another combination of calcium hydroxide and povidone-iodine against \textit{E. faecalis} was studied. Povidone-iodine (polyvinylpyrrolidone-iodine) is often used as an alternative agent because of its increased antibacterial activity, decreased potential to develop adverse reactions, easy availability, ease of handling, and cheap cost. Iodine has got a strong oxidizing property. It interacts with free sulfhydryl groups of bacterial enzymes resulting in disulfide linkages and hence effective against many root canal microbes. In this study, combination of calcium hydroxide and povidone-iodine did not give a significant reduction \textit{E. faecalis} in comparison with Ca(OH)\(_2\) + 2% CHX.

In our study, pH of calcium hydroxide mixed with saline was 12. Dentin itself has got buffer properties for the base, which may reduce the pH effect of the calcium hydroxide; hence, \textit{E. faecalis} cannot be eliminated, even in an environment with a pH reduced to 11.5. Calcium hydroxide combined with 2% CHX achieved a pH of 13, which explains for its better antibacterial action. There was no significant change in pH values of Ca(OH)\(_2\) paste in combination with povidone-iodine (pH > 12.5). Very little knowledge is available about the chemical reactions in mixtures of calcium hydroxide with either povidone-iodine or CHX. Basic chemical knowledge indicates that povidone-iodine is neutral with calcium hydroxide.

**Table 2: Comparison of mean CFU per millgram among four different groups**

| Groups         | N  | Mean    | Std. Deviation | Minimum | Maximum |
|----------------|----|---------|----------------|---------|---------|
| Group 1        | 10 | 0.076215| 0.0768555      | 0.0000  | 0.2352  |
| Group 2        | 10 | 0.534410| 0.3008136      | 0.2000  | 1.1250  |
| Group 3        | 10 | 2.230450| 0.7701219      | 1.3333  | 3.3750  |
| Group 4        | 10 | 4.440670| 0.8657095      | 3.4280  | 5.8333  |

**Table 3: Comparison of pH value of the medicaments among four different groups**

| Medicaments                  | pH |
|------------------------------|----|
| CHX                          | 7  |
| Povidone-iodine + saline     | 5  |
| Calcium hydroxide + CHX      | 12 |
| Calcium hydroxide + povidone-iodine | 12 |

**Conclusion**

This study concludes that combination of Ca(OH)\(_2\) + 2% CHX is most effective against \textit{E. faecalis}. Combinations of calcium hydroxide and 5% povidone-iodine showed
better antibacterial effect than calcium hydroxide and saline. Hence, this study demonstrated the synergistic action resulted from the mixture of calcium hydroxide and CHX. However, one must keep in mind that the antibacterial action of medicaments in vitro may be quite challenging in action in comparison to mixed cultures present in a dynamic biological condition, as usually occurs in vivo. Thus, direct application to clinical conditions must be advocated with proper care because of the obvious limitations of in vitro studies.

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Conflicts of interest
There are no conflicts of interest.

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