Antibacterial effect of triantibiotic mixture versus calcium hydroxide in combination with active agents against Enterococcus faecalis biofilm

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The aim of the present study was to compare the antibacterial effect of calcium hydroxide (CH), triantibiotic mixture (TAM), and CH in combination with chlorhexidine (CHX), sodium hypochlorite (NaOCl) or colchicine (COL) against Enterococcus faecalis (E. faecalis) in surface and deep dentinal tubules. Seventy five fresh single-rooted human teeth were infected and divided into five experimental groups (n=15). The experimental groups were treated with CH+distilled water, CH+CHX, CH+NaOCl, CH+COL+distilled water and TAM+distilled water. Dentin chips obtained from surface and deep dentin of these root canals were prepared and analyzed by counting the number of colony forming units. There was significant difference between groups in the surface dentin (p<0.05). TAM showed higher antibacterial activity compared to CH-containing groups. There was no significant difference between TAM and CH-containing groups in the deep dentin (p>0.05). CH-containing medications and TAM can be used as effective disinfectants in treatment of infected root canals.

Keywords: Calcium hydroxide, Colchicine, Enterococcus faecalis, Intracanal medication, Triantibiotic mixture

INTRODUCTION

The aim of root canal treatment is to eliminate the bacteria from the root canal system, and to provide suitable environment for periapical healing11. However, chemomechanical preparation alone of infected canals is not sufficient in many cases, and the use of intracanal medication is indicated12.

Calcium hydroxide (CH) is the most common and well known intracanal medication used in endodontics10. Its antibacterial property is attributed to high pH. Hydroxyl ions released from CH destroys bacterial cell membrane, and can penetrate into dentinal tubules41. Moreover, CH has anti-resorptive activity and promotes healing in periapical tissues41. Although CH has many advantages, it has limited antibacterial activity against some microorganisms such as Enterococcus faecalis (E. faecalis)41.

E. faecalis is a gram-positive facultative anaerobic bacterial species. It comprises a small proportion of the root canal flora in initial endodontic infections, but it is found in many of secondary or persistent endodontic infections15. This microorganism is resistant against CH action by mechanisms such as deep dentinal penetration ability, high pH tolerance, surviving in food deprivation conditions, and surviving without the presence of other microbial species8,18,19. Moreover, E. faecalis biofilm is gaining added attention in recent years. Biofilm is a mode of growth in which microorganisms can survive harsh environmental conditions. Characteristics of E. faecalis biofilm formation include increased adhering capacity, increased virulence factors, and increased resistance to antimicrobial agents15. This biofilm can serve as a steady source of bacterium for persistent chronic infections22.

To improve CH antibacterial effectiveness, it can be combined with active agents such as chlorhexidine (CHX) and sodium hypochlorite (NaOCl). CHX, one of the antibacterial agents effective against E. faecalis, destroys the bacteria by attaching to the bacterial cell wall13. CHX in low and high concentrations has anti-resorptive activity against some microorganisms such as Enterococcus faecalis (E. faecalis)41.

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Mediterranean fever, pericarditis, and Behcet’s disease. COL binds to tubulin, one of the main constituents of microtubule, inhibiting its polymerization20. Tubulin is essential to the process of mitosis; therefore, COL effectively acts as a mitotic poison and stops cell division20. In addition to anti-mitotic property, COL has anti-inflammatory and anti-pain properties21. Ahmad et al.20 demonstrated that COL had mild to moderate antibacterial and moderate to excellent antifungal properties.

Available data on COL antibacterial activity, and TAM antibacterial effectiveness in depths of dentinal tubules is limited. Therefore, the aim of the present study was to compare the ex vivo antibacterial effect of CH, TAM, and CH in combination with CHX, NaOCl or COL against E. faecalis in surface and deep dentinal tubules.

MATERIAL AND METHODS

One hundred and five freshly extracted, single-rooted human teeth with intact crown and root were used in this experimental study. The teeth were extracted because of periodontal or prosthetic reasons. The teeth were cleaned and disinfected by scaling and soaking in 2.5% NaOCl for 24 h. The experimental method used in this study was a modification of the model used by Haapasalo and Orstavik6. The cervical and apical portions of the roots were removed leaving eight mm long specimens. Gates Glidden (GG) drill size 3 (0.9 mm diameter) was used to enlarge each canal to create standard diameters in all specimens. The drills were discarded after a single use. The specimens were kept in distilled water during all procedures to prevent dehydration. To remove organic and inorganic debris, all specimens were immersed in an ultrasonic bath of 17% EDTA for three minutes, a bath of distilled water for five minutes and, a bath of 0.5% NaOCl for five minutes respectively. This was followed by irrigation with 5% sodium thiosulfate to neutralize NaOCl. The outer surfaces of the specimens were covered with an epoxy resin (3M Dental Products, Bracknell, UK).

The root specimens were autoclaved at 121°C for 20 min at 20 psi pressure. Then, they were placed in test tubes containing BHI broth (HiMedia, Mumbai, India) and incubated for 24 h at 37°C. Turbidity was checked to confirm sterility. To enhance BHI broth penetration into the dental tubules, the specimens were immersed in an ultrasonic bath of BHI broth for five minutes. Finally, the BHI broth was removed with a sterile syringe, and the lumen of specimen was blotted dry with sterile paper points. Then, one end of ninety specimens were sealed with temporary cement (Cimpat, Septodent, Saint-Maur-des-Fossés, France).

Pure E. faecalis cultures (ATCC 29212) were cultivated in BHI broth medium and then suspended in 4.0 mL of BHI. The cell suspension was adjusted spectrophotometrically to match the turbidity of 6.3×10⁸ CFU/mL of E. faecalis (equivalent to ≈2.0 McFarland standards). The lumens of the ninety specimens were infected with an inoculum of E. faecalis injected with a sterile syringe. Other ends of the infected specimens were sealed with temporary cement. The infected specimens were incubated at 37°C for 21 days. Fresh inoculum was added daily to the lumen of specimen to ensure viability of bacteria13. After this period, each lumen was irrigated with distilled water and dried with sterile paper points.

The infected specimens were randomly divided into five experimental and one positive control groups (n=15). Under aseptic conditions, one mL aliquot of intracanal medication (1.5 g powder per mL of liquid)20 was injected into the lumens of each canal by sterile syringe as follows:

- group 1: CH powder (Merck, Darmstadt, Germany) mixed with distilled water; group 2: CH powder mixed with 0.12% CHX (Merck, Darmstadt, Germany); group 3: CH powder mixed with 5.25% NaOCl (Merck, Darmstadt, Germany); group 4: CH powder plus COL (Sigma, MO, USA) mixed with distilled water (0.75 g CH plus 0.75 g COL per mL of distilled water); group 5: TAM (Clariant Life Sciences, Barcelona, Spain) mixed with distilled water (0.5 g ciprofloxacin plus 0.5 g metronidazole plus 0.5 g minocycline per mL of distilled water); group 6: distilled water (positive control group).

Fifteen remaining specimens without inoculation were set as negative control group.

All medicated and positive control specimens were placed in sterile petri dishes and covered with humid sterile gauzes and incubated at 37°C for seven days. Thereafter, the medications were removed with a sterile syringe and the canals were rinsed with 5 mL of distilled water. Subsequently, 0.5% citric acid for groups 1 and 4, 0.5% Tween 80 in 0.07% soy lecithin for group 2, and 0.6% sodium thiosulfate for group 3 were used as irrigation neutralizers. Finally, the canals were irrigated with 5 mL of distilled water and dried with sterile paper points.

Dentin chips from within the lumen of all infected and non-infected specimens were collected using the GG drills to test for bacterial survival. GG drills size 4 (1.1 mm diameter) and 5 (1.3 mm diameter) were used three times throughout the whole extension of lumen to create dentin shavings from the surface and deep dentin of the specimens respectively. The chips from each depth were immediately collected into microcentrifuge tubes containing one mL of BHI broth individually and mixed for one minute. Aliquots of 20 μL were seeded on the BHI blood agar and incubated at 37°C for 48 h. Then, CFUs were counted by a microbiologist.

In addition, aqueous solutions of CH-containing groups were prepared to a final concentration of 0.1 M. The pH was determined with digital pH meter (Broadley-James Irvine, California, USA), calibrated to pH 7 with standard buffer solution, immediately after solution preparation. Five reading per sample were taken and the mean was calculated.

The obtained data were verified with the Kolmogorov-Smirnov test for the normality of the data distribution and the Levene test for the homogeneity of
the variances. Statistical analysis was performed using the parametric one-way analysis of variance test and Tukey post hoc test. SPSS 15.0 Software (SPSS Inc., IL, USA) was used for statistical analysis. The statistical significance was set at a confidence level of 95%.

RESULTS
The results are summarized in Tables 1 and 2 and Figs. 1 and 2. No specimen of negative control group showed growth of *E. faecalis* on BHI blood agar medium. All groups were significantly different from the positive control group (*p*<0.05). The treatment with TAM presented the least number of CFUs. There were significant differences between TAM and CH-containing groups in the surface dentin (*p*<0.05), but these differences were not significant in the deep dentin (*p*>0.05). Although CH-COL group showed lower CFUs than other CH-containing groups, no significant differences in bacterial CFUs were observed between CH-containing groups in both depths (*p*>0.05). In addition, there was no statistically significant difference

Table 1  Mean (standard deviation) of Log CFU for all groups

|               | CH+DW | CH+CHX | CH+NaOCl | CH+COL+DW | TAM+DW | PC    |
|---------------|-------|--------|----------|-----------|--------|-------|
| Surface dentin| 3.01(1.23)a | 2.77(0.70)a | 2.90(1.14)a | 2.43(0.64)a | 1.32(0.39)b | 4.49(1.66)c |
| Deep dentin   | 1.35(0.42)a | 1.30(0.27)a | 1.38(0.42)a | 1.25(0.51)a | 1.02(0.62)a | 2.82(1.01)c |

Means with different superscript letters are statistically different (*p*<0.05).
CH: calcium hydroxide, DW: distilled water, CHX: chlorhexidine, NaOCl: sodium hypochlorite, COL: colchicine, TAM: triantibiotic mixture, PC: positive control, CFU: colony forming unit.

Table 2  Mean and standard deviation of pH for CH-containing groups

|               | CH+DW | CH+CHX | CH+NaOCl | CH+COL+DW |
|---------------|-------|--------|----------|-----------|
| Mean          | 12.63 | 12.66  | 12.60    | 12.62     |
| Standard Deviation | 0.002 | 0.003  | 0.003    | 0.002     |

CH: calcium hydroxide, DW: distilled water, CHX: chlorhexidine, NaOCl: sodium hypochlorite, COL: colchicine.

Fig. 1  Box plot of Log CFU of *E. faecalis* in surface dentin.
CH: calcium hydroxide, DW: distilled water, CHX: chlorhexidine, NaOCl: sodium hypochlorite, COL: colchicine, TAM: triantibiotic mixture, PC: positive control, NC: negative control, CFU: colony forming unit.

Fig. 2  Box plot of Log CFU of *E. faecalis* in deep dentin.
CH: calcium hydroxide, DW: distilled water, CHX: chlorhexidine, NaOCl: sodium hypochlorite, COL: colchicine, TAM: triantibiotic mixture, PC: positive control, NC: negative control, CFU: colony forming unit.
in the mean pH values among CH-containing group (p>0.05).

DISCUSSION

Microorganisms in the root canal system have an important role in pathogenesis of periapical destruction. Therefore, chemomechanical preparation and intracanal medications are used to disinfect the root canal system. CH is widely used as an intracanal medication due to its organic tissue dissolution potential, antibacterial activity, and anti-inflammatory capacity. Since CH is ineffective in elimination of some microorganisms such as E. faecalis, to improve its antibacterial activity, it has been combined with other materials. In the present study, to evaluate the combination potential of CH, it was combined with two established endodontic irrigants (CHX, NaOCl) and COL a medication with possible elimination of some microorganisms such as E. faecalis. Several studies have evaluated the antibacterial effect of CH alone or in combination with different materials using the dentin block model. However, there is still controversy on whether combination of CH with other active agents enhances its antibacterial potential.

One of the main limitations of the dentin block model is carry-over effect which is discussed extensively in the literature. In this study, the medicaments in groups 1 to 4 were neutralized with their specific solutions. However, there was no known neutralizer in the literature for TAM. Therefore, a pilot study was performed to assess whether there is any residual antibacterial activity of TAM in BHI blood agar before initiating this study. Similar to the present study, 30 single-rooted teeth were sterilized and infected. Fifteen teeth were treated with TAM, and the remaining teeth with distilled water. Dentin chips were obtained, seeded on the BHI blood agar, and incubated. Afterward, 0.5 McFarland standard suspension of E. faecalis was serially diluted, cultured on the BHI blood agar, and incubated. CFUs of new suspension were counted. The result showed that there were no significant differences between 2 groups (p>0.05). Therefore, TAM does not have any residual antibacterial activity on the BHI blood agar medium.

E. faecalis is a highly resistant bacterium to various harsh environmental conditions such as alkaline environment, bile salts, starvation, and many antibacterial agents. This strain easily penetrates into dentinal tubules. Since this bacterium is highly resistant against different antibacterial agents, it has been used to evaluate the antibacterial effectiveness of different intracanal medications in various studies. Accordingly, this strain was selected as a measure for antibacterial property of selected medications in the present investigation. George et al. demonstrated biofilm formation and deeper penetration of E. faecalis into the dentinal tubules when it was grown aerobically under nutrient-rich medium. Accordingly, in the present investigation, the lumen of the specimens was supplied with a fresh inoculum of E. faecalis in nutrient-rich medium every day for 21 days. This process ensures viability of bacterium, biofilm formation, and deeper dentinal tubule penetration of E. faecalis.

The results of the present study indicated that the antibacterial properties of CH did not improve when it was combined with CHX, NaOCl, or COL. This is in accordance with the findings of other reports. These results can be due to the similar pH values of CH solutions when combined with other irrigants or medications (Table 2). In contrast, many studies lend support to the CH combination procedures. According to these studies, this improved antibacterial effectiveness might be due to the additive or synergistic effect of CH mixed with selected materials.

The results of the present study showed that TAM, compared to other medications, had significantly better antibacterial activity against E. faecalis in the surface dentin. This observation is corroborated by Madhubala et al. study. They evaluated the intracanal antibacterial activity of several materials including CH and TAM. It was shown that TAM was significantly more effective than CH in first, second, and seventh days of dressing period against E. faecalis. TAM antibacterial properties might be due to the combination of antibiotics in the mixture. Metronidazole component of TAM has a wide bactericidal spectrum against obligate anaerobes. However, certain species are resistant to this antibiotic. Its antimicrobial action is enhanced when ciprofloxacin and minocycline are added to the mixture. Ciprofloxacin spectrum of activity includes most strains of gram positive and negative bacterial pathogens. Also, Minocycline is a tetracycline-based antibiotic with broader spectrum than the other members of its family. For the first time, in the present investigation, the antibacterial activity of TAM was evaluated in the depth of dentinal tubules. The results indicated that TAM had similar antibacterial effects to CH-containing groups in the depth of dentin. This might be due to the superior capacity of CH hydroxyl ions penetrating into dentinal tubules.

Ahmad et al. evaluated the antibacterial effect of COL on several microorganisms. They showed that COL had mild to moderate antibacterial effect on these species. However, these microorganisms are not prevalent in endodontic infections. In the present study, for the first time, COL in combination with CH was used as intracanal medication. Although bacterial growth in CH-COL group was less than other CH-containing
groups, this difference was not statistically significant. The superior *E. faecalis* inhibition in CH-COL group might be due to the synergistic effect of CH and COL.

There are limitations to the present study. First, the *in vitro* model used in this investigation cannot be a complete representation of the conditions in clinical environment. Second, endodontic infections are polymicrobial in nature, whereas, in the current study, a single microorganism was used to infect the root canals. Third, in clinical conditions, the remaining tissues and fluids in the root canal system may reduce the efficiency of intracanal medications. Fourth, the various concentrations of CH and other selected active agents may have different antibacterial effectiveness. Fifth, the sampling method may not be precise because the surface layer material is likely to mix with the deep layer.

In conclusion, under the limitations of this study, TAM can be considered as a potential useful intracanal medication in the root canal treatments, but further research should focus on TAM antibacterial effectiveness against other prevalent endodontic infection bacterial strains. Also, future investigations should evaluate COL antibacterial capabilities using different concentrations and methodologies.

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