Original Article

Efficacy of a Newly Introduced Intracanal Medicament (MTA Mixed with Chlorhexidine) Against *Enterococcus faecalis* Biofilm: An Ex Vivo Experiment

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Abstract

Background and Aim: This study evaluated the effectiveness of a newly introduced intracanal medicament by comparing its antibacterial performance against *Enterococcus (E.) faecalis* biofilm at varying dentinal depths with that of commonly used intracanal medicaments in an *ex vivo* model.

Materials and Methods: Thirty-eight single-root human extracted teeth were infected with *E. faecalis* following chemo-mechanical preparation. After 14 days of incubation, the root canals were disinfected using five different medicaments (n=6): triple antibiotic paste (TAP), double antibiotic paste (DAP), 2% Chlorhexidine digluconate (CHX) gel, calcium hydroxide [CH] +2% CHX and mineral trioxide aggregate (MTA) + 2% CHX and a control group. The medicaments were removed after 7 days, and dentin shavings were collected from root canals in three depths. The number of colony-forming units (CFU)/ml was counted for each sample.

Results: All tested medicaments exhibited antibacterial properties against *Enterococcus faecalis* biofilm. Among them, TAP demonstrated the highest efficacy in eradicating the bacterial biofilm across all three dentinal tubule depths, followed by DAP, with no statistically significant difference between the two. In contrast, the MTA/CHX mixture showed the lowest antibacterial activity and did not differ significantly from the control group ($p > 0.05$).

Conclusion: The use of antibiotics as intracanal medicaments can reduce *E. faecalis* biofilm burden. However, the mixture of MTA and CHX exhibits limited antibacterial efficacy within the root canal system.

Key Words: Anti-Bacterial Agents; Calcium Hydroxide; Chlorhexidine; Mineral Trioxide Aggregate; Drug Combinations; Root Canal Filling Materials

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Introduction

Apical periodontitis is an inflammatory condition caused by microbial infection within the root canal system, primarily resulting from bacterial invasion (1). The primary objective of endodontic treatment is to eliminate the bacterial load and its byproducts from the root canal system (2). Although chemo-mechanical

preparation significantly reduces the microbial load, residual bacteria have been detected within the dentinal tubules following endodontic procedures (3). During treatment, microorganisms may survive and proliferate within empty root canals due to the complex anatomy of the root canal system and their capacity to penetrate dentinal tubules and lateral canals (4).

Although successful healing of periapical lesions has been reported despite the presence of residual microorganisms (5), the persistence of these microorganisms becomes particularly critical in regenerative endodontic procedures, where complete disinfection is essential for establishing an optimal biological environment conductive to stem cell survival and continued root development (6,7). In necrotic immature teeth with thin dentinal walls, mechanical instrumentation is limited, thereby increasing reliance on chemical disinfection strategies to achieve adequate bacterial control (8). Therefore, identifying intracanal medicaments capable of eliminating bacteria residing deep within dentinal tubules remains a clinical challenge.

Enterococcus (E.) faecalis is a gram-positive bacterium commonly found in endodontic infections and periapical lesions. This microorganism may enter the root canal system during or after endodontic treatment, thereby causing reinfection. Its ability to survive harsh environmental conditions, penetrate deeply into dentinal tubules, and form resistant biofilms renders effective eradication particularly challenging and critically relevant to the success of root canal treatment (9–11).

Intracanal medicaments have been used to reduce the microbial population, neutralize canal contents, prevent leakage, and contamination between treatment sessions (11,12). Commonly used intracanal medicaments include calcium hydroxide (CH), chlorhexidine digluconate (CHX) (Morvabon, Tehran, Iran) gel, and antibiotic pastes (13-17). CH exerts its antibacterial effect through a high pH that inhibits bacterial growth; however, its efficacy against *E. faecalis* biofilm remains debated (18). Some studies suggest that a combination of CH and CHX gel has stronger antibacterial properties against *E. faecalis* than CH alone (19-22). CHX is a cationic agent with broad-spectrum antimicrobial properties and long persistence in dentinal tubules. Its gel formulation allows its use as an intracanal medicament either alone or in combination with other materials (23,24). TAP is commonly used as an intracanal medicament in regenerative endodontic treatments to eliminate microorganisms in deep areas of dentinal tubules and promote root development. It consists of three antibiotics (minocycline, metronidazole, and ciprofloxacin) (25). However, due to the risk of crown discoloration, minocycline can be removed from the composition to form a double antibiotic paste (DAP) (26).

MTA is a biocompatible material with favorable antibacterial properties. It may be combined with other antibacterial agents to enhance its antibacterial performance (27). Despite its favorable antibacterial properties, MTA has not been utilized as an intracanal medicament. A prolonged setting time is regarded as a limiting factor in most endodontic applications; however, by delaying the setting time to the greatest extent possible, this limitation could be transformed into an opportunity to employ MTA as an intracanal medicament. According to Mahmoud (28), mixing MTA with 2% CHX delays its setting time for up to 84 days and additionally elevates the pH, the rate of calcium ion release, and flowability, thereby providing the essential conditions necessary to achieve this purpose. However, despite these promising characteristics, the antibacterial efficacy of the MTA/CHX combination against *E. faecalis* biofilm, particularly at varying depths within dentinal tubules, has not been adequately investigated. This highlights a clear gap in the existing literature and limits evidence-based clinical decision-making regarding the use of this combination. Therefore, this study aimed to compare the antibacterial efficacy of MTA/CHX with commonly used intracanal medicaments against *E. faecalis* biofilm.

Materials and Methods

The study protocol was approved by the Research Ethics Committee of the Tehran University of Medical Sciences (No: REC.1399.255). This study was conducted in

accordance with the Declaration of Helsinki, and informed consent was obtained from all participants prior to tooth extraction. In this experimental study, 38 single-rooted human teeth, extracted for orthodontic or periodontal reasons and possessing straight root canals with closed apices, were included.

Specimen Preparation

All root canals of the teeth were negotiated using K-files size 10 (Dentsply Maillefer). The crowns of the teeth were cut perpendicular to their long axis to obtain 9 mm roots using a diamond disc (D+Z, Berlin, Germany) with a high-speed turbine under water irrigation. Subsequently, 3 mm of the apical part of the roots was removed to obtain a standard 6 mm cylinder from each specimen. Mechanical instrumentation of the root canal was performed using Protaper (Dentsply Maillefer, Ballaigues, Switzerland) rotary files (S1 to F3) following the the manufacturer's instructions, and the canals were irrigated with 1 mL of 5.25% NaOCl (Onemed, Cairan Dental, Kuala Lumpur, Malaysia) between each file during instrumentation. The internal diameters of the canals were then standardized using Gates-Glidden No. 1 and 2. Smear layer was removed using 1 mL of 17% Ethylenediaminetetraacetic acid (EDTA) (Vista Dental Products, WI, USA) and 1 mL of NaOCl, each for 3 min. Subsequently, 5 mL of sterile saline was used for final rinsing. The apical foramen was sealed with self-cure glass ionomer (GC Co., Tokyo, Japan). The root surfaces were then coated with two layers of nail varnish. To sterilize the samples, gamma radiation was applied at a dose of 40 kGy for 3 hours and 45 minutes. In the microbiology laboratory, six teeth were randomly selected as the negative controls and placed in a culture medium. These samples were then incubated at 37 °C for 48 hours to ensure complete sterilization. No bacterial growth was observed in any of the negative control samples.

Dentin inoculation with *E. faecalis* biofilm

A suspension of *E. faecalis* (ATCC 29212) was prepared in Trypticase Soy Broth (TSB) (Ibresco, Tehran, Iran) culture medium. A 20 µL volume of the 0.5 McFarland bacterial solution, containing a standard concentration of 1.5×10^8 colony-forming units (CFUs)/mL, was inoculated into the root canals. The samples were then placed in 1.5 ml sterile microtubes containing 1 mL of TSB medium, which was refreshed every 48 hours and were incubated for 14 days at 37 °C enabling the formation of microbial biofilms and their penetration into dentinal tubules.

SEM Evaluation

At the end of the incubation period, the presence of microorganism biofilms was confirmed by Scanning Electron Microscopy (SEM) (Nanoscience Instruments, Arizona, USA) in 2 randomly selected infected teeth (Figure 1).

Dentin Disinfection with Intracanal Medicaments. The samples were randomly allocated into five experimental groups and one positive control group using a computer-generated simple randomization method, and were disinfected with the following intracanal medicaments (n=6 per group):

1. TAP: 1.5 g triple antibiotic powder (containing metronidazole 500 mg, ciprofloxacin 500 mg and minocycline 500 mg) (Tehran Darou, Tehran, Iran) was mixed with 1.5 mL of normal saline to achieve a paste consistency.
2. DAP: 1.5 g of double antibiotic powder (containing Metronidazole 750 mg and Ciprofloxacin 750 mg) was mixed with 1.5 ml of sterile normal saline to form a paste.
3. CHX: 2% CHX gel
4. CH/CHX: A paste was prepared by combining 1 g CH powder (Golchadent, Tehran, Iran) with 1 mL of 2% CHX gel.
5. MTA/CHX: A mixture of mineral trioxide aggregate (MTA) and CHX gel 2% (2:1 ratio) was used. This mass ratio was obtained from the study by Mahmoud et al. (28) to prevent setting of MTA.

Accordingly, 1 g Retro MTA (bioMTA, Seoul, South Korea) and 0.5 g CHX gel 2% were mixed.

6. Positive Control: Normal saline served as the positive control to demonstrate the reduction of bacterial biofilm in other medication groups.

The root canals were filled with the medicaments using a lentulo spiral size 25

(Dentsply Maillefer, Ballaigues, Switzerland) at a speed of 600 rpm. The samples were sealed coronally using a thin layer of sterile rose wax and placed in 1.5 mL sterile microtubes containing TSB culture medium to prevent dehydration and incubated at 37 °C for 7 days to prepare for collecting dentin chips. Every 48 hours, the culture medium was replaced with fresh TSB under a biological safety cabinet.

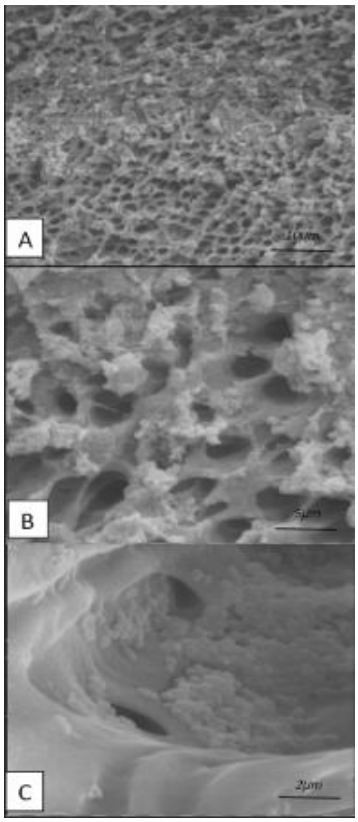

Figure 1. Images of *Enterococcus faecalis* biofilm formed on the root canal walls and inside dentinal tubules taken by Scanning Electron Microscopy (SEM) at magnifications of (A) 500, (B) 2000, and (C) 5000

Dentin Chip Collection

After 7 days of incubation, each sample was rinsed with 5 mL of sterile normal saline to remove the tested medication. Dentin chips were collected from three depths—D1, D2, and D3—using Gates-Glidden drills sizes 3, 4, and 5, respectively, along the root canal walls. The Gates-Glidden drills sequentially remove dentin chips from the inner surface of the root canal.

Bacterial Viability Quantification

Each microtube containing dentin samples received 100 µL of TSB medium and was incubated at 37 °C for 24 hours to increase the number of viable bacteria. The serial dilution method was then employed to count the number of *Enterococcus faecalis* (*E. faecalis*) colonies (CFU/mL).

Statistical Analysis

Data were analyzed using SPSS software, and the significance level was set at 0.05. The Kolmogorov–Smirnov test was used to assess data normality, and the results indicated a non-normal distribution. The experimental data were analyzed in two parts.

In the first analysis, each study group was considered as a fixed parameter, and the mean loads of residual microorganisms at different depths were compared to evaluate the antibacterial potency of each medicament across different dentin depths. Because the data were not normally distributed, the Friedman test was used to analyze differences across depths.

In the second analysis, bacterial counts were compared between different intracanal medicament groups at each dentinal depth. As the data were not normally distributed, the Kruskal–Wallis test was applied to assess differences among the groups.

Results

Scanning electron microscopy (SEM) confirmed the presence of *Enterococcus faecalis* biofilm on the root canal walls and inside the dentinal tubules at magnifications of 500×, 2000×, and 5000× (Figures1 A–C). Figure 2 illustrates the mean residual bacterial counts (log scale) for each intracanal medicament across three dentinal depths (D1‑D3). Normal saline showed the highest bacterial counts at all depths ($p<0.05$). TAP and DAP demonstrated the lowest residual bacterial counts at all depths, indicating greater bacterial reduction compared with the other groups ($p<0.05$). Table 1

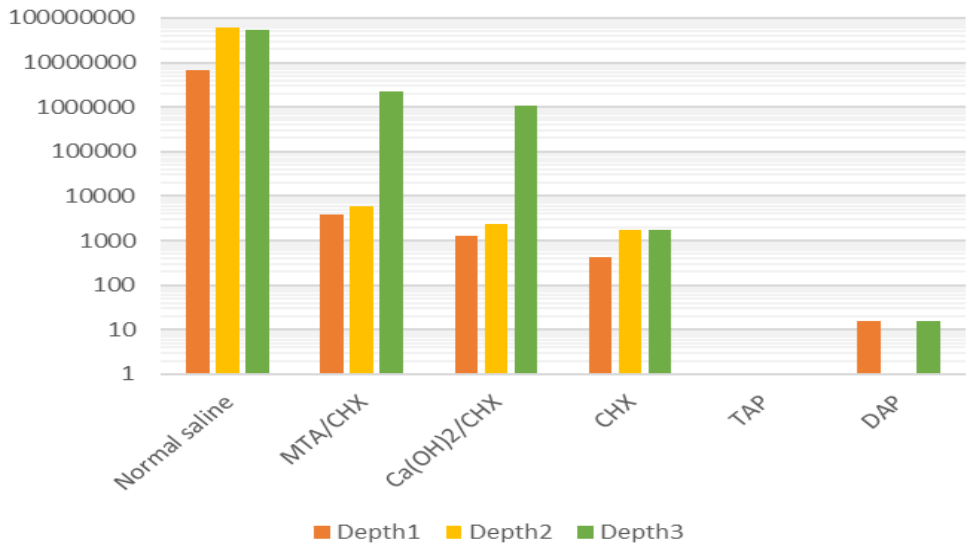

Figure 2. Mean Log10 colony-forming units (CFU/mL) of study groups at different dentinal tubule depths

Table 1. Comparing groups at D1, D2, D3 obtaining dentin shavings with GG NO. 3, 4 and 5, respectively

Sample 1 – Sample 2	P value		
	D_1	D_2	D_3
TAP - Saline	< 0.001	< 0.001	< 0.001
TAP - MTA + 2% CHX	= 0.004	= 0.007	= 0.001
TAP - CH + 2% CHX	= 0.10	= 0.046	= 0.013
TAP - 2% CHX	= 0.10	= 0.019	= 0.005
TAP - DAP	= 0.76	= 1.00	= 0.81
DAP - Saline	< 0.001	< 0.001	< 0.001
DAP - MTA + 2% CHX	= 0.011	= 0.007	= 0.003
DAP - CH + 2% CHX	= 0.17	= 0.046	= 0.024
DAP - 2% CHX	= 0.18	= 0.019	= 0.010
2% CHX - Saline	= 0.006	= 0.035	= 0.76
2% CHX - MTA + 2% CHX	= 0.22	= 0.74	= 0.66
2% CHX - CH + 2% CHX	= 0.98	= 0.72	= 0.73
CH + 2% CHX - Saline	= 0.006	= 0.014	= 0.035
CH + 2% CHX - MTA + 2% CHX	= 0.23	= 0.49	= 0.44
MTA + 2% CHX - Saline	= 0.12	= 0.075	= 0.18

MTA: Mineral trioxide aggregate, CHX: Chlorhexidine, CH: Calcium Hydroxide, TAP: Triple antibiotic paste, DAP: Double antibiotic paste, D1:Depth 1, D2:Depth 2, D3:Depth 3.

presents pairwise comparisons of bacterial reduction among the tested intracanal medicaments at three dentinal depths (D1–D3). At depth 1, significant bacterial reduction was observed for TAP, DAP, CHX and CH/CHX compared with saline ($p < 0.001$, < 0.001, 0.006 and 0.006, respectively). TAP and DAP demonstrated greater bacterial reduction than the MTA + 2% CHX at this depth ($p = 0.004$ and 0.011 respectively), whereas MTA/CHX did not differ significantly from saline ($p = 0.12$).

At depths 2 and 3, TAP and DAP showed significantly greater bacterial reduction compared with saline and non-antibiotic medicaments ($p < 0.05$), while no significant difference was detected between TAP and DAP ($p = 1.000$ and 0.81). The MTA/CHX mixture did not differ significantly from saline at these depths ($p = 0.075$ and 0.18).

Discussion

The present study compared the antibacterial efficacy of MTA/CHX with commonly used intracanal medicaments against *E. faecalis* biofilm at three dentinal depths, providing insight into their relative performance under controlled laboratory conditions.

Antibiotics are utilized as one of the most critical intracanal medicaments in regenerative endodontic procedures and in addressing resistant endodontic infections (29, 30). However, due to bacterial resistance and the possibility of tooth discoloration, efforts are being made to replace them with other substances such as CHX and CH. Studies showed controversial results about the antibacterial effect of CH on *E. faecalis*; nevertheless, it has been demonstrated that by adding 2% CHX gel, its antimicrobial property against this bacterium is enhanced (31-35). It can be due to its high penetration power into dentinal tubules and its strong antibacterial property (23). These findings confirmed the results of a previous work by Zancan et al (25) , Arruda et al. (35) and Ghabraei et al. (31) which measured the percentage of *E. faecalis* biofilm elimination by several drug groups, despite differences in the study method, TAP group followed by DAP showed the lowest percentage of viable bacteria, demonstrating a significant difference compared to the mixture of CH and CHX.

MTA has not been used as an intracanal medicament, despite its favorable antibacterial properties. A prolonged setting time is regarded as a limiting factor in most of its endodontic applications; however, by delaying the setting time to the greatest extent possible, this limitation could be converted into an opportunity to employ MTA as an intracanal medicament (28, 36). According to Mahmoud (28), the admixture of MTA with 2% chlorhexidine (CHX) postpones its setting time for up to 84 days and additionally elevates the pH, the rate of calcium ion release, and flowability, thereby providing the primary conditions requisite for this purpose. Based on the aforementioned study and in order to evaluate the antibacterial efficacy of this compound, the present study was designed.

The present study was designed based on the findings reported by Mahmoud (28), who proposed the combination of these two materials as an intracanal medicament. The aim was to directly challenge the antibacterial efficacy of this combination, a critical characteristic of intracanal medications in endodontic treatment, by comparing it with other commonly used intracanal agents. If this important property is validated, it would emphasize the potential of this combination as a new intracanal medicament. A key finding of this study was the limited antibacterial efficacy of the MTA/CHX combination against *E. faecalis* biofilm compared to other medications, as well as the decrease in antibacterial potency with increasing dentin depth. This finding suggests that the MTA/2% CHX combination may possess limited penetration into the dentinal tubules of the root canal system. The ability of a material to penetrate deeply into the tubules enhances its effectiveness by allowing it to reach and eliminate bacteria in hard-to-reach areas, to prevent their proliferation, and to reduce the risk of infection (37). This may be related to physicochemical interactions between MTA and CHX, changes in viscosity after mixing, or precipitation phenomena that limit penetration into dentinal tubules, although

these mechanisms were not directly investigated in this study.

Importantly, the findings of the present study should be interpreted within the context of its limitations. This was an *in vitro* investigation using a single bacterial species (*E. faecalis*), which cannot fully replicate the polymicrobial nature of endodontic infections. The sample size, limited incubation period, and evaluation under static laboratory conditions further restrict direct extrapolation to clinical scenarios. Therefore, no definitive claims regarding clinical effectiveness can be made based solely on these findings.

Within these limitations, the present study provides comparative laboratory evidence indicating that MTA/CHX exhibits inferior antibiofilm activity relative to established intracanal medicaments, particularly antibiotic pastes. Future studies should explore the underlying mechanisms affecting MTA/CHX antibacterial performance, assess its behavior against multispecies biofilms, and evaluate its penetration dynamics prior to any consideration of potential clinical application

Conclusion

In conclusion, the present *in vitro* study demonstrated that antibiotic-based medicaments produced a greater reduction of *E. faecalis* biofilm than non-antibiotic formulations, whereas the MTA/2%CHX mixture exhibited comparatively lower antibiofilm activity under the conditions tested. Furthermore, the antibacterial performance of MTA/2%CHX combination decreased with increasing dentinal depth, indicating limited efficacy against bacteria residing in deeper dentinal tubules. These findings suggest that, within the constraints of the present laboratory model, MTA/2%CHX may be less efficacious than established intracanal medicaments for biofilm reduction.

Given the *in vitro* nature of the present study, the utilization of a single bacterial species, and the limited incubation period, further investigations are warranted. Future investigations should evaluate the antibacterial activity of MTA/CHX against multispecies biofilms, assess prolonged exposure durations, and examine its performance in *ex vivo* or clinical models prior to any consideration of clinical implications.

Declarations

Ethical Considerations

The study protocol was approved by the |Research Ethics Committee of the Tehran |University of Medical Sciences (Approval No: IR.TUMS.REC.1399.255). The study was |conducted in accordance with the Declaration of Helsinki, and informed consent was obtained from all participants prior to tooth extraction.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing Interest

The authors declare no conflict of interest pertaining to this study.

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Authors' Contributions

Sholeh Ghabraei (S.G.), Mahsa Sobhi Afshar (M.S.A.), and Mohaddeseh Haghighi (M.H.) contributed to conceptualization, resources, validation, visualization, data curation, and methodology. Mohaddeseh Haghighi (M.H.) contributed to investigation and writing—original draft preparation. Sholeh Ghabraei (S.G.) contributed to project administration. Sholeh Ghabraei (S.G.), Mahsa Sobhi Afshar (M.S.A.), Mohaddeseh Haghighi (M.H.), Fatemeh Malekpour (F.M.), and Farzaneh Afkhami (F.A.) contributed to writing—review and editing. All authors read and approved the final manuscript.

Declaration of Generative Artificial Intelligence (AI) Utilization

During the preparation of this manuscript, the authors used a large language model (OpenAI's ChatGPT) for language polishing, grammar correction, punctuation standardization, and

stylistic improvements to enhance clarity and formality. All AI-generated suggestions were critically reviewed, edited, and approved by the authors. The authors assume full responsibility for the final content, accuracy, and integrity of the manuscript.

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