Evaluation of the Antibacterial Efficacy of Daptomycin, Gentamicin, and Calcium Hydroxide—Antibiotic Combinations on *Enterococcus faecalis* Dentinal Biofilm: An *In Vitro* Study

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Abstract

Aim: To evaluate the antibacterial efficacy of calcium hydroxide (CH) with antibiotic combinations: daptomycin and gentamicin against *Enterococcus faecalis* (*E. faecalis*) dentinal biofilm.

Materials and methods: Sixty freshly extracted single-rooted mandibular premolars were inoculated with *E. faecalis* (ATCC 29212) ($n = 30$) (group A) and clinical isolates ($n = 30$) (group B) for 3 weeks to form a biofilm. The tooth samples of groups A and B were randomly divided into three subgroups of 10 each, groups 1A and 1B (CH), groups 2A and 2B (CH+G), groups 3A and 3B (CH+D), depending on the medicaments to be placed for one week. The difference between initial and final CFU was calculated and statistically analyzed.

Results: Among the clinical isolates, CH-antibiotic combinations were more effective than CH alone, which was statistically significant ($p = 0.006$).

Conclusion: The dentinal biofilm of clinical isolates of *E. faecalis* strains exhibited more reduction in bacterial colonies with CH in combination with antibiotics (D and G).

Clinical significance: Daptomycin and gentamicin when used as an intra-canal medicament in combination with CH are effective in eliminating *E. faecalis*.

Keywords: Calcium hydroxide, Daptomycin, Dentinal biofilm, *E. faecalis*, Gentamicin.

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Introduction

The successful outcome of endodontic treatment relies on the reduction or elimination of microbes within the root canal. However, the complexity of the root canal system poses a challenge.[1] Ideally, irrigants and intra-canal medicaments should reach the natural complexities, remove the smear layer, and exert antimicrobial activity. However, intra-canal medicaments play an important role by remaining in the root canal between appointments to eliminate surviving bacteria.[2]

Calcium hydroxide (CH) is widely used as an intracanal medicament due to its high alkaline pH of 12.5 and the limited cytotoxicity to the tissue in direct contact. But CH exhibits reduced antimicrobial activity due to low solubility, low diffusability, and difficulty to significantly increase the pH due to dentin buffering resulting in poor elimination of bacteria.[3]

Antibiotics are used as intra-canal medicaments due to their effectiveness in systemic route of administration. Studies have shown that CH with triple antibiotic paste is more effective than CH alone.[4,5] Earlier investigations evaluated the effect of various antibiotic combinations, such as Grossman's poly-antibiotic paste, which contains penicillin, bacitracin or chloramphenicol, and streptomycin; tri-antibiotic mixture TAM (ciprofloxacin, metronidazole, and minocycline) against several endodontic pathogens including *Enterococcus faecalis (E. faecalis)*.[6] The disadvantages of these mixtures include limited antibacterial efficacy and discoloration caused by minocycline.[7]

Gentamicin (G) is an aminoglycoside that exhibits bactericidal action by inhibiting bacterial protein synthesis, with comparatively

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low level of resistance. Hence, it is effective against a wide variety of bacteria including *E. faecalis*.[8] Daptomycin (D) at high doses demonstrates bactericidal activity against Enterococci species by disrupting the cell membrane and is routinely used in treatment of bacterial endocarditis.[9]

Therefore, the purpose of our study was to evaluate the synergistic antibacterial effect of CH with G and D against clinical

isolates and ATCC 29212 strain of *E. faecalis.* Though there are studies on CH with various combinations including antibiotics; this was the first study to assess the antimicrobial property of CH in combination with D and G.

MATERIALS AND METHODS

This study was reviewed and approved by the Institutional Ethical Committee (IEC) of Sree Balaji Dental College and Hospital, Chennai: approval number-SBDCECM 104/16/18.

Preparation of the Test Solutions

Commercially available CH paste (Dentocal™, Anabond Stedman Pharma Research Pvt Ltd, Chennai, India) was used.

G (Gentalab, Laborate Pvt Ltd, Panipat, Haryana, India) was procured in the injectable form at a concentration of 80 mg/2 mL, which was diluted to obtain a concentration of 40 mg/mL for the present study.

D was procured in powder form (Cubicin, OSO Bio-pharmaceuticals Ltd, Albuquerque, New Mexico, USA). A stock solution was prepared in sterile distilled water at a concentration of 60 mg/mL for this study.

Screening for Antibacterial Activity and Determination of Minimum Bactericidal Concentration (MBC)

The antibacterial ability of the test solutions against *E. faecalis* a standard strain, American Type Culture Collection (ATCC 29212), and a clinical strain (SBEF 2) isolated from a post-treatment root canal infection maintained in the Department of Microbiology was assessed by agar well diffusion assay and disc-diffusion assay.

For the agar well diffusion text, lawn cultures of the bacterial isolates were prepared on separate Mueller Hinton Agar (MHA) (Hi media Laboratories Pvt Ltd, Mumbai, India) plates. The assay was performed in triplicate (three MHA plates). Wells of 8-mm diameter were punched using sterile cork borer and 50 µL of each test solution (CH; CH+G; CH+D; CH+G+D) was added in the labeled wells and the plates were incubated at 37 °C for 24 hours. For CH alone and CH combined with G and D, four wells per plate were used separately for ATCC strain (Fig. 1A) and clinical strain (Fig. 1B).

Disk-diffusion method was adopted to determine the antibacterial efficacy of the antibiotics; G (without CH) and D
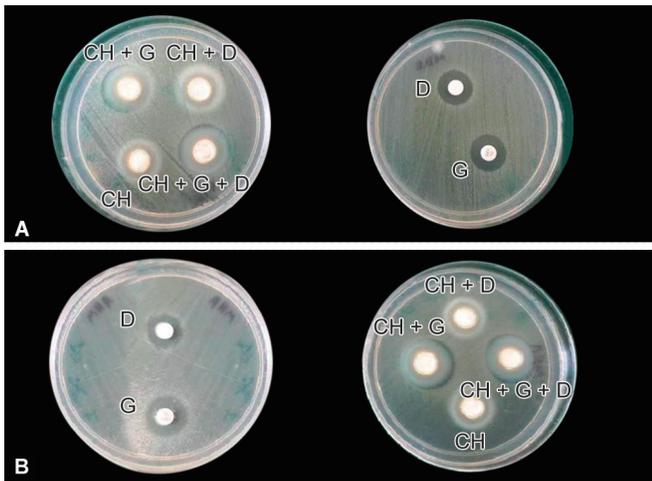

Fig. 1: Agar-diffusion assay and disc-diffusion assay. (A) *E. faecalis* ATCC 29212; (B) *E. faecalis* clinical isolate. CH: calcium hydroxide, G: gentamicin, D: daptomycin

(without CH) impregnated on sterile filter paper disc (Hi media Laboratories Pvt Ltd, Mumbai, India). For G and D, two discs per plate were used (Fig. 1). After overnight incubation, the diameter of the zone of inhibition around the wells/discs was measured and recorded in millimeters (mm). The assay was performed in triplicates. The minimum inhibitory concentration (MIC) was determined by broth microdilution method in sterile disposable 96-well microtiter plates. The minimum bactericidal concentration (MBC) that inhibits approximately 99% of bacterial growth was determined by spot inoculation of 5 µL of the culture from each well of the microtiter plate onto an MHA plate and incubated for 18 hours at 37 °C.[10,11]

Selection and Preparation of Tooth Samples

Sixty intact, non-carious, single-rooted human mandibular premolars with fully formed apices freshly extracted for orthodontic treatment were selected for the study. The teeth were cleaned and stored in normal saline to prevent dehydration until the analysis. Both mesiodistal and buccolingual digital radiographs of all teeth were obtained using intraoral periapical radiography photostimuable phosphor plate system (Kavo Scan Exam One, Kavo Dental Ltd, Mi Healthcare, Knowsley, England, UK) to confirm the presence of single canal. All the specimens were decoronated to 2–3 mm below the cemento-enamel junction (CEJ) using a safe-sided diamond disc to standardize root length to 10 mm. A size 10 K-file (Mani Inc, Tochigi, Japan) was used to scout and establish patency till the tip of the file was seen at the apical foramen. The working length was corrected by reducing 1 mm from the radiographic apex.[12] The coronal third was prepared by Gates-Glidden drills (sizes—1, 2, 3, and 4) (Mani Inc, Tochigi, Japan). The samples were then instrumented with ProTaper Universal rotary system (Dentsply, Maillefer, Switzerland, USA) in a crown-down technique.

Two mL of 3% NaOCl (Prime Dental Products Pvt Ltd, Bhiwandi, Maharashtra, India) for 1 minute and 17% EDTA (Pulpdent Corporation, Massachusetts, USA) for 1 minute was used as the working solution during instrumentation, delivered by a 30-gauge side-vented needle placed as far apically as possible into the canal without binding. The apical third of each canal was prepared up to size F5 (5% taper 50 size) to allow adequate flushing and penetration of the irrigating solution.

After instrumentation, the enlarged apical foramen was sealed with epoxy resin to prevent bacterial leakage and the tooth samples were mounted vertically in acrylic blocks to simulate the position of the tooth in the oral cavity.

Sterilization of Teeth Samples

The mounted samples were placed in clean glass test tubes (5 tooth/test tube) containing 5 mL of sterile distilled water and autoclaved at 121 °C at 15 lbs pressure for 20 minutes. After sterilization, the tubes were placed in an incubator at 37 °C for 48 hours and checked for turbidity.

Biofilm Formation

The sterilized samples mounted on acrylic blocks (1 mounted teeth/well) were transferred aseptically into the wells of sterile 24-well tissue culture plates (DSMZ- German collection of micro-organisms and cell cultures, Zellkulter, Germany). They were randomly divided into two groups, A and B (*n* = 30 in each group). Group A was inoculated with *E. faecalis* ATCC 29212, while Group B with *E. faecalis* clinical isolate (10 µL of bacterial culture/tooth) followed by incubation at 37 °C to form a 3-week dentinal biofilm.[13]

Initial Microbiological Sampling and Determination of Initial Bacterial Count

At the end of the third week, the initial microbiological sampling was performed by flooding the canals with 5 mL sterile saline with a double-vented close-ended 27-gauge needle to full length of the canal followed by the insertion of a size 50 Hedstrom file (Mani Inc, Tochigi, Japan) into the canal to scrape the bacteria adherent to the dentin. The file was manually agitated in microfuge tubes (Tarsons India Pvt Ltd, Kolkata, India) containing 1 mL of sterile saline to dissolve the debris. Three sterile absorbent paper points were placed in the lumen of each canal for 60 seconds and transferred into same microfuge tube and vortexed for 60 seconds in a vortex mixer (Spinx, Tarsons India Pvt Ltd, Kolkata, India).

Colony count was performed for all 60 samples to establish the level of contamination before application of medicament by spread plate method. Briefly, 10 μL of the sample was transferred onto sterile MHA plates using micropipettes. The inoculum was spread uniformly using sterile disposable plastic loops (Hi-FlexiLoop4, HiMedia Laboratories, Mumbai, India). The plates were incubated aerobically for 24 hours at 37 °C. After incubation, the numbers of colonies on the agar plate were counted using a digital colony counter (Deep vision, India), and the colony-forming units per mL was calculated using the formula, cfu/mL = No of colonies × 100.

Placement of Intracanal Medicaments

The final concentration of the antibiotics, G and D admixed with the intra-canal medicament (CH), was adjusted to six times the MIC of the planktonic cells. The tooth samples of group A, *E. faecalis* (ATCC 29212), and group B, *E. faecalis* clinical isolate, were randomly divided into three subgroups of ($n = 10$) in each subgroup: 1 A – CH; 2 A – CH+G; 3 A – CH+D; 1 B – CH; 2 B – CH+G; 3 B – CH+D.

The respective medicament was placed inside the root canals using lentulo spiral size 30 and condensed with hand pluggers. Cavit G (3 M ESPE, Minnesota, USA) was placed at a thickness of 2 mm and kept undisturbed for 7 days.

Final Microbiological Sampling and Determination of Final Bacterial Count

After 7 days, the final microbiological sampling was done as described for the initial sampling and for all the 60 samples, final colony count was determined by spread plate method. The antibacterial activity of the intra-canal medicaments was measured by comparing the reduction in colony counts before and after intra-canal medication.[14]

STATISTICAL ANALYSIS

It was performed using Statistical Package for the Social Sciences (SPSS) software version 17. The Wilcoxon signed-rank test was used to assess the statistical difference in the mean initial count and mean colony count within each subgroup. Kruskal–Wallis test and chi-square test were performed to assess the statistical significance in the final colony count between the subgroups. Mann–Whitney U-test was performed to assess the statistical difference in the reduction in colony count between subgroups.

RESULTS

Agar WellDiffusion Assay

Initial screening of the antibacterial activity of CH; CH+G; CH+D; CH+G+D combination against *E. faecalis* strains (ATCC 29212 and Clinical isolate) revealed that CH exhibited discrete zone of inhibition against both the strains of *E. faecalis* (Fig. 1).

When CH was combined with G and D, an increase in the diameter of the zones of inhibition was observed (Table 1). However, there was no significant difference between CH+G and CH+G+D.

Minimum Inhibitory Concentration

The MIC of G against both *E. faecalis* ATCC 29212 strain and clinical isolate was 8 μg/mL. However, the MIC of D against ATCC 29212 and clinical isolate was 2 μg/mL and 4 μg/mL, respectively (Table 2).

Reduction in Colony Count

Table 3 shows the mean colony count before (initial) and after placement of intra-canal medicament (final) among the groups at the end of 7 days. Wilcoxon signed-rank test showed statistically significant reduction in the colony count after the placement of intra-canal medicament in all the subgroups ($p = 0.005$), except subgroup 1B ($p = 0.008$) (Table 3, Fig. 2 and Graphs 1 and 2).

Comparison between the Subgroups

Kruskal–Wallis test and Chi-square test determined the statistical significance in the final colony count (7 days after intra-canal

Table 1: Diameter (in mm) of the zone of the inhibition

| | Mean zone of inhibition (mm) | |
| | Bacterial strain | |
Groups	E. faecalis ATCC 29212	E. faecalis Clinical strain (SBEF-2)
CH	12.67	12.00
CH+G	20	20.67
CH+D	12.33	12.00
CH+G+D	19.67	20.67
G	16	14.67
D	14.67	13.33

Table 2: Minimum inhibitory concentration of the test solutions

Bacterial strain	CH+G (μg/mL)	CH+D (μg/mL)	CH+G+D (μg/mL)	G (μg/mL)	D (μg/mL)
E. faecalis ATCC 29212	4	4	8	8	2
E. faecalis Clinical isolate (SBEF-2)	4	4	8	8	4

Table 3: Mean colony count—initial and final (after placement of intra-canal medicament for 7 days)

Bacterial isolate	Subgroups	Intra-canal medicament	Mean initial count	Mean final count	p-value
ATCC E. faecalis 29212 (Group A)	1A	CH	31,770	270	0.005*
	2A	CH+G	21,500	230	0.005*
	3A	CH+D	12,160	160	0.005*
E. faecalis clinical isolate (Group B)	1B	CH	24,800	400	0.008
	2B	CH+G	10,290	140	0.005*
	3B	CH+D	17,340	170	0.005*

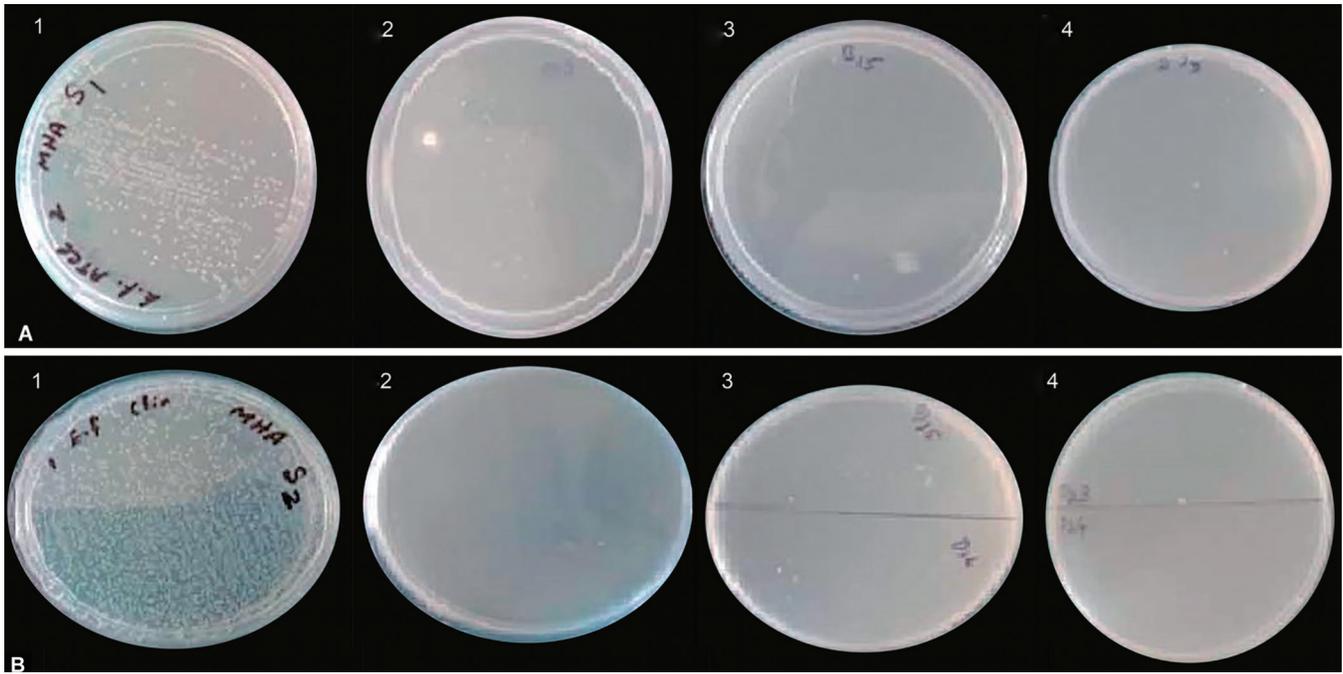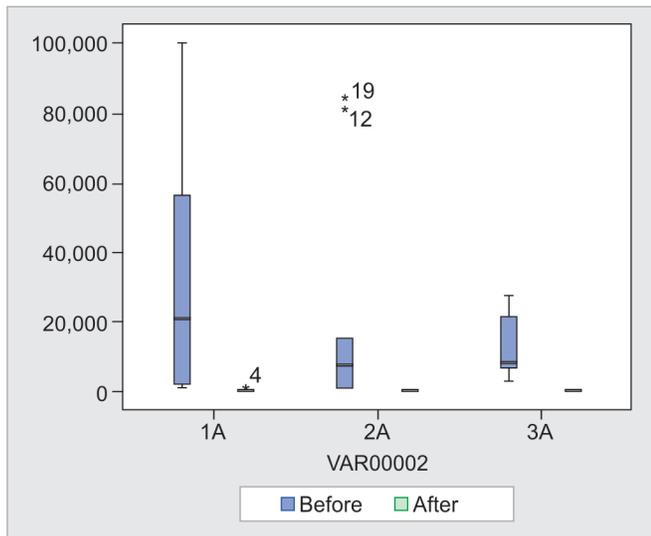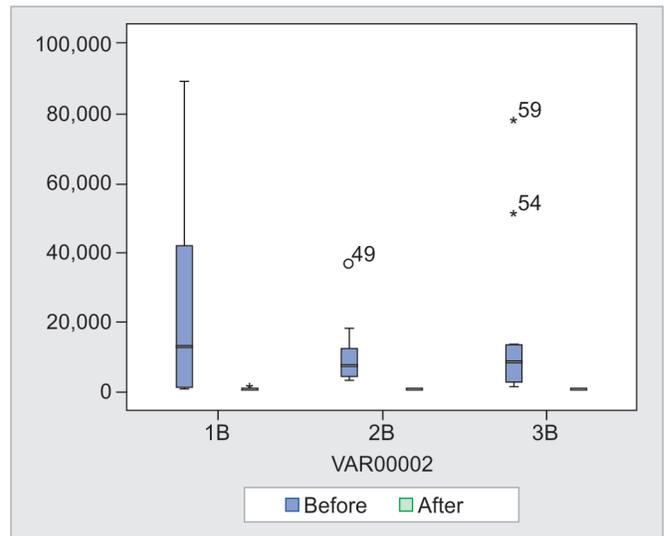*p-value = 0.005 indicates statistically significant

Fig. 2: Initial and final colony count. (A) *E. faecalis* ATCC 29212; (B) *E. faecalis* clinical isolate. (1) Initial colony count; (2) 7 days after placement of CH; (3) 7 days after placement of CH+gentamicin; (4) 7 days after placement of CH+daptomycin

Graph 1: Reduction in colony count (before vs after) in Group A (ATCC 29212)

Graph 2: Reduction in colony count (before vs after) in Group B (clinical isolate)

medicament) between the subgroups (1A, 2A, and 3A) of group A and subgroups (1B, 2B, and 3B) of group B. Nevertheless, there was no significant difference in the mean final count between the subgroups of group A ($p = 0.349$) and group B ($p = 0.015$) (Table 4).

Mann–Whitney U-test was performed to assess the difference in the inhibition of growth of *E. faecalis* after 7 days between subgroups of the same main group (with different intra-canal medicaments). Among the ATCC strain, no significant difference was observed in the final colony count between the subgroups 1A and

Table 4: Comparison between subgroups, final colony count, statistical analysis, Kruskal–Wallis test and Chi-square test

	E. faecalis ATCC 29212		*E. faecalis Clinical isolate*	
	Before	*After*	*Before*	*After*
Chi-square	1.253	2.105	0.101	8.410
df	2	2	2	2
p-value	0.535	0.349	0.951	0.015

Table 5: Comparison between subgroups, final colony count, statistical analysis, Mann–Whitney U-test, and Wilcoxon signed-rank test

	1A		1B	
	Before	*After*	*Before*	*After*
2A	0.520	0.176	–	–
3A	0.289	0.527	–	–
2B	–	–	0.820	0.030*
3B	–	–	0.762	0.006**

*Statistically significant

2A (*p* = 0.176) and between the subgroups 1A and 1C (*p* = 0.527). However, among the clinical isolates, significant difference was observed in the reduction in colony count between the subgroups 1B (CH) and 2B (CH+G), (*p* = 0.030). Also, significant reduction in colony count was observed between the subgroups 1B (CH) and 3B (CH+D) (*p* = 0.006). However, no significant difference was observed in the reduction in colony count between the subgroups 2B (CH+G) and 3B (CH+D) (*p* = 0.752) (Table 5).

DISCUSSION

Inter-appointment intra-canal medication has contributed to favorable outcomes when treating endodontic infections. The need for intra-canal medication is greater in those cases where bacteria are resistant to routine endodontic treatment and where the therapy cannot be successfully completed due to the presence of pain or continuous exudates.[15] Although there are several intra-canal medicaments, CH paste continues to be the medication of choice between appointments to reduce bacteria in root canal systems owing to its biological action. CH due to its high alkalinity (pH 12.5) can hydrolyze bacterial lipo-polysaccharides and neutralize the residual effect after bacterial cell lysis.[16] Nevertheless, a few reports indicate minimal antimicrobial action against *E. faecalis*, even after prolonged periods of medication with CH due to buffering capacity of dentin and proton pump mechanism that alters the pH on the outer surface of dentin from 12.5 to 8.[3,17] Hence as suggested by Siqueira and Lopes, hydroxyl ions from CH should diffuse into the dentin at sufficient concentrations, exceeding the dentin buffering capacity reaching high pH levels to destroy bacteria in the dentinal tubules.[3]

In our study, a significant reduction in colony count was observed between CH and CH+D/G, which could be attributed to the reduction in pH. Our results are in line with Molander and Dahle, who showed addition of antimicrobial agents to CH would enable synergistic antimicrobial effect against *E. faecalis in vivo*.[18] Our results suggest that combination of G/D with CH could be an effective intra-canal medicament.

In the current study, the limited antibacterial efficacy of CH (alone) against *E. faecalis* could be attributed to (1) proton pump inhibitory mechanism of *E. faecalis* that carries protons to the interior of the cell acidifying the cytoplasm thereby neutralizing the alkaline environment created by CH, (2) buffering capacity of dentin, (3) pH (8.6–10.3) required for the biological action of CH, (4) low solubility of CH and hence longer dissolution time and limited depth of penetration of CH into the dentinal tubules.[3–5,16]

According to our results, compared to CH+G combination, CH+D combination was more effective in inhibiting *E. faecalis*. The lipophilic D tail is inserted into the bacterial cell membrane. It causes calcium-dependent rapid membrane depolarization of the bacterial cell membrane and a potassium ion efflux. This is followed

by arrest of DNA, RNA, and protein synthesis resulting in bacterial cell death.[19] Basically, *E. faecalis,* due to its proton pump inhibitory mechanism, carries protons to the interior of the cell, acidifying the cytoplasm which is an important mechanism that enables the bacterium to survive in harsh alkaline environment,[20] whereas D alters the internal pH of the cell by potassium ion efflux mechanism *(E. faecalis* membrane potential and multiple cellular functions are disrupted followed by membrane depolarization involving the external release of potassium ions from the cell membrane) creating an internal alkaline environment thereby enhancing the antimicrobial property of CH.[21]

Thus, CH in combination with antibiotics (D and G) had synergistic antimicrobial effect against clinical isolates of *E. faecalis,* which is in agreement with other studies were CH with various antibiotic combinations like triple antibiotic paste, erythromycin silver zeolite were used and proved to be effective and have proved the superior antimicrobial efficacy of CH against clinical isolates of *E. faecalis*.[18,22] An in vitro study by de Freitas et al. proved that the association of NSAIDs or antibiotic did not interfere with the pH of CH paste and increased the antimicrobial action of CH paste against the biofilm of *E. faecalis*.[23]

G, being an β-lactam aminoglycoside, in combination with CH and D exhibited significant synergistic bactericidal activity against *E. faecalis* biofilm. The mechanism of antibacterial activity of G includes the following: inhibition of bacterial protein synthesis by binding to the bacterial 30S ribosomal subunit; alkalization and modification of the cationic concentration of the bacterial cell membrane, this causes the inhibition of bacterial cell wall synthesis. Others mechanisms include biofilm formation preventing activity by reducing the expression of genes necessary for bacterial adhesion and inhibition of bacterial cell division thereby preventing bacterial growth. Previous in vitro and in vivo research has proved the synergistic activity of G in combination with other antibiotics was more effective in eradication of *E. faecalis* biofilm than the solitary use of G alone.[24,25]

The drawbacks and limitations of this study include the following: the sample size can be increased and more number of clinical bacterial strains can be tested rather than using only one clinical isolate; this in vitro study evaluated the antibacterial efficacy on 3-week biofilm; however, further *in vivo* studies on 6 weeks bio-mineralized biofilm are required to ascertain the effect of CH in combination with G and D against *E. faecalis*.

CONCLUSION

Within the limitation of the study, it can be concluded that dentinal biofilm of clinical isolates of *E. faecalis* strains exhibited more reduction in bacterial colonies with CH in combination with antibiotics (D and G).

CLINICAL SIGNIFICANCE

D and G when used as an intra-canal medicament in combination with CH are effective in eliminating *E. faecalis.*

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