Original Article

A Comparative Evaluation of the Influence of Three Different Vehicles on the Antimicrobial Efficacy of Triple Antibiotic Paste against *Enterococcus faecalis*: An *In vitro* Study

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Abstract

Introduction: The root canal is a hub of numerous microorganisms. Routine endodontic procedures fail to remove the resistant microorganisms such as *Enterococcus faecalis*. **Aim:** The aim of the present study was to evaluate the influence of different vehicles on the antimicrobial efficacy of triple antibiotic paste (TAP) on *E. faecalis* infected root canals. **Materials and Methods:** Eighty single-rooted and freshly extracted human teeth were prepared in radicular portion, and pure culture of *E. faecalis* (ATCC® 29212™) inoculum was injected into canals of tooth blocks and incubated for 21 days. Tooth blocks were divided into five groups. Each experimental group was then medicated with 0.1 ml of TAP and no medication was added for control groups. After 21 days of incubation at 37°C, colony-forming units per milliliter (CFU/ml) were counted for each group. **Results:** Group II treated with TAP mixed with propylene glycol revealed a maximum reduction in CFU/ml, and that was followed by Group I and Group III, where TAP was mixed with 2% chlorhexidine (CHX) and 0.9% normal saline, respectively. Data were compared and analyzed using statistics software. The results were considered statistically significant for $P < 0.05$. There was a statistically significant difference in CFU/ml between propylene glycol and positive control group, between CHX and positive control group, between saline and positive control group. **Conclusions:** The propylene glycol group with TAP was the most effective vehicle for the elimination of *E. faecalis* from canals of tooth blocks, followed by 2% CHX solution as the second vehicle of choice over 0.9% normal saline.

Keywords: *Antimicrobial efficacy, chlorhexidine solution, colony-forming units per milliliter, propylene glycol, triple antibiotic paste*

Introduction

The biological aim of endodontic treatment is to facilitate the healing of the periapical tissues after eliminating bacteria from the root canal system.[1] Failure to heal is usually a result of residual infection or recontamination of the root canal system by microorganisms, which can penetrate about 300 μm deep into the dentinal tubules.[2,3]

Cleaning and shaping of the root canal system reduce the bacterial population but produces a smear layer that contains bacteria.[4] Aerobic and facultative anaerobic microorganisms are found in higher frequency in failed cases.[5] It has been reported that *Enterococcus faecalis* is more likely to be found in failed cases, which also appeared to be one of the most resistant intracanal microorganisms.[6]

Intracanal medication serves the purpose to eliminate the microbes that have the potential to influence the treatment outcome. Calcium hydroxide (CH), which was shown to be the intracanal medication of choice, is not effective against *E. faecalis*.[7] Studies investigating the antimicrobial activity of different concentrations of chlorhexidine (CHX) and sodium hypochlorite (NaOCl) against *E. faecalis* concluded that CHX in the liquid form at all concentrations tested (0.2%, 1%, and 2%) and NaOCl (5.25%) were the most effective irrigants against *E. faecalis*.[8,9]

Local application of antibiotics is considered to be a more effective mode of delivering antibiotics in endodontic practice. Because of the intricacy of the root canal infection, it is improbable that any single antibiotic could facilitate effective sterilization of

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the canal. More likely, a combination would be needed to address the diverse flora encountered.

Therefore, the present study was designed to evaluate the antibacterial efficacy of 3Mix, namely Triple antibiotic paste (TAP) containing a mixture of three antibiotics, including ciprofloxacin, metronidazole and minocycline against *E. faecalis* when mixed with three different vehicles viz. 2% CHX solution, propylene glycol, and 0.9% normal saline. The null hypothesis of the study tested was that there would be no difference in the influence of the three different vehicles on the antimicrobial efficacy of TAP against *E. faecalis.*

Materials and Methods

This study was carried out as an endodontic microbiological study conducted at the MGM Hospital, Navi Mumbai, India for 8 months. Ethical approval was obtained from the Institutional Review Board before the commencement of the study.

Preparation of specimen

Eighty single-rooted freshly extracted intact human teeth were used in this study. Permanent teeth with intact apices, no visible fracture lines, having single root with straight single canal were included in the study. Deciduous teeth, single rooted teeth with multiple canals, teeth with restorations or which are endodontically treated, and teeth having sharp curvatures of the root, root caries, or resorptions were excluded from the study. The crowns of the teeth were sectioned 1 mm below the cemento-enamel junction, and tooth blocks of 7 mm length were obtained by sectioning the apices accordingly [Figure 1]. Biomechanical preparation of the root canals was done by hand instrumentation till #50 K-files (Mani, Japan) followed by Gates-Glidden drills till #3 size (ISO size 090, 32 mm, Mani, Japan).

Organic and inorganic debris and the smear layer were removed by treatment in an ultrasonic bath in 5% NaOCl, followed by17% ethylene diamine tetraacetic acid for 5 min each. The tooth blocks were then placed in flasks containing Brain-Heart Infusion broth (BHI broth) and autoclaved for 30 min at 121°C.[10] Sterility of the tooth blocks was checked by the use of turbidity test. The tooth blocks were then mounted in sterile Petri dishes using decontaminated sticky wax. Two coats of nail varnish were applied to protect the external surfaces of all the tooth blocks. The aseptic environment was maintained throughout the experiment by working in a laminar airflow cabinet.

Preparation of inoculum

Pure culture of *E. faecalis* American Type Culture Collection (ATCC® 29212™) was grown overnight in brain heart infusion broth, and a suspension of bacterial inoculum was created. McFarland barium sulfate ($BaSO_4$) standard (approximately 10^8 colony forming units per milliliter [CFUs/ml]) was used to measure the optical density and calibrate the values using spectrophotometry at 600 nm wavelength. The turbidity of the prepared bacterial inoculum was standardized against 0.5 McFarland standard, which was used to contaminate the tooth blocks.

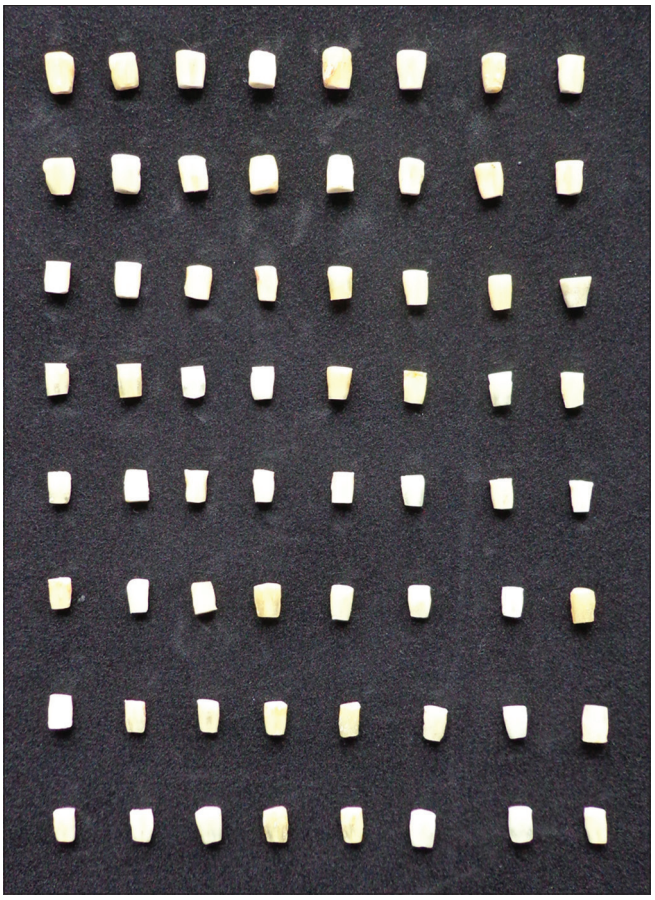

Figure 1: Tooth blocks of 7 mm length obtained by sectioning the crown and apices of single rooted freshly extracted intact human teeth

Inoculation of *Enterococcus faecalis* (ATCC® 29212™) in tooth blocks

Eighty tooth blocks were divided into five groups - 3 experimental groups (Groups I, II, III; n = 20 each); and positive and negative control groups (Groups IV and V, respectively; n = 10 each), and subjected to inoculation with *E. faecalis* [Figure 2]. The experimental groups were followed as Group I: TAP + 2% CHX solution, Group II: TAP + propylene glycol, Group III: TAP + 0.9% Normal Saline, Group IV: Positive control (infected with *E. faecalis* strain and no medication placed), Group V: Negative control (only sterile Brain Heart Infusion broth was placed and no medication placed).

Fresh inoculum of 0.1 ml *E. faecalis* grown in Brain heart infusion broth were added every alternate day to maintain the viability of the organism in Groups I, II, III, and IV. The Group V received only sterile Brain Heart Infusion broth. Specimens from all the groups were incubated at 37°C for 21 days. Supernatant from the tooth blocks of all the groups

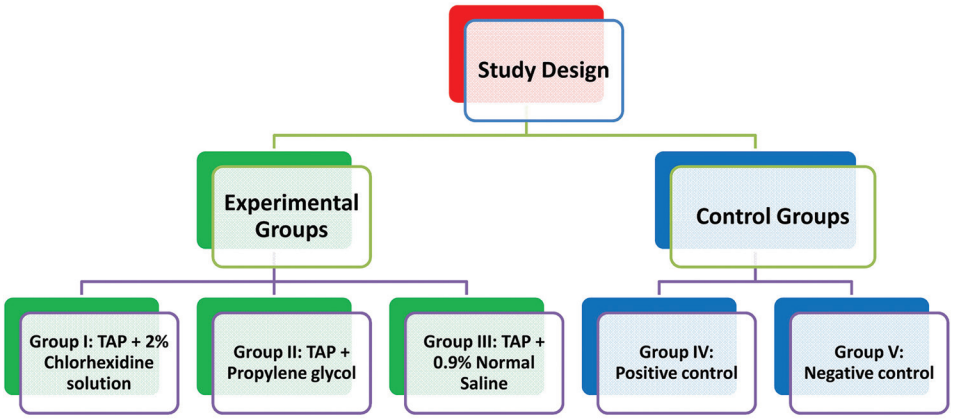

Figure 2: Study design of five groups - 3 experimental groups (Groups I, II, III); positive and negative control groups (Groups IV and V) respectively

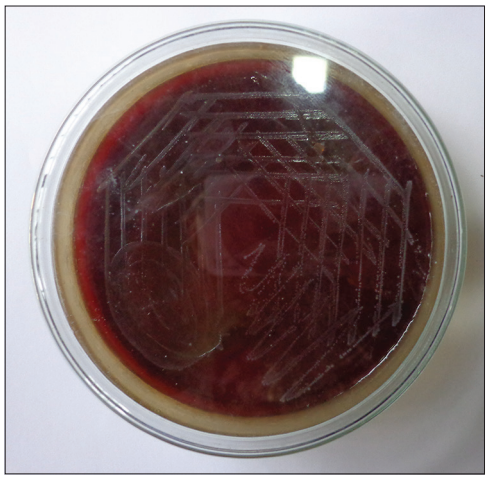

Figure 3: Streak culture test showing *Enterococcus faecalis* on blood agar plate

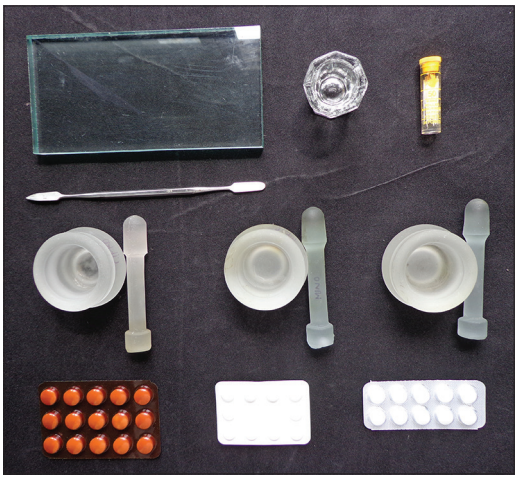

Figure 4: Figure showing glass slab, cement spatula, dappen dish, absorbent points #50, mortar pestle and intracanal medication: (L-R) Metronidazole, minocycline hydrochloride, ciprofloxacin hydrochloride

was further subcultured weekly on 5% Sheep blood agar to confirm the viability and the purity of the inoculum using the Streak culture method [Figure 3]. Additionally, the test organism was confirmed from all the groups by all of the following tests - Gram staining, Catalase test, Bile Esculin test, 6.5% NaCl test, L-pyrrolidonyl-β-naphthylamide test, Hanging drop test, Arginine dihydrolase test, Carbohydrate fermentation tests viz. Sorbose test, Arabinose test, Raffinose test, Lactose test, Sucrose test, Sorbitol test, Mannitol test and Growth in pyruvate.[11]

Preparation of the triple antibiotic paste

Commercially prepared chemotherapeutic agents, namely, Ciprofloxacin (250 mg), Metronidazole (400 mg), and Minocycline (100 mg) were pulverized into a fine powder using Mortar Pestle [Figure 4]. 50 mg of each drug was taken in dappen dish to achieve a ratio of 1:1:1 by weight.[11] This powder was mixed with 1 ml of the desired carrier medium, namely 2% CHX, propylene glycol, and 0.9% normal saline for the three experimental groups on glass slab using cement spatula [Figure 4]. A paste of 0.1 ml TAP containing 5 mg ciprofloxacin, 5 mg metronidazole, and 5 mg minocycline mixed with respective carrier viz., 2% CHX, propylene glycol and 0.9% Normal Saline for the three experimental groups was then placed in the tooth blocks.

Placement of triple antibiotic paste in the tooth blocks

At the end of the inoculation period, each canal was thoroughly rinsed with 0.9% sterile saline thrice and blotted dry using Absorbent points #50 [Figure 4]. Under aseptic conditions, the canals of the Groups I, II, and III were filled with the respective intracanal medication using #35 lentulo spiral paste carrier. The specimens of Groups I, II, and III were sealed coronally with decontaminated sticky wax and incubated at 37°C for 21 days. No medication was placed in Groups IV and V.

Microbiological sampling

Groups IV and V were subjected to microbiological sampling immediately after the inoculation period, while the Groups I, II, and III were subjected to microbiological sampling after the medication period. The tooth blocks were removed from petri dishes for microbiological sampling

after 21 days. Inner dentin of 300 microns [Figure 5] from root canals was then sampled using no. 6 Gates-Glidden drill (ISO size 150) fitted in X-Smart at a rotational speed of 500 rpm and 5 Newton Meter (N·m) torque to obtain dentin powder from the tooth blocks. The dentin powder was collected in 2.7 ml cryovials containing 1 ml Brain Heart Infusion broth.

Assessment of microbial growth

The tubes were vortexed for 30 s and dilutions were serially made using sterile normal saline in 4 test tubes to a concentration of 10^{-4}. Subcultures were performed by inoculating droplets of 100 µL from the last tube on solid medium, namely 5% sheep blood agar plate and incubated for 24 h at 37°C to allow the growth of any bacteria harbored in the dentin. The Petri plates were assessed for the presence of bacterial growth. Growth measured as the number of CFUs formed by inoculation on the solid medium by Spread Plate method. The following equation was used to calculate the number of CFU per ml from the original aliquot/sample:

Colony forming unit per milliliter = Average number of colonies for a dilution x dilution factor

Two-fold dilution of bacterial suspensions was prepared in four test tubes for all the experimental and negative control groups. For the positive control group, 10-fold dilution of bacterial suspensions were prepared in four test tubes. Normal saline was used as diluent. With a fresh sterile pipette, 1 ml of the first dilution was transferred into the next tube, and the remaining dilutions were done in a similar way.

Results

Maximum CFUs were observed in the positive control group [Figure 6], whereas no bacterial growth was noticed in the negative control group. Reduction in CFUs of *E. faecalis* was prominent in all the experimental groups i.e., I, II, and III [Figure 7]. The maximum reduction in CFUs was seen in Group II, followed by Group I and lastly, in Group III [Table 1].

Data were compared and analyzed using IBM SPSS statistical software version 20 (Armonk, New York, United States). The normality of data was tested using the Kolmogorov–Smirnov test and Shapiro–Wilk test and was found to be normally distributed since all the *P* values were $P > 0.05$ for the above tests [Table 2].

CFUs/ml was compared between groups using one-way Analysis of Variance (ANOVA) test [Table 3]. Figure 6 represents the mean values of CFU/ml and a standard deviation in each of the experimental and control groups. F-stat was found to be 26597 ($P < 0.05$), which indicates that the mean CFU/ml of at least one group is significantly different from the other groups. The results were considered statistically significant when the $P < 0.05$.

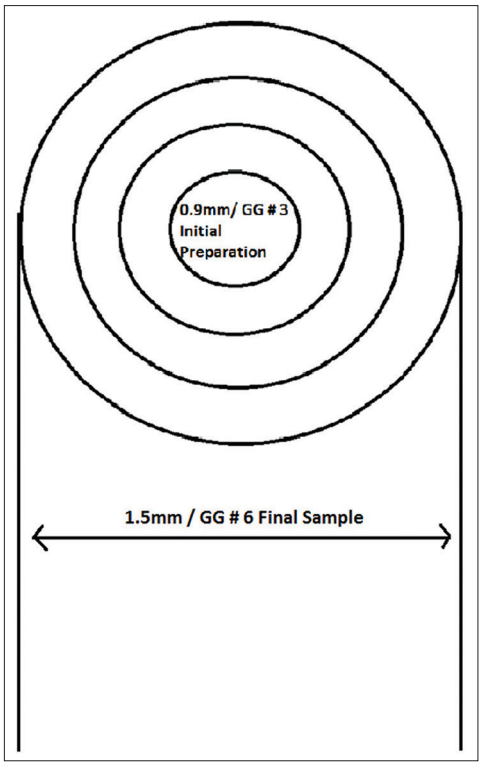

Figure 5: Schematic representation of the circumferential sampling of the dentin. Root canals initially prepared with No. 3 Gates-Glidden drill were enlarged with No. 6 Gates-Glidden drill and the dentin shavings were collected for an analysis of bacterial growth

Further, the pair-wise comparison was made using the least significance difference test. There was no statistically significant difference in CFU/ml between propylene glycol and CHX groups ($P = 0.27$). All three experimental groups showed statistically significant inhibition of growth in comparison with the positive control group. There was statistically significant difference observed in CFU/ml between Group II and Group III ($P = 0.02$) and between the control groups.

The results of the present study are in disagreement with the null hypothesis, which stated that there will be no difference in the influence of different vehicles on the antimicrobial efficacy of TAP against *E. faecalis.*

Discussion

It has been validated that the high-grade mechanical cleaning and shaping of the root canal, organized with the use of antibacterial irrigation will reduce 50% to 70% of infection from canals while the remaining canals hold vital bacteria.[12] *E. faecalis* has been identified in cases of failed endodontic treatment, and it has proven to be difficult to eradicate during retreatment.[7]

An intracanal medication with an effective antibacterial action is recommended to predictably eliminate bacteria from the entire root canal system. Studies have reported that *E. faecalis* is resistant to CH.[13] Sato *et al*. reported the prospect of the mixture of ciprofloxacin, metronidazole and

minocycline to have the potential to kill bacteria from the inner layers of root canal dentine *in situ*.[14] Hoshino *et al*. in 1996 investigated the antibacterial effect of a mixture of ciprofloxacin, metronidazole, and minocycline on bacteria taken from infected dentine of root canal walls.[15]

In the present study, an attempt was made to evaluate the susceptibility of *E. faecalis* to lower concentrations of the antibiotic drugs as compared to those used in another study.[16] *E. faecalis* ATCC® 29212™ was selected for the study as they are well-characterized microbial strains standardized for quality control research identification. The

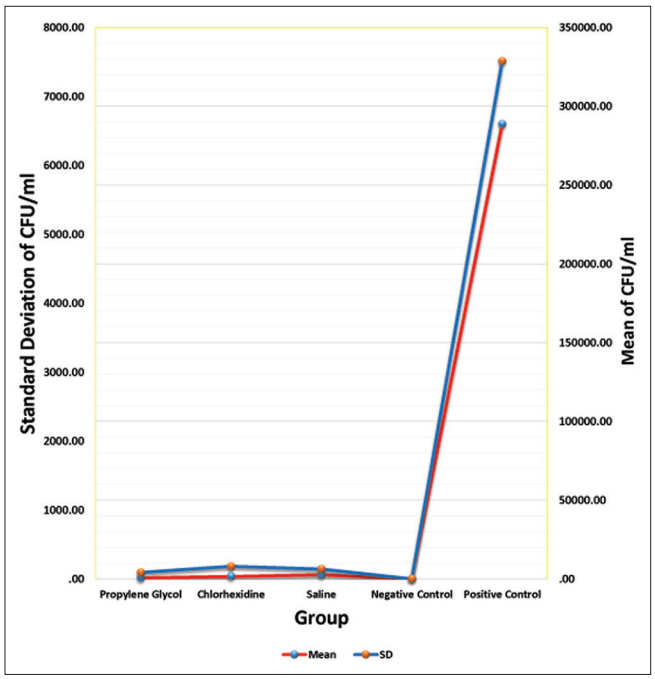

Figure 6: Frequency of the mean values and standard deviation of colony forming units per milliliter in each of the experimental and control groups

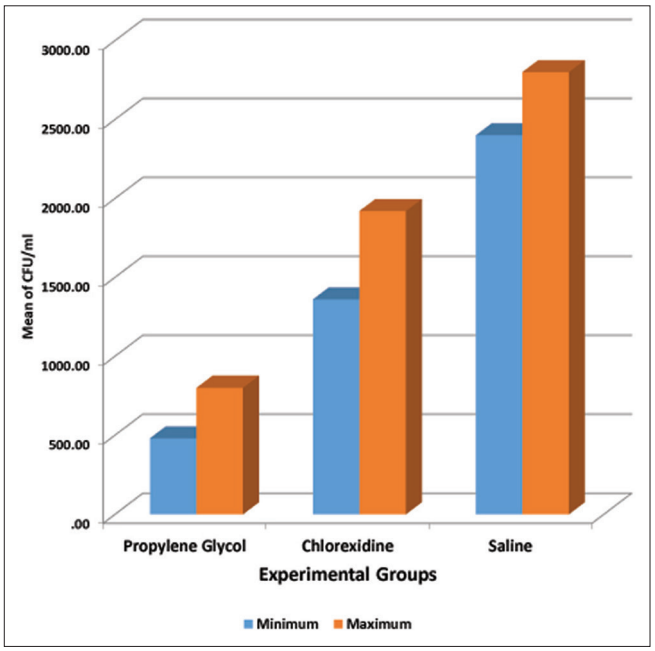

Figure 7: Comparison of the mean values of colony forming units per milliliter in the experimental groups

Table 1: Number of colony forming units per millilitre from each tooth block subculture in Groups I, II, III, IV and V estimated after 21 days of incubation

Sample name	CFU/ml				
	Group I: Chlorhexidine	Group II: Propylene Glycol	Group III: 0.9% Saline	Group IV: Positive control	Group V: Negative Control
A	1920	480	2480	291,200	0
B	1440	640	2720	300,800	0
C	1360	560	2640	281,600	0
D	1520	560	2480	284,800	0
E	1440	640	2800	291,200	0
F	1680	720	2720	297,600	0
G	1680	640	2560	284,800	0
H	1760	800	2720	294,400	0
I	1360	640	2800	281,600	0
J	1440	560	2480	278,400	0
K	1440	720	2400		
L	1520	640	2720		
M	1840	800	2560		
N	1520	560	2480		
O	1680	640	2400		
P	1440	560	2720		
Q	1360	640	2800		
R	1840	560	2480		
S	1680	800	2560		
T	1360	720	2400		

Sample name A to T indicates the sample labels in alphabetical order. CFU/ml: Colony forming units per milliliter

experimental model used in this study was adapted from the study by Basrani *et al*. for infection and disinfection of dentinal tubules.[10]

Among the three antibiotics, metronidazole is a nitroimidazole compound and it exhibits a wide bactericidal spectrum against anaerobes, which are common in oral sites. Two other antibacterial drugs viz., ciprofloxacin and minocycline, was mixed with metronidazole (3Mix: Sato *et al*.) in an effort to eliminate all the bacteria.[14] Minocycline is a broad-spectrum tetracycline antibiotic and has a broader spectrum than the other members of the group. Minocycline is the most effective component of the TAP against *E. faecalis*.[17]

The antibiotic mixture has to be mixed with an appropriate vehicle for optimizing intracanal placement. It helps the medicine to penetrate through the infected dentin and eliminate the viable bacteria from the deeper recesses of the radicular dentin. Thus, different vehicles were utilized in the present study to deliver antibiotic drugs. In the specimens belonging to the Group I, the TAP was mixed with 2% CHX solution which has some inherent antimicrobial properties; in the Group II, the vehicle used was a viscous substance, propylene glycol and in the Group III vehicle used was an aqueous substance, 0.9% saline.

Assessment of *E. faecalis* was quantified by measuring the number CFU/ml. The results obtained from the present study have revealed that all the experimental groups containing TAP mixed with different vehicles were effective in reducing the CFUs of *E. faecalis* from primarily infected dentin blocks. The maximum reduction in CFUs was seen in Group II, followed by Group I and the least in Group III.

The use of propylene glycol vehicle in the Group II has reduced the CFU/ml to the maximum extent compared to other two vehicles, which could have been facilitated by its high molecular weight that minimizes the dispersion of TAP into the dentin and maintains the paste in the area for longer intervals leading to prolonged action of the paste.

TAP mixed with 2% CHX solution was found to be better than the saline group but not as effective as observed in the propylene glycol group. CHX is a large cationic biguanide and has been widely used as an antiseptic agent for the irrigation of root canals as well as a medicament during root canal treatment. CHX is an efficient antimicrobial agent along with an effective anti-inflammatory agent. CHX decreases the inflammatory activity of the major virulence factor of *E. faecalis*, namely lipoteichoic acid.

The lowest reduction in CFUs was seen in Group III, which could be due to higher ionic dissociation caused by the lower viscosity of saline. Furthermore, the lower molecular weight of saline increases the dispersion of medication into the tissue and causes rapid antimicrobial action in the desired area.

Although TAP has been reported to be an effective antibiotic against *E. faecalis,* certain drawbacks have been observed with it. Among the three antibiotics used, minocycline has been proven to elicit tooth discoloration.[18] Minocycline binds to calcium ions to form an insoluble agent via the process of chelation.[19] Various methods were attempted, including replacing minocycline from TAP to combat discoloration. Doxycycline-containing TAP was found to have discoloration of lesser intensity when compared to minocycline.[20] Karczewski *et al*. in their study suggested that Clindamycin-modified triple antibiotic (metronidazole, ciprofloxacin, and clindamycin) polymer (polydioxanone or PDS) nanofibers might be a viable alternative to minocycline-based antibiotic pastes to counter severe discolorations caused by minocycline.[21]

TAP can adversely affect the dentin leading to demineralization, along with decreased microhardness and fracture resistance.[22] A recent study suggested that TAP caused superficial collagen degradation of radicular dentin after 2–4 weeks of exposure.[23] Berkhoff *et al*. concluded

Table 2: Normality of data was tested using Kolmogorov-Smirnov and Shapiro-Wilk test

Tests of normality (variable CFU/ml)						
Group CFU/ml	**Kolmogorov-Smirnov[a]**			**Shapiro-Wilk**		
	Statistic	**df**	**Sig**	**Statistic**	**df**	**Sig**
Propylene glycol	0.217	20	0.064	0.904	20	0.058
Chlorexidine	0.204	20	0.059	0.890	20	0.056
Saline	0.207	20	0.058	0.885	20	0.062
Positive control	0.195	10	0.200*	0.944	10	0.603

"Sig" is the significance for the test (or the *P*). df: Degrees of freedom; CFU/ml: Colony forming units per milliliter. * This is a lower bound of the true significance, [a]Lilliefors Significance Correction

Table 3: One-Way ANOVA table for comparison of colony forming units per milliliter between groups

Comparison of CFU/ml in various groups								
	n	**Mean**	**SD**	**SE mean**	**Minimum**	**Maximum**	***F*-statistics**	***P***
Propylene Glycol	20	644.00	91.67	20.50	480.00	800.00	26597.28	<0.001
Chlorhexidine	20	1564.00	180.71	40.41	1360.00	1920.00		
Saline	20	2596.00	143.28	32.04	2400.00	2800.00		
Negative control	10	0.00	0.00	0.00	0.00	0.00		
Positive control	10	288640.00	7512.24	2375.58	278400.00	300800.00		

CFU/ml: Colony forming units per milliliter; SD: Standard deviation; SE: Standard error

that TAP could not be removed successfully from the root canals, and almost >80% of the TAP is left back in the root canal system irrespective of the irrigation procedure used.[24] The residues of TAP may also adversely affect the bonding of root canal sealers to dentin and Gutta-percha obturation, causing gap formation and subsequent microleakage. In a recent study, Turkaydin *et al*. demonstrated that XP-Endo Finisher significantly eliminated more amounts of remaining TAP when compared with passive ultrasonic irrigation and needle irrigation from root canal walls which has been assessed using scanning electron microscopy.[25]

The results of a recent study indicated that the application of TAP as an intracanal medicament reduced the sealing efficiency of NeoMTA Plus over a period.[26] The values of bond strength of intracanal medicaments declined as the treatment time was extended. The duration of the medicament application has to be monitored during treatments.

The present *in-vitro* microbiological study demonstrates the effectiveness of TAP for sterilization of infected root dentine with *E. faecalis* using different vehicles. However, techniques have to be evolved to adequately remove the TAP from the canals, as this might influence the outcome of the endodontic therapy.

There were certain limitations of the study. As it is an *in vitro* study, the *in vivo* effects still needs to be confirmed to assess the exact effectiveness of the antimicrobial combinations. The effects of antimicrobial agents in tooth blocks taken from the mid root region is certainly more when compared to apical parts of root canals where the penetration is less.

Conclusions

Within the limitations of the present *in vitro* study, it can be concluded that TAP mixed with propylene glycol and 2% CHX solution is effective against *E. faecalis,* and it can be clinically used for challenging and failed endodontic cases. Further endodontic research needs to be carried out to identify the implications of high-level ciprofloxacin-resistant *E. faecalis* in root canal infections. Further research is needed to evolve techniques for complete removal of TAP following intracanal placement, especially from apical parts of root canals, optimize maximum concentration and formulation of these drugs to prevent cytotoxicity, if any, while creating a microenvironment that fosters periapical healing of tissue for maximizing the antimicrobial effect. *In vivo* effects of these formulations also need to be ascertained after confirming the persistence of clinical microbes colonizing in the root canals of persistent infections and failed cases.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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