Original Article

A Scanning Electron Microscope Evaluation of Smear Layer Removal and Antimicrobial Action of Mixture of Tetracycline, Acid and Detergent, Sodium Hypochlorite, Ethylenediaminetetraacetic Acid, and Chlorhexidine Gluconate: An *In vitro* Study

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ABSTRACT

Objectives: The main objective is to evaluate the efficiency in removal of smear layer of mixture of tetracycline, acid and detergent (MTAD), sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA) and chlorhexidine gluconate by scanning electron microscope (SEM) evaluation and also to evaluate the antimicrobial action of the same irrigants against standard culture strains of *Enterococcus faecalis*.

Materials and Methods: This study included 60 extracted permanent teeth with single root canal. The sample was categorized into five groups with 12 teeth in each group. Root canals were enlarged till size 40 with K-files. One group was kept as control and irrigated only with saline. Other four groups used 5% NaOCl as irrigant during instrumentation and MTAD, 5% NaOCl, 17% EDTA, and 2% chlorhexidine gluconate as final rinse. Teeth were split and examined under SEM. To test the antibacterial action, the zone of inhibition method using agar plates was used. Obtained data were statistically analyzed by SPSS version 17 (SPSS Inc., Chicago, IL, USA).

Results: MTAD and 17% EDTA removed smear layer from all regions of the root canals. About 5% NaOCl and 2% chlorhexidine gluconate were ineffective in removing the smear layer. The mean zone of inhibition formed by the irrigants was in the following order; MTAD (40.5 mm), 2% chlorhexidine gluconate (29.375 mm), 17% EDTA (24.125 mm), 5% NaOCl (22.125 mm), and saline (zero).

Conclusion: MTAD showed high smear layer removal efficacy, but no significant difference was found to that of 17% EDTA. As the dimensions of the zones of inhibition showed, MTAD has got highest antibacterial action against *E. faecalis*, followed by 2% chlorhexidine gluconate, 17% EDTA, and 5% NaOCl. However, the exact correlation of *in vitro* study results to clinical conditions is impossible due to the variables involved.

Keywords: *Acid and detergent, enterococcus faecalis, mixture of tetracycline, scanning electron microscope, smear layer, zone of inhibition*

Introduction

The complex root canal system precludes the absolute elimination of the bacteria. Facultative bacteria such as enterococci, nonmutans streptococci, and lactobacilli are more probable to endure

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chemomechanical instrumentation and irrigation medication.[1,2]

According to Mader *et al*., smear layer is made up of a superficial layer on the root canal walls about 1–2 μm in thickness and a deep layer of about 40 μm packed into the dentinal tubules.[3] Few consider it may be valuable as it lessens the dentin permeability, thereby preventing the bacterial penetration into the dentinal tubules. Several methods used for smear layer removal are mechanical, chemical, and lasers of which chemical method using different irrigating solutions is the most popular one. Of the several root canal irrigants, saline, sodium hypochlorite (NaOCl), and ethylenediaminetetraacetic acid (EDTA) are the frequently used ones.[3-5]

Recently irrigating solutions such as chlorhexidine gluconate and a mixture of tetracycline, acid and detergent (MTAD), and their combinations are in use. We carried our study to evaluate the efficiency in removal of smear layer of MTAD, NaOCl, EDTA, and chlorhexidine gluconate by scanning electron microscope (SEM) evaluation and also to evaluate the antimicrobial action of the same irrigants against standard culture strains of *Enterococcus faecalis*.[5]

Materials and Methods

Sixty extracted permanent mandibular premolars with single root canal, and fully developed apices were included. The study period was between March 2016 and December 2016 at Coorg Institute of Dental Sciences, Virajpet, Karnataka, India, after obtaining institutional ethical committee approval (Reference No. 152/CIDSV/IRB-E/2016). The sample size and procedure were based on Attur *et al*. study (2016) with some modifications. The irrigants used were MTAD, 5% NaOCl, 17% EDTA, and 2% chlorhexidine gluconate. The smear layer removal efficacy was evaluated using SEM analysis, and antimicrobial action was tested by zone of inhibition method on agar plates inoculated with *E. faecalis*.

Inclusion criteria

1. Teeth with straight roots
2. Teeth with fully formed apices
3. Noncarious teeth.

Exclusion criteria

1. Teeth with previous coronal restoration
2. Endodontically treated teeth
3. Fractured teeth.

Evaluation of smear layer removal efficacy of irrigants

Instrumentation

After preparing conventional access cavities for each tooth, coronal flare was given for the preparation using Gates-Glidden burs #2–4. With a 10 K file, working length was determined and teeth with working length ranging between 21 and 24 mm were used in this study. Root canals were then enlarged to the working length with 40 K-files [Figure 1].

Irrigation during instrumentation

Canal irrigation was done with 2 ml of 5% NaOCL (except for control group where saline was used for irrigation during instrumentation) using hypodermic syringe and side-vented 30-gauge needle. The total time of chemomechanical canal preparation was between 15 and 17 min.

Grouping of teeth

After the instrumentation, sample was categorized into different groups. Control group consisted of 12 teeth, and the remaining teeth were randomly divided into four experimental group of 12 each [Table 1].

Irrigation after instrumentation

All the canals were primarily irrigated with 1 ml of one of the agents. To make sure consistent and direct contact of each irrigating solution with the walls of root canals, a #15 barbed broach was rapped with cotton and soaked with the same irrigating agent and then placed into the canal. Then, each canal was irrigated with 4 ml of the

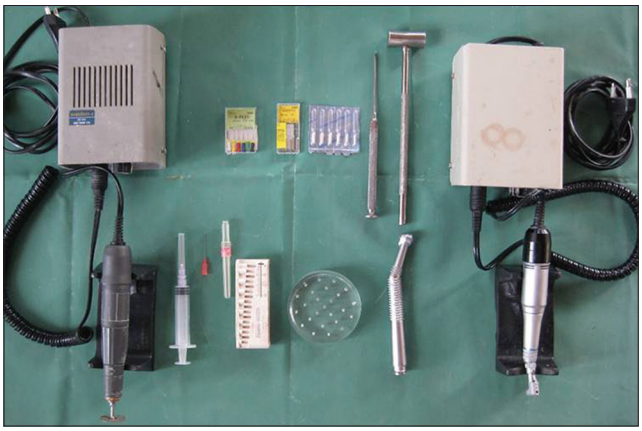


Figure 1: Armamentarium used to prepare samples for scanning electron microscope evaluation

Table 1: Groups of teeth samples based on the final irrigant used

Group	Irrigation during instrumentation	Irrigation after instrumentation
A (control)	Saline	Saline
B	5% NaOCl	MTAD
C	5% NaOCl	5% NaOCl
D	5% NaOCl	17% EDTA
E	5% NaOCl	2% chlorhexidine gluconate

NaOCl=Sodium hypochlorite, EDTA=Ethylenediaminetetraacetic acid, MTAD=Mixture of tetracycline, acid and detergent

same agent. For about 5 min, every canal was exposed to the final irrigating solution. Finally, 10 ml of saline was used to end the action of irrigant used, and canals were dried with paper points.

- Group A (Saline): After instrumentation teeth were irrigated with 5 ml of saline for 5 min and canals were dried with paper points
- Group B (MTAD): Final flush in this group was done with 5 ml of BioPure MTAD for a time of 5 min, then 10 ml of saline was used to terminate the action of MTAD. Then, canals were dried using paper points
- Group C (5% NaOCl): Final flush was done with 5% NaOCl for 5 min, then 10 ml of saline was used to stop the action of NaOCl
- Group D (17% EDTA): Final irrigation was done with 17% EDTA for 5 min, and irrigation with 10 ml of saline was done to terminate action of EDTA
- Group E (2% chlorhexidine gluconate): Final irrigation in this group was done with 2% chlorhexidine gluconate for 5 min and with 10 ml of saline.

Longitudinal grooves were then made on the labial and lingual surfaces of the tooth, and roots were split along the grooves with chisel and mallet [Figure 2]. One half of each tooth was taken for SEM analysis, and the other half was discarded.

Specimen preparation for scanning electron microscopic analysis

The specimen was fixed in 3% glutaraldehyde and kept overnight at 4°C, and then dehydrated in sequential concentrations of ethyl alcohol solutions (30%, 50%, 70%, 90%, and 100%) for about a total of 3½ h. Then, the specimens were put in isoamyl acetate for 15 min. Later, they were dried using critical point dryer (Hitachi –2). The specimens were mounted on an aluminum stub with a double-sided adhesive, with canal surface facing upward, then placed in the ion-sputtering unit (Hitachi, E-101) Vacuum dried and then sputter coated with gold-palladium

Scanning electron microscope evaluation

All the specimens were then viewed through SEM (S–2400) in the coronal, middle, and the apical areas of the root canal for the evaluation of smear layer efficacy of the irrigants. Photographs at magnification of ×1000 were taken at 12 representative areas for each group [Figures 3-7].

Methods of evaluation

Based on the SEM evaluation, the specimens were assessed for the presence or absence of smear layer using the following rating system [Table 2].

Evaluation of antimicrobial action of irrigants

Method of study

An overnight culture of *E. faecalis* (ATCC 29212) was standardized to 0.11 optical density measured at 570 nm. Using a cotton swab, the standardized bacterial culture was spread into trypticase soy agar (TSA) plate to provide an even lawn of cells. One-quarter inch sterile filter paper was placed into five different areas of the TSA plate. At the bottom of the plate, the filter papers

Table 2: Smear layer-scoring scheme

Score	Measure
1	Absent smear layer - There is absolutely no smear layer on canal surface and with clean and open tubules
2	Moderate smear layer - There is absolutely no smear layer on canal surface and with debris over tubules
3	Heavy smear layer - There is smear layer over root canal surface and tubules

Figure 2: Split tooth after ion sputtering

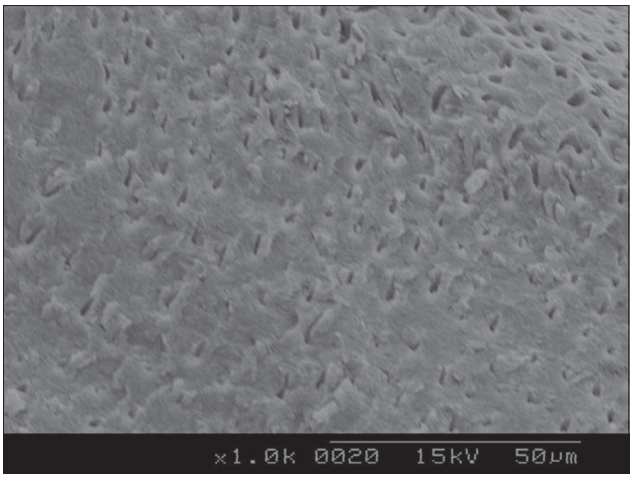


Figure 3: Scanning electron microscope findings after saline irrigation at coronal third of tooth

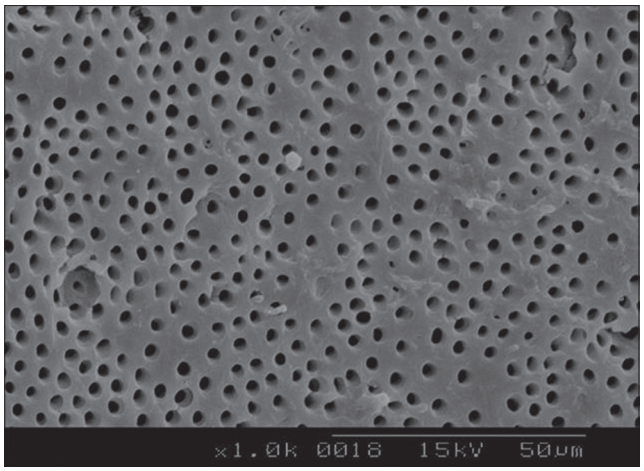


Figure 4: Scanning electron microscope findings after tetracycline, acid and detergent irrigation at coronal third of tooth

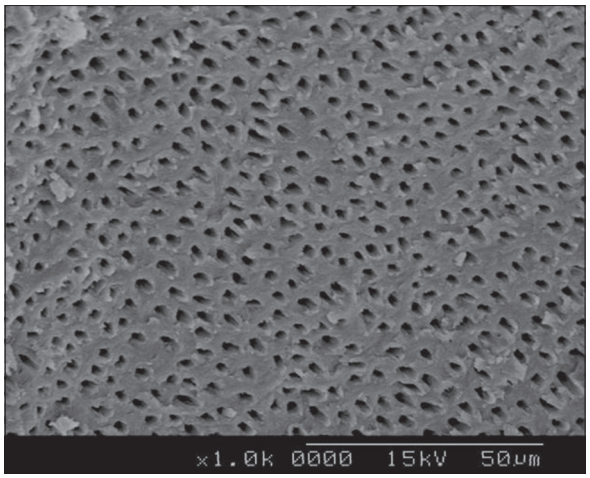


Figure 5: Scanning electron microscope findings after 5% sodium hypochlorite irrigation at coronal third of tooth

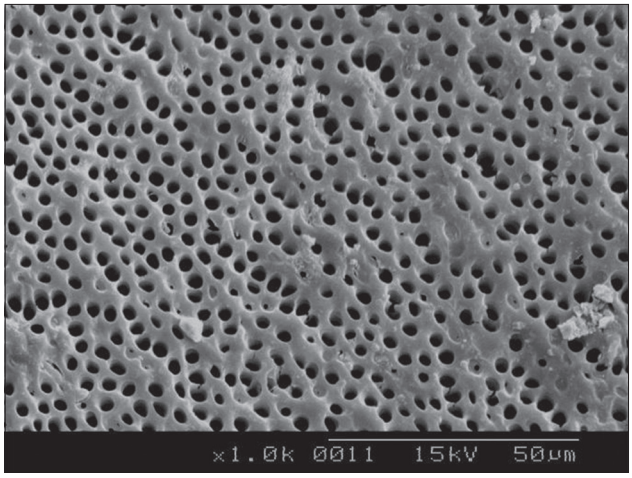


Figure 6: Scanning electron microscope findings after ethylenediaminetetraacetic acid irrigation at coronal third of tooth

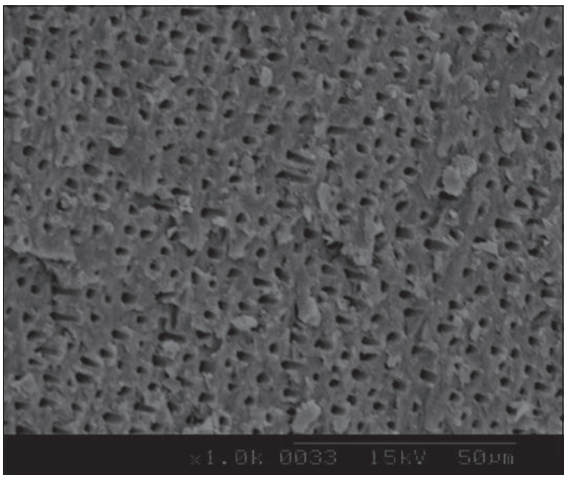


Figure 7: Scanning electron microscope findings after 2% chlorhexidine gluconate irrigation at coronal third of tooth

were marked as Saline, MTAD, 5% NaOCl, 17% EDTA, and 2% chlorhexidine gluconate. Twenty microliters of saline (control), MTAD, 5% NaOCl, 17% EDTA, and 2% chlorhexidine gluconate were added into corresponding filter papers using micropipette. For each irrigant, separate disposable tips for the micropipette were used to prevent contamination. Eight replicates were prepared and incubated overnight at 37°C for 24 h for each of the test solution. After 24 h, the TSA plates were taken from the incubator and zone of inhibition were measured across the diameter [Figure 8].

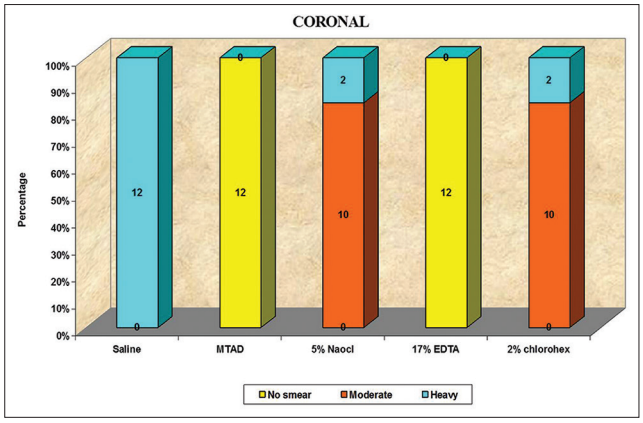
Results and Observations

The zone of inhibition formed by each irrigant was measured in millimeters.

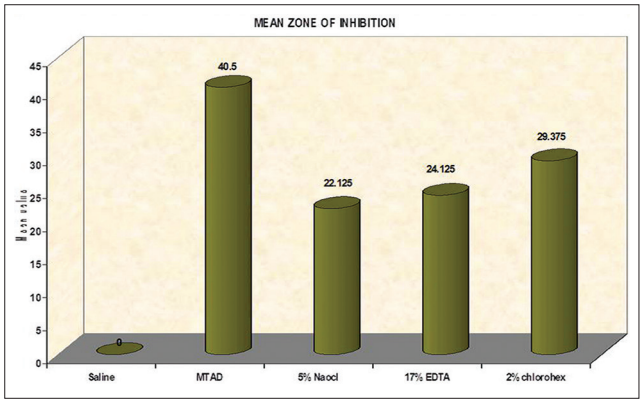
- Group A (Saline): Heavy smear layer in the coronal, middle, and apical thirds of all the specimens and whole of the root canal surface
- Group B (MTAD): No smear layer was observed in the coronal, middle, and the apical portion of all the specimens, except for apical third of two specimens which showed moderate smear layer
- Group C (5% NaOCl): Coronal third of eight specimens showed moderate smear layer, and in the remaining two specimens, the coronal third showed heavy smear layer. The middle and the apical areas of all the specimens showed heavy smear layer
- Group D (17% EDTA): No smear layer in coronal and middle thirds of all the specimens. In the apical third, nine specimens showed no smear layer, and three specimens showed moderate smear layer
- Group E (2% chlorhexidine gluconate): Coronal third of eight specimens showed moderate smear layer and remaining two specimen showed heavy smear layer. Middle and the apical thirds of all the specimens showed heavy smear layer.

The results were analyzed statistically using Chi-square test [Tables 3-6 and Graphs 1, 2] (SPSS version-15,

Figure 8: Trypticase soy agar-plate showing zones of inhibition



Graph 1: Bar diagram comparing smear layer removal efficacy of irrigants



Graph 2: Comparison between mean zone of inhibition of different irrigants

SPSS Inc., Chicago, IL, USA and Excel). There was a significant difference in the effectiveness of different irrigation regimes in removing smear layer at the coronal, middle, and apical third ($P < 0.001$).

We found that in Group A, no difference in the ability of smear layer removal by the irrigant from all the three regions. In Group B, no significant difference in ability of irrigant in removing smear layer from all three regions of the root canal ($P = 0.12$). In Group C, very highly significant and marked difference in the ability of smear layer removal from coronal compared to middle and apical thirds ($P < 0.001$). In Group D, there was a significant difference in the ability of smear layer removal from coronal and middle thirds compared to apical thirds ($P = 0.038$). In Group E, very highly significant and marked difference in the ability of irrigant to remove smear layer from coronal compared to middle and apical regions of the canal ($P < 0.001$).

The mean zone of inhibition produced by MTAD was 40.50 which is highest of all the test irrigants. It was followed by 2% chlorhexidine gluconate (29.375), 17% EDTA (24.125), 5% NaOCl (22.125), and saline (zero) in decreasing order. Statistical analysis using Kruskal–Wallis teat (H) showed very high significant difference ($P = 0.001$) between the zones formed by different irrigants [Table 7].

Comparison between different irrigants

Comparison between different irrigants was done using Mann–Whitney U-test (Z). The mean zones of inhibition were in the following order: MTAD (40.5 mm) >2% chlorhexidine gluconate (29.375 mm) >17% EDTA (24.125 mm) >5% NaOCl (22.125 mm) >Saline (zero).

Discussion

Studies have shown that smear layer prevents proper adaptation of softened gutta-percha to the root canal walls. When smear layer was removed a significant enhance of adhesive strength and microleakage resistance of AH 26 sealer was observed.[5-8]

However, Timpawat *et al*. found more apical microleakage when smear layer was removed. It has also been showed that patent dentinal tubules are necessary for reducing the irrigating time to achieve disinfection.[6]

We found that NaOCL alone was not able to remove smear layer completely. Our findings are in accordance with Yamada *et al*. and Torabinejad *et al*. This might be due to the fact that NaOCL dissolves the organic component and leaves the smear layer of inorganic tissue.[8]

We found that following the use of EDTA, the smear layer was completely removed from coronal and middle thirds of all the specimen but was less effective in the apical one third, the difference being statistically significant. This might be due to nonpenetration of the irrigant into the narrow apical region of teeth. Our findings are in accordance with Perez and Rouqueyrol-Pourcel[9,10] Dogan *et al*. reported that EDTA may make NaOCL ineffective by reducing the availability of chlorine.[11]

Table 3: Comparison between coronal thirds of different groups

	Groups					Total	*P*
	Saline	MTAD	5% NaOCl	17% EDTA	2% Chlorhexidine		
1.0 count %	0 (0%)	12 (100.0%)	0 (0.0%)	12 (100%)	0 (0.0%)	24 (40.0%)	<0.001*
2.00 count %	0 (0.0%)	0 (0.0%)	10 (83.3%)	0 (0.0%)	10 (83.3%)	20 (33.3%)	
3.00 count %	12 (100.0%)	0 (0.0%)	2 (16.7%)	0 (0.0%)	2 (16.7%)	16 (26.7%)	
Total count %	12 (100.0%)	12 (100.0%)	12 (100.0%)	12 (100.0%)	12 (100.0%)	60 100.0%	

χ^2=97.5, *P*<0.001 VHS

Table 4: Comparison between middle thirds of different groups

	Groups					Total	*P*
	Saline	MTAD	5% NaOCl	17% EDTA	2% Chlorhexidine		
1.00 count %	0 (0.0%)	12 (100.0%)	0 (0.0%)	12 (100.0%)	0 (0.0%)	24 (40.0%)	<0.001*
3.00 count %	12 (100.0%)	0 (0.0%)	12 (100.0%)	0 (0.0%)	12 (100.0%)	36 (60.0%)	
Total count %	12 (100.0%)	12 (100.0%)	12 (100.0%)	12 (100.0%)	12 (100.0%)	60 (100%)	

χ^2=60, *P*<0.001 VHS

Table 5: Comparison between apical thirds of different groups

	Groups					Total	*P*
	Saline	MTAD	5% NaOCl	17% EDTA	2% Chlorhexidine		
1.00 count %	0 (0.0%)	10 (83.3%)	0 (0.0%)	9 (75.0%)	0 (0.0%)	19 (31.7%)	<0.001*
2.00 count %	0 (0.0%)	2 (16.7%)	0 (0.0%)	3 (25.0%)	0 (0.0%)	5 (8.3%)	
3.00 count %	12 (100.0%)	0 (0.0%)	12 (100.0%)	0 (0.0%)	12 (100.0%)	36 (60.0%)	
Total count %	12 (100.0%)	12 (100.0%)	12 (100.0%)	12 (100.0%)	12 (100.0%)	60 (100%)	

χ^2=60.632, *P*<0.001 VHS

Table 6: Chi-square tests-comparison between 3 regions

Group Pearson χ^2	Value	*P*
MTAD Pearson χ^2	4.235	0.120 (NS)
5% NaOCl Pearson χ^2	27.692	0.001 (VHS)
17% EDTA Pearson χ^2	6.545	0.038 (significant)
2% chlorhexidine Pearson χ^2	27.692	0.001 (VHS)

NS=Not significant, VHS=Very highly significant, NaOCl=Sodium hypochlorite, EDTA=Ethylenediaminetetraacetic acid, MTAD=Mixture of tetracycline, acid and detergent

We found 2% chlorhexidine gluconate to be ineffective in removal of the smear layer in all regions of the root canal. Our finding is in accordance with agreement with Yamashita *et al*.[2]

MTAD was first introduced by Torabinejad *et al*. and they found that it effectively removes smear layer, when it is used as a final rinse, with NaOCL as an initial irrigant. According to them the tetracycline part of MTAD removes the smear layer and other debris and detergent Tween-80, reduces the surface tension of the irrigant, thereby aiding in better penetration of the irrigant.[12]

We found that the mean zone of inhibition produced by 5% NaOCl against *E. faecalis* was less compared to other irrigant solutions tested, which was very highly significant compared to MTAD and 2% chlorhexidine gluconate and not significant compared to 17% EDTA. This is in accordance with Radcliffe *et al*. and Shabahang and Torabinejad. This might be due to less availability of free chlorine in NaOCl that is stored for a long time.[13,14]

We found the antimicrobial action of 17% EDTA against *E. faecalis* had a mean zone of inhibition slightly >5% NaOCl solution, but the difference was not statistically significant. This is in agreement with Siqueira *et al*. and Sahar-Helft and Stabholtz. However, the zone of inhibition in the present study was less compared to MTAD, and 2% chlorhexidine gluconate and the difference was very highly significant.[15,16]

We found the antimicrobial action of 2% chlorhexidine gluconate against *E. faecalis* had a mean zone of inhibition higher than that produced by 5% NaOCl and 17% EDTA (very highly significant statistically) signifying a clear-cut antibacterial action of chlorhexidine. This is in agreement with Gomes *et al*. and in contrast to Siqueira *et al*. and Suchithra. This variation might be due to the difference in methodologies and strains of organisms used, but to be effective, smear layer must be removed before its application.[15,17,18]

BioPure MTAD revealed the largest zone of inhibition. Our findings are in agreement with Shabahang *et al*. and Yadav *et al*. Its antibacterial action was found after a short period and is due to the doxycycline

Table 7: Zone of inhibition-mean and standard deviation

	n	Mean	SD	Minimum	Maximum
Saline	8	0.0000	0.0000	0.00	0.00
MTAD	8	40.5000	0.53452	40.00	41.00
5% NaOCl	8	22.1250	2.10017	19.00	24.00
17% EDTA	8	24.1250	2.03101	22.00	26.00
2% chlorhexidine	8	29.3750	0.51755	29.00	30.00

[a]*H*=36.415, *P*=0.001 - VHS. SD=Standard deviation, NaOCl=Sodium hypochlorite, EDTA=Ethylenediaminetetraacetic acid, MTAD=Mixture of tetracycline, acid and detergent, VHS=Very highly significant

component.[19,20] However, Kho and Baumgartner did not find any significant difference in antibacterial efficacy among 1.3% NaOCl/BioPure MTAD and 5.25% NaOCl/15% EDTA in the apical 5 mm of the root canals.[21,22]

Shahravan *et al*. carried out a systematic review between 1975 and 2005 to find out if removal of smear layer lessens leakage after endodontic treatment of teeth *in vitro*. They found that there was a reduction of leakage after removal of smear layer (*z*-score = 0.37, *z* = 2.31, *P* = 0.021).[22]

The major limitation of our study is its *in vitro* nature, as it cannot exactly represent natural teeth in the oral environment. Still, there are many factors that raise controversies such as optimum time of contact of the irrigant, mechanism of action of irrigants, and smear layer removal is not the single factor influencing success of endodontic therapy.

A precise correlation of the *in vitro* study results to clinical conditions is not possible. Hence, the future *in vivo* research should be carried out in ideal clinical situations with more latest irrigant solutions, on a larger sample, and for a longer duration as *in vitro* studies will not exactly reflect *in vivo* environment.

To summarize, we found that there was complete removal of smear layer by MTAD and 2% chlorhexidine gluconate had better antimicrobial action.

Conclusion

We found MTAD has the highest efficiency in removal of the smear layer from all regions of the root canal, without any significant difference in its efficacy between coronal, middle, and apical thirds. We also found that MTAD showed comparatively large mean zone of inhibition against *E. faecalis*, followed by 2% chlorhexidine gluconate, 17% EDTA, and 5% NaOCl.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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