Evaluation of Smear Layer Removal and Antimicrobial Efficacy of HybenX Against *Enterococcus Faecalis* Biofilm

Keywords

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ABSTRACT

To evaluate the antibacterial efficacy of HybenX, an endodontic irrigant against Enterococcus faecalis biofilm and determine its efficacy in removal of smear layer. Forty root canals were inoculated with E. faecalis for three weeks, divided into 4 groups and irrigated with: Group1: 5 ml 2.5% sodium hypochlorite; Group2: 5 ml 2% chlorhexidine gluconate; Group3: 5 ml HybenX; Group4: 5 ml distilled water (control). Dentin shavings were collected and plated on agar plates, followed by colony forming unit (CFU) determination. For smear layer removal examination, 30 single rooted teeth were instrumented and divided into 3 groups and treated with: GroupI: 5 ml of 17% EDTA; GroupII:5 ml of HybenX; and GroupIII: 5 ml of distilled water. Samples were then subjected to SEM examination. All irrigants showed a significant reduction in CFUs compared to the control group (P< .001), but none compared to each other. Regarding smear layer removal in the apical third, EDTA removed smear layer more effectively than HybenX (P = .014). HybenX demonstrated good antimicrobial efficacy against E. faecalis biofilm and removed the smear layer effectively in coronal and middle third of the root canal system. HybenX can be considered as a promising irrigating agent in root canal treatment of infected teeth.

INTRODUCTION

The outcome of root canal therapy depends on effective elimination of bacterial biofilm from root canal system.[1] This is traditionally achieved by a combination of mechanical instrumentation, use of disinfecting irrigating solutions and placement of intracanal medicaments. It has been demonstrated that, quality of the root canal treatment and post endodontic restorations are essential prerequisite for the success of treatment.[2] In order to achieve quality root canal treatment, disinfection of the root canal system plays an important role. It has been reported that 35% of the root canal system remain untouched during mechanical instrumentation regardless of the instrumentation technique used.[3] Hence, irrigation plays a vital role in disinfecting the root canal system. The common recovery of *Enterococcus faecalis* from root canals of teeth in which previous treatment has failed is notable.[4] *E. faecalis* have been reported to be resistant to alkaline pH and often been found to survive in root canals as single organism.[5,6] It has also been reported to be resistant to alkaline stress due to its ability to penetrate deep into dentinal tubules.[5]

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Chlorhexidine gluconate (CHX) is one of the effective antimicrobial endodontic irrigants.[7] The substantivity property of CHX may prevent colonization of microorganisms on dentinal surface.[8] It even continued to prevent reinfection of dentine for up to 72 hours.[9] However, CHX does not have ability to remove smear layer effectively and dissolve pulp tissue.[10,11] Sodium hypochlorite (NaOCl) remains as the main endodontic irrigant because of its broad spectrum antimicrobial action, ability to dissolve the pulp tissue and remove organic part of smear layer.[12] Several studies have reported that CHX and NaOCl are effective against *E. faecalis*.[8,13] Mechanical instrumentation of root canal system produces smear layer, which consists of inorganic dentine debris, pulp tissue, odontoblastic process, necrotic debris, microorganisms and their metabolic products.[14] The radicular smear layer presents differences in relation to the coronal smear layer that affect the adhesive procedure. In root dentin, the smear layer is formed in two stages.[15,16] The smear layer has been defined as 'any debris, calcific in nature, produced by reduction or instrumentation of dentin, enamel or cementum',[17] or as a 'contaminant'[18] that precludes interaction with the underlying pure tooth tissue.[19] Despite its different composition the thickness of smear layer ranges from 0.5 to 2 μm, in addition to a deeper layer packed into the dentin tubules to a depth of up to 40 μm, obstructing their orifices thus forming the so-called *smear plugs*. In addition to the 'traditional' smear layer produced by manual or rotary instrumentation of the root canal walls, the subsequent preparation of the post space using post drills resulted in an additional and even thicker smear layer composed of debris and sealer/gutta-percha remnants that significantly influenced the adhesion of fiber posts.[20] In fact, the action of the drills used to remove the root- filling material to create post space produces a new smear layer rich in sealer and gutta-percha remnants that are plasticized by the frictional heat of the drill and this may diminish the penetration and chemical action of the agents used to bond fiber posts.

The morphological features, composition and thickness of the smear layer are determined by the type of endodontic instrument used, the method of irrigation and tooth substrate at which it is formed.[19,21] Coronal smear layers reflect the substructure of dentine matrix composition while endodontic smear layer contains inorganic and organic substances that include also fragments of odontoblastic process, microorganisms and necrotic material.[22]

Studies have demonstrated that removal of smear layer improves the fluid tight seal of root canal system, eliminates the bacteria within dentinal tubules and also helps in penetration of intra canal medicaments, irrigating agents and sealers into the dentinal tubule.[14,23-25] Combined application of ethylenediaminetetraacetic acid (EDTA) and NaOCl is commonly recommended for effective removal of smear layer from root canal system.[12,14]

Recently, a novel antimicrobial agent "HybenX" (Epien Medical, Saint Paul, MN, USA) has been marketed as a focal irrigating solution. It is used as an adjunctive treatment method with the potential to remove residual biofilm from oral tissue surfaces.[26] It consists of mono-sulfonated and bis-sulfonated hydroxybenzenesulfonic acid (HBSA) and mono- abd bis-sulfonated hydroxymethoxybenzenesulfonic acid (HMBSA) together with free sulfuric acid, water and a colorant. Manufacturers of HybenX claim that, it is an effective solution against plaque biofilm. It was shown that HybenX with scaling and root planning had greater reduction in clinical, microbial and inflammatory mediators compared to scaling and root planning alone.[27] Furthermore HybenX was very effective against acute periodontal abscess,[28] while killing intratubular bacteria biofilms.[29]

The aim of this study was to evaluate the antibacterial efficacy of HybenX against *E. faecalis* biofilm and also to determine its efficacy as a final irrigant in removal of smear layer from the instrumented root canal system. The null hypothesis tested was that HybenX has no antibacterial efficacy against endodontic biofilm and it would not affect the removal of smear layer from root canal system.

MATERIALS AND METHOD

EVALUATION OF ANTIMICROBIAL EFFICACY

Ethical clearance for the use of human teeth for the experiments performed in the present study was obtained from the institutional review board (IEC 199/2015 on 14/04/2015). 42 extracted human single rooted teeth with mature apices were selected. Teeth with caries, cracks and dilacerations (observed under magnifying loupes) were excluded.

PREPARATION OF TEETH SAMPLES

Teeth were cleaned with 2.5% NaOCl (Vista Dental; WI, USA) and stored in 0.2% sodium azide (Sigma Chemical, St. Louis, MO) at 4 ºC until the experiment. Periapical radiographs were taken in buccolingual and mesiodistal directions, to confirm the presence of a single canal and absence of anatomical variations. Teeth were decoronated and length of the roots were standardized to 17 mm. Apical patency was established using #10 K file (Mani Inc- Tochigi Ken, Japan). The working length was established by inserting a No.10K file (Mani Inc- Tochigi Ken, Japan) into each root canal until it was just visible at apical foramen which was observed under magnifying loupes (Carl Zeiss Meditec, Göschwitzer Straße, Jena, Germany) and by subtracting 1mm from this point. Canals were then prepared to F3 size using Protaper universal instruments (Dentsply, Ballaigues, Switzerland). Irrigation was performed with 5 ml of 2.5% NaOCl between each instrument change and final irrigation was performed using 5 ml of 17% EDTA (Merck; Darmstadt, Germany) for one minute. Finally, the canals were rinsed with 5 ml of distilled water to remove any precipitate

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if formed. All the irrigation was performed using 28-gauge needle (NaviTip; Ultradent, UT, USA) placed 1-2 mm short of working length. Shallow grooves were then prepared using a diamond disc (Horico Dental, Berlin, Germany) on mesial and distal sides of all roots without perforating the canal to facilitate splitting of the sample. All the samples were then coated with two layers of nail varnish and apical patency was checked again. Then all samples were sterilized using ethylene oxide. The root apices were closed with sticky wax (Saffroshine; Solapur, Mumbai, India) and each root was placed in a glass vial filled with soft putty impression material (Aquasil Dentsply Sirona; Konstanz, Germany) to mimic a closed end system for irrigation.

MICROBIAL STRAIN

Standard strain of *E. faecalis* (ATCC 29212) from stock collection was transferred and maintained in Muller Hinton Agar (MHA; Himedia Laboratories, Mumbai, India). Subcultures were made to ensure purity.

INOCULUM PREPARATION

2 to 3 well isolated colonies was transferred to 3 ml of Muller Hinton Broth tubes (MHB; Himedia Laboratories, Mumbai, India) and incubated at 37 °C for 3-4 hours with intermittent shaking at 150 rpm in a water bath to ensure the logarithmic phase cultures. For inoculation of the teeth samples, the optical density was measured using spectrophotometer (Mettler Toledo, Mumbai, India) to 1.8 nm with final cell concentration of 1.5×10^5 colony forming units (cfu)/ml.

BIOFILM FORMATION IN ROOT CANALS

The 5 µl inoculum was injected in the root canals and incubated at 35 °C for 3 weeks in 100% relative humidity. Each day the left over inoculum was aspirated and replenished with fresh preparation.

ASSESSMENT OF BIOFILM FORMATION

Two inoculated teeth samples were randomly selected and subjected to scanning electron microscope (SEM) examination to confirm the biofilm formation and its characteristics. The samples were split longitudinally using a straight chisel and were dehydrated using ascending grades of ethyl alcohol (25%, 50%, 75% and 100%) for 15 min. The samples were then mounted on metallic stubs, gold sputtered using an ion sputter, and examined under SEM (JEOL, Tokyo, Japan) at 3,000× magnification and 5KV.

TREATMENT OF TEETH SAMPLES WITH BIOFILMS

Remaining forty samples were then divided into three experimental and one control group (n=10).

Group 1→ 5 ml of 2.5% NaOCl for 5 min.

Group 2→ 5 ml of 2% CHX for 5 min.

Group 3→ 5 ml of HybenX for 5 min.

Group 4→ 5 ml of distilled water for 5 min (Control).

All the irrigation was performed using 28 gauge needle (Vista Dental Products, WI, USA) which was placed 1-2 mm short of working length. All procedures were done under aseptic conditions.

ANALYSIS OF COLONY FORMING UNITS (CFUS)

After irrigation with test solutions for the specified time intervals, final irrigation was performed with distilled water and sodium thiosulfate (5%) or Tween 80 (Merck, Darmstadt, Germany) to inactivate NaOCl and CHX respectively. Remaining samples were longitudinally split with the help of a straight chisel, to expose the root canals. Dentine shavings were collected from the entire root canal surface using a sterile stainless steel round bur at a depth of 0.5 mm. Two samples of shavings were collected from buccal and lingual halves of each root. The dentinal shavings were then transferred to 1 ml Brain heart infusion broth (BHI) (MHB; Himedia Laboratories, Mumbai, India) in centrifuge tube (Labcon, Petaluma, California, USA) and incubated in an orbital incubator (Scigenics Biootech, Neelankarai, Chennai, India) (120 rpm) at 37 °C under aerobic conditions for 6 hours to enrich the bacterial cells before plating. The enriched samples were then serially diluted and plated in triplicates on BHI agar plates. Plates were incubated for 12 hours, and the colony forming units (CFUs) was determined. The results were statistically analyzed using the One-way ANOVA and Duncan multiple range test.

EVALUATION OF SMEAR LAYER REMOVAL EFFICIENCY

PREPARATION OF THE SPECIMENS

Thirty extracted human single rooted teeth were selected. Teeth were cleaned with 2.5% NaOCl (Vista Dental, USA) and stored in 0.2% sodium azide (Sigma Chemical, St. Louis, MO) at 4 °C until the experiment. Radiographs were taken in buccolingual and mesiodistal directions, using periapical films to confirm the presence of a single canal and absence of anatomical variations. Teeth were decoronated and length of the roots were standardized to 15 mm. Apical patency was established using #10 K file (Mani Inc- Tochigi Ken; Japan). The working length was established by inserting a No.10K file (Mani Inc- Tochigi Ken; Japan) into each root canal until it was just visible at apical foramen (observed under magnifying loupes) and by subtracting 1mm from this point. Canals were then prepared to F3 size using Protaper instruments (Dentsply; Ballaigues, Switzerland). Irrigation was performed with 2.5% NaOCl between each instrument change to remove the organic part of the smear layer formed.

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IRRIGATION TECHNIQUE

The specimens were then randomly divided into 3 groups (n=10) based on irrigation regimen.

Group I: EDTA group: Each specimen was treated with 5 ml of 17% EDTA for 1 minute.

Group II: HybenX group: Each specimen was treated with 5 ml of HybenX for 1 minute.

Group III: Control group: Each specimen was treated with 5 ml of saline for 1 minute.

All irrigating solutions were introduced into the canal using 28 gauge needle (Vista Dental Products, USA) which was placed 1-2 mm short of working length. The root canals were finally irrigated with 5 mL of distilled water to remove any precipitate that might have been formed. The canals were then dried with sterile paper points (Dentsply, Ballaigues, Switzerland) and longitudinal grooves were prepared on buccal and lingual surfaces of each root by using a diamond disc (Horico Dental, Germany) at a slow speed without penetrating the canal. The roots were then split into two halves using a straight chisel. Then each half of a specimen in each respective group was subjected to SEM analysis to evaluate for the presence or absence of smear layer.

SEM ANALYSIS

The specimens were dehydrated using ascending grades of ethyl alcohol (25%, 50%, 75% and 100%) for 15 min. Samples were then mounted on metallic stubs, gold sputtered using an ion sputter, and examined under SEM. Photomicrographs were taken to observe the surface morphology at 1,000× magnification and 10 KV of the canal walls at the coronal (10-12 mm from apex), middle (6-7 mm from apex), and apical (1-2 mm from apex) thirds of each specimen. Two independent evaluators who were unaware of the experimental groups evaluated the images obtained. The images were scored according to the Torabinejad criteria [17]: 1 = no smear layer (no smear layer on the surface of the root canal; all tubules were clean and open); 2 = moderate smear layer (no smear layer on the surface of the root canal, but tubules contained debris); and 3 = heavy smear layer (smear layer covered the root canal surface and the tubules). The procedures related to teeth preparation and smear layer evaluation were performed by a single operator who was specialized in endodontic procedures. The microbial analysis was conducted by an experienced microbiologist.

STATISTICAL ANALYSIS

For the antimicrobial activity, group comparison was performed using Kruskal Wallis test and inter group comparison was done using Mann Whitney U test. In smear layer removal efficacy, the inter-examiner's reliability was verified by using the kappa test. The data of the scores for the presence or absence for smear layer was statistically analyzed using Pearson Chi square test. The analysis was undertaken using SPSS software, version 20 (SPSS, IL, USA) with the significance level preset at $\alpha = 0.05$.

RESULTS

ASSESSMENT OF ANTIMICROBIAL ACTIVITY OF HYBENX

The SEM observation revealed colonization of *E. faecalis* on root canal dentine surface. The mean CFU levels in infected root canals after treatment with experimental irrigants is demonstrated in (*Figure 1*). All the test irrigants used in this study showed a significant reduction in CFUs when compared to control group (distilled water) ($P < .001$). There was no statistical significant difference between CHX and HybenX ($P = .317$); CHX and NaOCl ($P = .146$) and HybenX and NaOCl ($P = .542$) in reducing *E. faecalis* populations.

ASSESSMENT OF SMEAR LAYER REMOVAL EFFICACY OF HYBENX

Kappa test results showed that there was no statistically significant difference between the two examiners values for scoring the smear layer in coronal (0.40), middle (0.25) and apical (0.08) thirds for both HybenX and 17% EDTA groups. The comparative percentage of smear layer removal by test irrigants at various thirds of root canal is demonstrated in (*Figure 2*). There was no significant difference between HybenX and 17% EDTA group in removal of smear layer in coronal and middle thirds ($P > .05$). However, in apical third of the root canal system, 17% EDTA (*Figure 3*) removed smear layer more effectively than HybenX ($P = .014$). In HybenX treated group, the specimens had moderate amount of smear layer present in apical third (*Figure 4*). In the control (saline) group, all specimens were heavily smeared in coronal, middle and apical thirds of root canal system.

DISCUSSION

This study evaluated for the antimicrobial efficacy of a novel root canal disinfectant. The hypothesis of this study was rejected since HybenX had antimicrobial efficacy against *E. faecalis* biofilm and also removed the smear layer from root canal system. The endodontic infections are invariably polymicrobial in nature consisting of *Streptococci*, *E. faecalis*, *F. nucleatum*, *Prevotella* species whose diversity may vary according to the type of infection.[30] The intracanal microbiota exists both as a planktonic and in biofilm forms.[31] The biofilm mode of growth allows bacteria to survive unfavorable environmental and nutritional conditions.[32] It has also been reported that, bacteria present in intraradicular biofilm is 100-1000 times more resistant to antimicrobial agents than their planktonic counterpart.[33] Hence, bacterial biofilm model was used in this study to assess the antimicrobial

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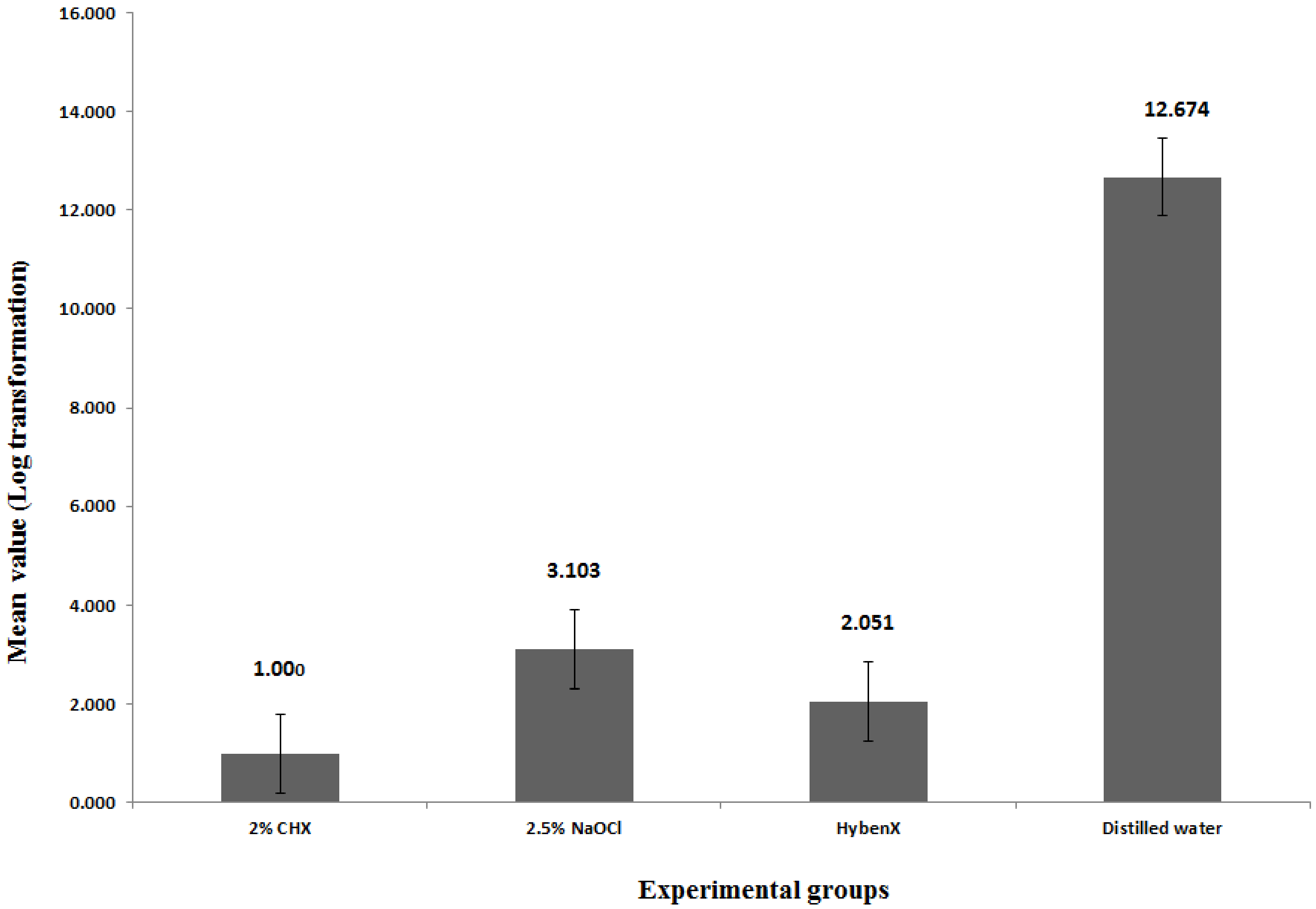

Figure 1: Mean CFU levels in infected root canals after treatment with 17% EDTA, HybenX and Saline.

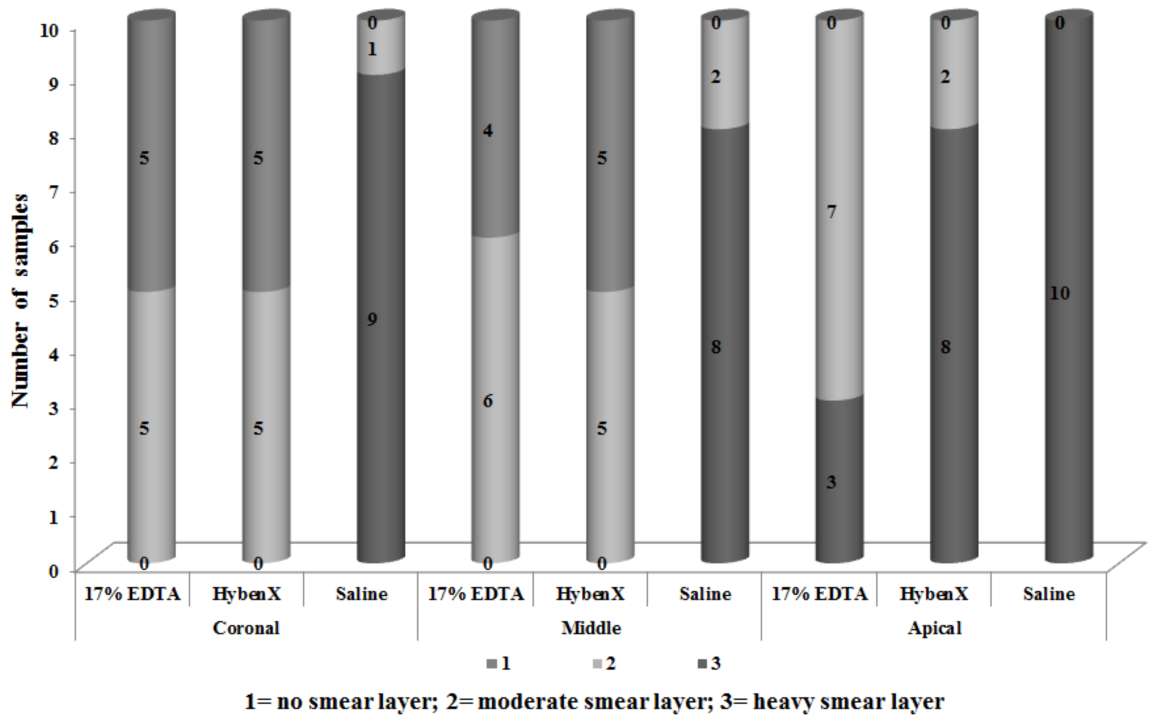

Figure 2: Number of samples with (1= no; 2= moderate and 3= heavy smear layer) among the test irrigants (17% EDTA, HybenX and Saline) at coronal, middle and apical thirds of the root canal system.

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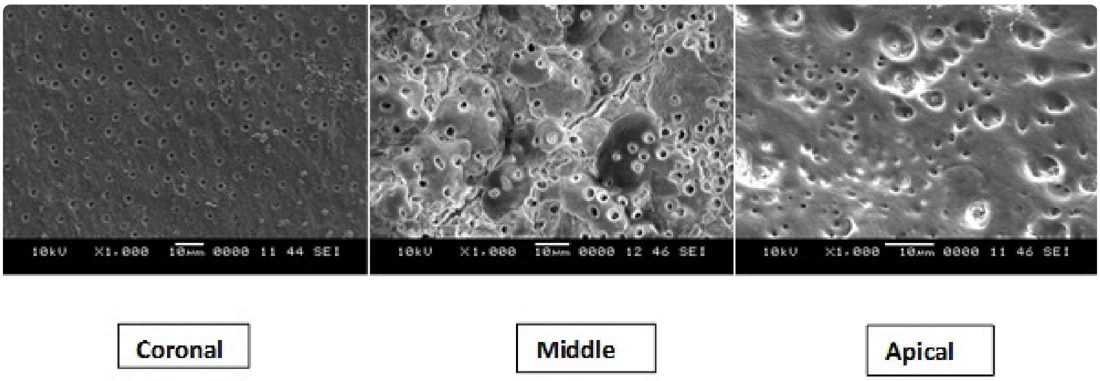

Figure 3a-c: Photomicrographs of root canal walls instrumented with 17% EDTA a) coronal b) middle and c) apical third.

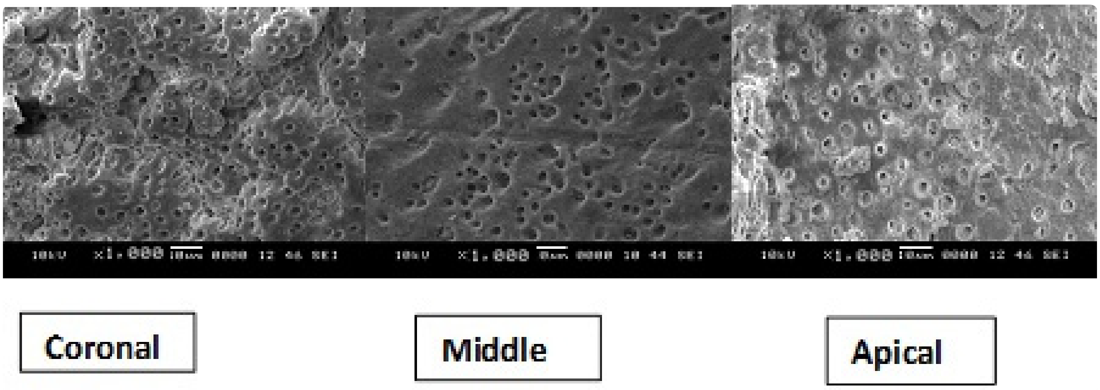

Figure 4a-c: Photomicrographs of root canal walls instrumented with HybenX a) coronal b) middle and c) apical third.

activity of the endodontic irrigating agents.[34] In the present study, HybenX demonstrated antimicrobial activity against *E. faecalis* biofilm, which was equivalent to CHX and NaOCl. This may be attributed to the "contact desiccation" process of HybenX due to the presence of concentrated liquid sulfates and sulfonates present in it.[35] The attraction between the water's positive hydrogen pole and sulfate negative oxygen surface that enables the sulfate molecule to act as a desiccant. Once it comes in contact with the biofilm, HybenX leads to denaturation and coagulation of bacteria present in the biofilm. Once the water from biofilm is removed, they tend to collapse and contract together.[36] This denaturing activity aggressively assists the mechanical action of HybenX during irrigation in removing the biofilm. The main advantage of HybenX over the other root canal disinfectant is remote chance of any microorganism being resistant to it. This is mainly due to its mechanism of action which is based on physical change to microenvironment. Another advantage is that, water-sulfate attraction in HybenX is such that, precipitates only pathogenic bacteria in the biofilm, and do not damage to the normal tissues of the oral cavity.[35] CHX and NaOCl in the present study showed similar antimicrobial activity in reducing CFUs which is in accordance with the previous studies.[37]

All the sample were subjected to same amount of BHI broth and incubation time so as to ascertain equal enrichment process to all sample representing a real situation. The enrichment process was included for two reasons; primarily, since the process of using a sterile round bur at a depth of 0.5 mm to collect dentine shaving collection are likely to attribute to desiccation which may hamper the microbial cell viability, secondly direct CFU determination after vortexing the shavings in buffer or water alone would not address the recovery of cell wall deficient forms of bacterial cells (L forms) and thus need to be enriched for resuscitation followed by CFU enumeration.

Studies have reported the high reoccurrence of *E. faecalis* from failed root filled teeth.[4,38] It has shown to survive under extended periods of starved conditions.[39] Hence, this microorganism was chosen in the present study. The root canals in the present study were incubated for 21 days, as described by previous studies.[40] It has been proven that, *E. faecalis* has the ability to invade the dentinal tubules after 21 days of incubation.[40]

The results of smear layer evaluation revealed that, HybenX was as effective as 17% EDTA in removal of the smear layer in the coronal and middle third of the root canal system. This may be attributed to the acidic nature of sulfuric acid present in the HybenX, which has got a demineralizing effect. However, in the apical third, the smear layer removal efficacy of

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HybenX was not as efficient as 17% EDTA. This could be attributed to the less pronounced action of HybenX on the sclerosed dentin present in the apical third of the root canal system.[41] Saline, which was used as an irrigant in control group, had no effect on the smear layer removal in all thirds of the root canal system. This is in accordance with the results of one other study.[42]

One minute interval for irrigation was used in the present study for smear layer removal. This is in accordance with various other studies.[43] Also, it has been reported that, EDTA when used for more than one minute causes erosion of the peritubular and intertubular dentin.[44]

Partial or total removal of the smear layer is essential for self-etch and etch&rinse adhesive strategies for bonding of the coronal filling upon coronal dentin or in case of post application upon intraradicular dentin. The bond established by the etch-and-rinse adhesive systems relies only on micromechanical retention between the demineralized dentin matrix and the polymerized adhesive system. The penetration of the adhesive into the funnelled dentin tubules creates the so-called resin tags, particularly extended when the adhesive is applied in endodontically treated teeth since no adverse pulpal pressure is present. When self-etch adhesive systems are used, the smear layer is only partially demineralized, depending on the pH and pKa of the etching acidic solution. In relation to the etching ability of the adhesives, self-etch systems are classified as mild, intermediate and strong.[45] Strong self-etch adhesives are able to completely dissolve the smear layer similar to the etch-and-rinse strategy,[46] while intermediate to mild systems modify the smear layer and demineralize the dentin matrix leaving residual hydroxyapatite crystals on the collagen fibrils providing additional chemical bond with adhesive monomer,[47] that cannot be obtained with etch-and-rinse adhesives since they fully demineralize the collagen.

The limitations of this study include the type of teeth used (single rooted teeth) and the culturing technique employed for microbiologic assessment. Further, in *ex-vivo* studies using multispecies biofilm should be performed in teeth with more complex root canal anatomy using molecular diagnostic techniques to validate the use of HybenX in root canal therapy.

CONCLUSIONS

From this study, the following could be concluded:

1. HybenX is efficient in reducing *Enterococcus faecalis* biofilm.
2. HybenX had also good smear layer removal efficiency from root canal system and therefore, it can be considered as a promising irrigating agent in infected root canals.

DISCLOSURE

The authors declare that they have no conflict of interest.

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