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Original Article

Dentistry Section

The Effect of Addition of an EPS Degrading Enzyme with and without Detergent to 2% Chlorhexidine on Disruption of *Enterococcus faecalis* Biofilm: A Confocal Laser Scanning Microscopic Study

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ABSTRACT

Background: *Enterococcus faecalis* is one of the most commonly occurring organisms retrieved from root canal treated teeth that show refractory apical periodontitis. Though it is well known that the ability of *E. faecalis* to form a matrix-encased biofilm contributes to its pathogenicity, the role of extracellular dextran and DNA in biofilm formation and its effect on the susceptibility of the biofilm to chlorhexidine remains poorly understood. It was hypothesized that the addition of an Extracellular Polymeric Substance (EPS) degrading enzyme along with a detergent to chlorhexidine may increase the susceptibility of the *E. faecalis* biofilm.

Aim: To evaluate the sensitivity of *Enterococcus faecalis* biofilms treated with DNase enzyme and their susceptibility to 2% chlorhexidine used alone or in conjunction with a detergent in a dentin disinfection model and examine under confocal laser scanning microscopy (CLSM).

Materials and Methods: Semi cylindrical shaped dentin specimens were infected with *E. faecalis* and incubated for 24 hours. Following incubation, the infected dentin specimens were exposed for 3 minutes to the four disinfecting solutions and grouped accordingly. {Group I- Sterile saline, Group II- 2% Chlorhexidine (CHX), Group III– Dnase1 Enzyme + 2% CHX, Group IV- DNase1 Enzyme + 2% CHX & Tween 80. Bacterial viability was then assessed by staining the specimens and examining under CLSM to analyse the proportion of dead and live bacteria within the dentinal tubules.

Results: The Groups II, III and IV showed statistically significant ($p<0.05$) percentage of dead bacteria compared to the control (Group I). However there was no significant difference in the killing effectiveness within the experimental groups (II-IV) at ($p<0.05$).

Conclusion: EPS degrading enzyme (DNase I) disrupts the biofilm and increases the susceptibility of *E.faecalis* when exposed to 2% Chlorhexidine and the use of a surfactant with this combination significantly contributes to improving the antibacterial efficacy.

Keywords: Bacterial viability, Confocal laser scanning microscope, Dentinal tubules, DNase1, Tween 80

INTRODUCTION

Enterococcus faecalis is an anaerobic gram-positive bacteria, has ability to survive under harsh conditions and can penetrate into the dentinal tubules [1,2]. Pinheiro et al., used RT-qPCR assay and identified 77.8% of *E. faecalis* from teeth with persistent/secondary intraradicular infections [3]. Zhang et al., proved that *E. faecalis* was highly related to persistent intraradicular infections compared with untreated chronic periapical periodontitis [4].

Biofilms represent bacterial colonies enveloped within an extracellular matrix composed of polysaccharides, proteins, and nucleic acids. The bacteria growing in this environment have advantages such as: i) cell – cell communication which helps in survival and virulence; ii) genetic exchange between cells, which include genes encoding virulence and bacterial resistance against antibiotics; and iii) protection from environmental stress and host defence [5].Thus *E. faecalis* biofilms pose a severe challenge to eradication during root canal treatment procedures.

Thomas et al., provided primary evidence for the pivotal role of extracellular DNA (eDNA) in *E. faecalis* biofilms. eDNA is a quintessential component of the extracellular polymeric substance of bacterial biofilms, providing structural stability and resistance against antimicrobial agents [6]. Li et al., had evaluated the effect of dextran-degrading enzyme DNase I on *E. faecalis* biofilm and found that it inhibited biofilm formation, decreased its adhesion to dentin and increased its susceptibility to 2% Chlorhexidine [7]. The EPS degrading enzyme could possess the ability to act as biofilm degrading agent, which in turn could improve the ability of the antibacterial agent to act effectively on the *E. faecalis* biofilm [7].

CHX is a broad-spectrum antimicrobial agent and effective against both gram-positive and gram-negative organisms [8,9]. It possesses the property of substantivity [10]. Substantivity is defined as the prolonged adherence of CHX to oral surfaces, in this case the dentinal tubules and root canal walls, and its slow release at effective doses that guarantees the persistence of its antimicrobial activity. Recently Ma et al., proved that chlorhexidine might be more effective in improving the antibacterial activities of alkaline root canal medicaments against *E. faecalis* when compared to NaOCl [11]. The essential function of any irrigating solution in endodontic therapy is to eliminate the infecting organism from the root canal system [12]. The addition of surface active agents to the disinfectants helps to reduce the surface tension and increase the wettability of the solutions, thereby increasing their efficacy [13-16]. Irrigants should contact with the dentin walls by physicochemical property called wettability [17]. Wang et al., proved that adding detergent to disinfecting agents would increase its antibacterial efficacy [18].

A new non-invasive dentin disinfection model was proposed by Ma et al., in 2011 in an in-vitro study to establish bacterial presence within the dentinal tubules [19]. In comparison to bacterial invasion models based on culturing techniques that cannot be replicated in the clinical setup [20], this new model provided penetration of bacteria deep into the dentinal tubules by centrifugation and has

been implemented in our study to standardize the methodology. The power of centrifugation forces allows for a strong bacterial invasion into the dentin and allows for standardized measurements of live/dead bacteria when used with the CLSM to measure the efficacy of the disinfecting solutions. Confocal Laser Scanning Microscopy (CLSM) presents two distinct advantages over any other microscopic system in its ability to control the depth of field with elimination or reduction of background information from the focal plane thereby avoiding image degradation *and* its potential to obtain serial optical sections from thick specimens [21]. CLSM helps in visualizing the live and dead bacteria, and has the ability to penetrate 10 µm below the surface of the specimen. Del Carpio-Perochena A et al., used CLSM to measure the cell viability and bacterial volume on polymicrobial biofilms [22].

Hence the aim of this study was to evaluate the susceptibility of *E.faecalis* biofilms to 2% chlorhexidine that was treated with DNase enzyme, and used alone or in conjunction with a detergent in a dentin disinfection model and examine under Confocal Laser Scanning Microscopy (CLSM).

MATERIALS AND METHODS

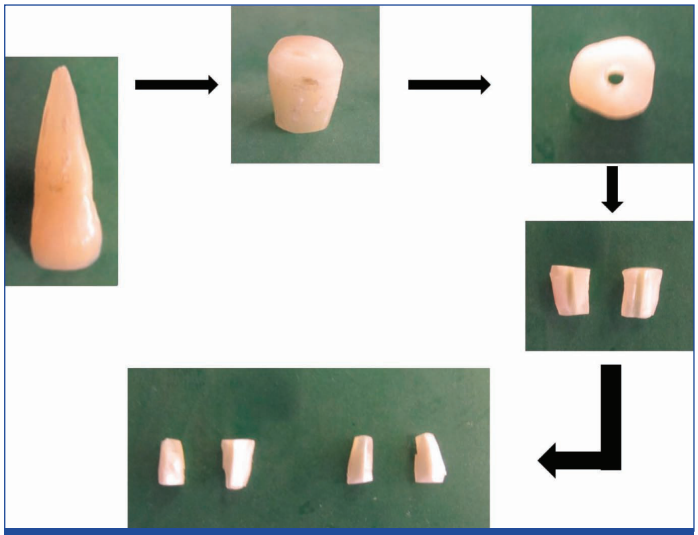
Specimen Preparation

This study was carried out jointly at the Department of Conservative dentistry and Endodontics and the Central research facility of Sri Ramachandra University in the year 2012 after obtaining ethical clearance from the institutional review board. Eight extracted caries free single rooted teeth were used in this study. The teeth were stored in 0.01% sodium hypochlorite solution before use in order to clear them off the soft tissue debris. Based on a previously mentioned protocol [19], a root dentin block, with a length of 4 mm was horizontally sectioned from each tooth at 1 mm below the cementoenamel junction by a 0.6-mm-thick precision diamond saw (Isomet 5000; Buehler Ltd, LakeBluff, IL) at 1000 rpm under water cooling. Each cylindrical dentin block was fractured by first making a thin groove in the middle of the specimen by using low speed handpiece with a small round bur (Tulsa Dentsply) and then fracturing the specimen with a blade and a hammer into 2 semi cylindrical halves. Root canals were enlarged to 1.5 mm with a Gates Glidden drill (Mani Inc, Japan) at 300 rpm under water cooling. Sixteen semi cylindrical halves were thus vertically fractured from the 8 dentin blocks, followed by final shaping and refining of the specimens to obtain a specimen size measuring 4x4x2 mm. The outer surfaces of the 16 semi cylindrical halves were ground by 600-grit silicon carbide paper (Carbine, Buehler Ltd) to achieve a standard thickness of 2 mm and to remove the root surface cementum. The dentin specimens were then shaped by a water-cooled low-speed handpiece with a fine carbide bur (Tulsa Dentsply) at 300 rpm to make the specimen fit the inner wall of a filter tube with 0.45-mm pore size (Pall Corporation, Ann Arbor, MI).

The smear layer on both sides of the specimen was removed by immersion in 5.25% NaOCl (Prime Dental Products, Mumbai, India) and 17% EDTA (Prime Dental Products, Mumbai, India) individually for 4 minutes. The specimens were then rinsed in sterile water for 1 minute. The dentin specimens, following preparation, were placed canal sides up at the bottom of the upper chamber of a microfiltration tube (Pall Corporation, Ann Arbor, MI) and the space between the dentin block and inner tube was sealed by a composite filling material (Synergy D6, Coltene Whaledent) and light cured for 20 seconds [Table/Fig-1].

Dentin Infection

E. faecalis (American Type Culture Collection 29212), used as the test organism was grown at 37ºC on brain-heart infusion (BHI) agar plates overnight. The organism was standardized spectrophotometrically to McFarlands scale of 0.5 to form $3x10^6$ colony-forming units/mL.



[Table/Fig-1]: Specimen preparation and disinfection

The *E.faecalis* suspension was further sequentially centrifuged into the dentinal tubules. The infected dentin specimens within the tubes were incubated at 37ºC in brain-heart infusion broth in air for 24 hours to allow bacterial recovery following centrifugation [19].

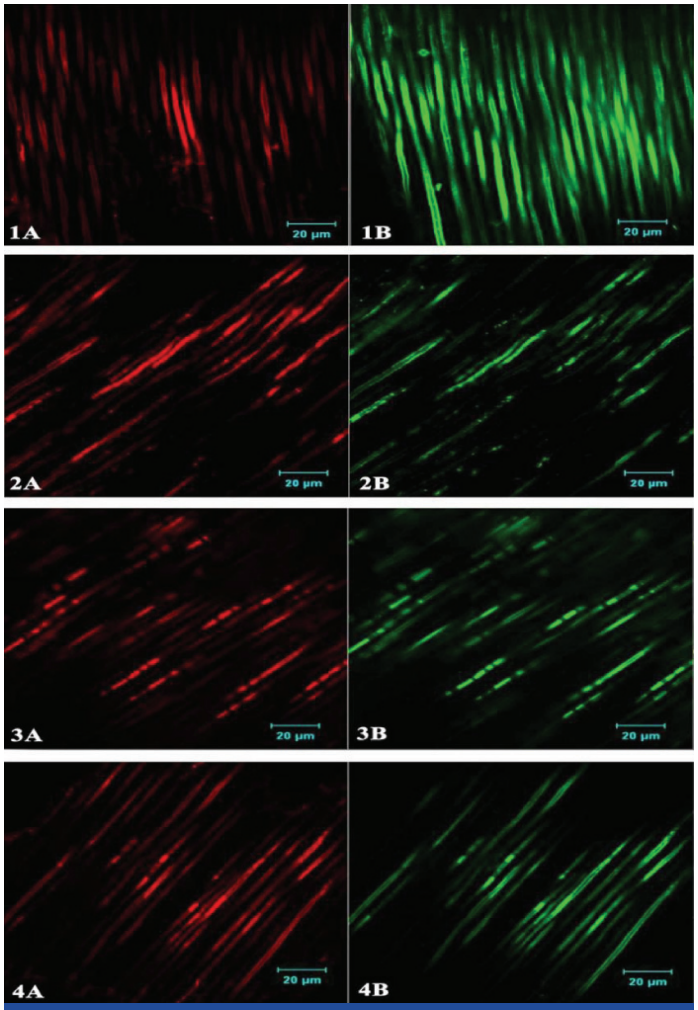
Dentin Disinfection

The dentin specimens were removed from the tubes and rinsed in phosphate buffered saline (PBS) for 1minute to remove the broth followed by air-drying. The outer cemental surfaces were coated (2 coats) with clear nail varnish (Lakme). The 16 dentin blocks were randomly assigned into 4groups with 4 specimens in each: Group I-Sterile Saline (Control), GroupII-2% Chlorhexidine (CHX) (ASEP RC, Anabond-Stedman, India), Group III-DNase1 enzyme (Sigma, Activity of 2,200 Kunizunits/mg) + 2% CHX, Group IV- DNase1 Enzyme + {(2% CHX with Tween 80 ("polyoxyethylenesorbitanmonooleate") in a ratio of 1:1}. Tween 80 is a detergent and is a nonionic surfactant that helps in reducing the surface tension. A 50µL of each of the disinfecting solutions were placed on the root canal side of each dentin specimens for 3 minutes. In groups III & IV, the dentin specimens were incubated for 10 minutes in 1mL BHI broth containing 100 µg/mL of DNase1, following which the disinfecting solutions were applied. The specimens were then rinsed thoroughly in PBS buffer for 1 minute and fractured vertically through the root canal into 2 halves there by exposing a fresh longitudinally fractured dentin wall for CLSM examination [19].

CLSM Examination

The Confocal microscopy was performed using a LSM710 Metalaser scanning confocal microscope (Carl Zeiss) having a 63x oil immersion objective. The images were acquired and analysed using the AIM software (Carl Zeiss 4.2 version SP.1). For viewing under the confocal microscope, the washed dentin specimens were stained in the dark at room temperature with Fluorescein Diacetate (Sigma) (green fluorescence) and Propidium iodide (Sigma) (red fluorescence) to differentiate the live and dead cells in the bacterial biofilm. The specimens were washed twice with PBS buffer to remove the excess dye. The specimens were then transferred to glass cover slips, and covered with immersion oil before image acquisition. Confocal illumination was performed using an argon laser with excitation wavelength of 543nm for red and 488 nm for green fluorescence. Simultaneous dual-channel imaging mode was used to display both green and red fluorescence. For each group 8 dentin blocks were used for image analysis and each sample was scanned in 5 areas. The AIM software was used to acquire and analyse the images by removing background fluorescence and quantifying the live/dead ratios of the cells within the infected dentinal tubules. The volume of (red to

[Table/Fig-2]: Confocal armamentarium and bacterial stains used in the study



[Table/Fig-3]: Figures from 1-4 represent the 4 groups- Group I: Sterile Saline, Group II: 2% CHX, Group III: 2% CHX+DNase I, Group IV: 2% CHX+Tween 80+DNase I. A represents dead bacteria and B represents live bacteria

green) fluorescence to red fluorescence indicated the proportion of killed cells for each disinfecting solution [Table/Fig-2].

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS software (SPSS for Windows version 16, SPSS Inc, Chicago, IL). The acquired images from CLSM were stacked and analysed using one-way ANOVA and multiple comparisons using Turkey HSD test. A $p<0.05$ was considered to be statistical significant.

RESULTS

An even penetration of *E.faecalis* extending deep into the dentinal tubules of the specimens was observed by CLSM. The control group, (Group1), treated with sterile saline, showed maximum presence of live bacteria within the dentinal tubules. The Groups II, III and IV showed statistically significant ($p<0.05$) percentage of killing effectiveness compared to the control (GroupI), with no significant difference in the killing effectiveness within the experimental groups (II-IV) although group III showed higher percentage of dead bacteria compared to groups II& IV after an exposure time of 3 minutes [Table/Fig-3,4].

Groups	Mean & Standard Deviation (Absolute Fluorescence)		Percentage (Killing Effectiveness)	
	Live	Dead	Live	Dead
Sterile Saline	8913.63±1930.890	676.38±197.414	92.71%	6.07%
2% CHX	3060.50±881.169	5385.25±2324.059	38.86%	61.1%
2%CHX + DNase I	2000.75±849.904	6584.88±2091.893	24.08%	75.85%
2%CHX + Tween 80+DNase I	1533.63±702.296	3858.63±733.964	27.39%	73.50%

[Table/Fig-4]: Percentage of killing effectiveness of *E. faecalis* cell volume in a dentin disinfection model treated with disinfectant solution

DISCUSSION

Enterococci are gram-positive cocci and facultative anaerobes that can occur either in glyorin pairs, or even as short chains [23]. *E.faecalis* has been associated with different forms of periradicular disease which ranges from primary endodontic infections to persistent periradicular infections [24]. *E. faecalis* possess a number of virulence factors like lytic enzymes, pheromones, aggregation substance, cytolysin, and lipoteichoic acid [25]. These enable them to survive very challenging environments by resisting all the antimicrobial measures that are used such as irrigants and intracanal medicaments. In addition *E.faecalis* possess the ability to suppress the action of lymphocytes, thereby contributing to endodontic failure [26]. The presence of serineprotease, gelatinase, and collagen-binding protein (Ace) provides the ability to bind and adhere to dentin [27]. However, the most important factor of *E.faecalis* is its ability to form a biofilm that prevents it from degradation by enabling the bacterial colony to become 1000 times more resistant to antibodies, phagocytosis, and antimicrobial agents compared to planktonic bacteria [1,28].

The extracellular matrix of biofilms is composed of proteins, carbohydrates and nucleic acids [5,29]. It has been thought that the presence of extracellular polymeric substances (EPS's) in the bacterial biofilm imparts the bacterial colony resistance against immune system and antibacterial agents [30,31]. The EPS matrix provides the biofilm with mechanical stability and a cohesive, interconnected

3D polymer network. Also, the biofilm matrix serves as an external digestive system by trapping the extracellular enzymes close to the cells which enables them to metabolize dissolved, colloidal and solid biopolymers [31]. In recent years, dextran and eDNA have been identified in the matrix of *E.faecalis* biofilms suggesting their role in the development of bacterial communities. Dextran belongs to a class of extracellularly formed glucans that is produced by bacteria while eDNA in biofilms is presumably derived from cell lysis [6,32].

From the results of our study it was seen that the dentin biofilms irrigated with 2% chlorhexidine have shown the least percentage of killing effectiveness. In the field of endodontics, CHX has been used as an irrigant with proven substantivity because of its ability to adhere to hydroxyapatite of dentin [33,34] and its effectiveness against endodontic pathogens. However, biofilms within root canals are resistant to the antibacterial effect of CHX. Hence in this study chlorhexidine when used alone has performed poorly. The reason for the superior disinfection of Group III and IV could be attributed to the ability of the DNase1 enzyme to disrupt the bacterial biofilm making it susceptible to the antibacterial effect of 2% chlorhexidine. Thus in the presence of DNase1, it may have been possible for CHX to act more directly on the bacterial biofilm, thereby increasing its bactericidal potential.

Wettability of the disinfecting solutions used during irrigation has also been shown to play a major role in facilitating their penetration into the intricacies of the root canal system [35]. It has been defined as the force between molecules that tends to reduce the surface area of a liquid [36]. This decrease in the surface tension which is achieved by the addition of chemical compounds known as detergents [37] which can disintegrate the cohesive forces and promote the destruction of EPS matrix and bacterial cell membrane [38]. Detergents such as Cetrimide and Tween 80 have been employed in endodontic irrigants such as CHX-Plus and MTAD respectively to decrease their surface tension [39]. Previous studies have revealed that addition of detergents with inherent antibacterial activity to the disinfecting solutions have significantly increased their antibacterial effect on dentin biofilms [18]. The results of our study have also shown a marginal increase in the antibacterial efficacy in the surfactant added group (Group IV) compared with Group III (2% chlorhexidine + DNase1 enzyme) though not statistically significant. The surfactant (Tween 80) used in the present study did not possess any inherent antibacterial properties but still has shown improved antibacterial activity when added to 2% chlorhexidine. The addition of DNase1 enzyme which disrupts the bacterial biofilm has made it susceptible to the antibacterial effect of 2% chlorhexidine. In addition the use of a surfactant along with the DNase I enzyme has enabled deeper penetration of 2% chlorhexidine into the dentinal tubules by lowering the surface tension and increasing the wettability of the chlorhexidine. Hence the superior performance of the experimental groups can be explained. The results of our study have thus shown a definite increase in antibacterial activity with the use of both DNase1enzyme and the surfactant along with 2% Chlorhexidine.

CONCLUSION

Within the limitations of our study, that include the use of only one single strain of *E. faecalis* biofilm and the inherent difficulty of extrapolating the results obtained by an in-vitro investigation, to an invivo clinical setup, it can be concluded that, the use of an EPS degrading enzyme (DNase I) disrupts the biofilm and increases the susceptibility of *E. faecalis* when exposed to 2% chlorhexidine and the use of a surfactant with this combination significantly contributes to improving the antibacterial efficacy.

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