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Original Article

Antibiofilm efficacy of root-end filling materials against *Enterococcus faecalis* - An *in vitro* study

Geeta Hiremath, Madhu Singh, Bhushan Kulkarni[1], Rajeev Potdar[1], Balaram Naik

Department of Conservative Dentistry and Endodontics, SDM College of Dental Sciences and Hospital, Constituent Unit of Shri Dharmasthala Manjunatheshwara University, Dharwad, [1]Department of Biotechnology, P C Jabins Science College, Hubli, Karnataka, India

ABSTRACT

Aim of the Study: This study aims to evaluate the antibiofilm activity of root end materials against *Enterococcus faecalis*.

Materials and Methods: Mineral trioxide aggregate (MTA), MTA plus and Biodentine were conjugated with chitosan gel and tested against the 3-day biofilm of *E. faecalis*. The incubated plates were stained using crystal violet stain and the optical density of adherent stained biofilm was read at 590 nm using ELISA auto reader.

Results: There was a mean clinical reduction in the biofilms of the conjugates as compared to their individual counter parts. There was a statistically significant difference seen between the groups (MTA Plus – Chitosan Conjugate) and (MTA – Chitosan Conjugate) with $P = 0.0495$.

Conclusion: The conjugates did perform better in inhibiting the biofilm activity of *E. faecalis*. Although all conjugates formed were not statistically significant.

Keywords: Biofilm, chitosan conjugate, root-end materials

INTRODUCTION

Biofilm is a complex structure adhering to surfaces that are regularly in contact with water, consisting of colonies of bacteria and usually other microorganisms such as yeasts, fungi, and protozoa that secrete a mucilaginous protective coating in which they are encased. Donlan and Costerton in 2002 defined biofilm as "A microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other are embedded in a matrix of extra poly saccharide (EPS) that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription."

Endodontic biofilm is established to be less diverse compared to the oral biofilm. The progression of infection alters the nutritional and environmental status within the root canal. The root canal environment apparently becomes more anaerobic and the nutritional level will be depleted. These changes will offer a tough ecological niche for the surviving microorganisms.[1]

Intracanal microbial biofilms are formed on the root canal dentine of an endodontically infected tooth. Nair documented a detailed description on the intracanal bacterial biofilm in 1987. He suggested that the intracanal microbiota in an endodontically infected teeth existed as both loose collection and biofilm structures, made up of cocci, rods, and filamentous bacteria. Intracanal biofilms displayed characteristic bacteria-dentine wall relationship and distinct patterns in the organization of microbes in the biofilm.[2]

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Address for correspondence: Dr. Geeta Hiremath, Department of Conservative Dentistry and Endodontics, SDM College of Dental Sciences and Hospital, Constituent Unit of Shri Dharmasthala University, Dharwad, Karnataka, India.
E-mail: geethahiremath7@yahoo.co.in

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Enterococcus faecalis are Gram-positive cocci, facultative anaerobes and are commonly detected in asymptomatic, persistent endodontic infections. Its prevalence in such infections ranges from 24% to 77%. Studies have established the ability of *E. faecalis* to resist starvation and develop biofilms under nutrient-deprived conditions. Biofilms formed by *E. faecalis* are able to resist destruction by enabling the bacteria to become 1000 times more resistant to phagocytosis, antibodies, and antimicrobials than nonbiofilm producing bacteria. Root canal failures are likely to occur due to the persistence of bacteria especially *E. faecalis* and their by-products in root canal system. This bacterium appears to be highly resistant to the anti-bacterial effect of $Ca(OH)_2$. Evans *et al*. reported that *E. faecalis* was resistant to $Ca(OH)_2$ at a pH of 11.1 but was unable to survive at a pH >11.5.[3]

Root canal failure when treated surgically comprises mainly the removal of periapical pathologies leading to regeneration of healthy and functional periodontal tissues. Root end filling procedure is a step, which is performed in these surgeries to prevent the invasion of irritants from infected root canals into the periapical tissues. In addition to improving the sealing of the existing root canal filling, these materials should possess antimicrobial properties to prevent the movement of bacteria and their products from the root canal system to the periapical tissues for the success of the endodontic surgeries.

A number of biomaterials have been developed by various manufacturers for use as a root-end filling material such as mineral trioxide aggregate (MTA) and Biodentine. MTA was first introduced in 1990s has been proven to be a superior material regarding with its excellent sealing ability, biocompatibility and hard tissue forming capacity. It also possesses ideal properties such as its antimicrobial effect, dimensional stability, radiopacity, and tolerance to moisture. Although MTA is considered to have ideal properties, it has disadvantage of having longer setting time, difficulty in handling and high-cost. These shortcomings of MTA led to the continuous efforts in developing the newer materials such as MTA Plus and Biodentine.

A widely used material due to its biodegradable, nontoxic, nonantigenic, and biocompatible properties is a biopolymer isolated from shellfish, crab and shrimp, called chitosan, which is reported to exhibit numerous health-related beneficial effects, including strong antimicrobial property. Chitosan also can accelerate the wound healing, inhibit bacteria growth, and alleviate pain and can be used as vehicle. The inherent capacity of *E. faecalis* to resist the bactericidal action of many antimicrobial agents, along with its ability to form distinct biofilm under tough environmental and nutrient conditions over root-end filling materials, made the basis for this study.

Hence, the aim of this study is to evaluate and compare efficacy of MTA, MTA Plus, Biodentine, Chitosan, and their conjugates on *E. faecalis* biofilm.

MATERIALS AND METHODS

The sample size was calculated to be 10 in each group with an alpha error 5% and 80% power of test.

The materials used in this study were MTA (MTA Angelus), MTA Plus (Prevest Denpro, India), Biodentine (Septodont), and Chitosan (Sigma Aldrich, India).

Bacteria

Gram-positive bacterium tested-*E. faecalis* ATCC 29212 (American Type Culture Collection strain) was used in the study.

Growing the biofilm

A 3-day biofilm was generated in a 96 well microtiter plate. Biofilm was grown at 37°C (2 ml Brain Heart Infusion Broth [BHI] containing 0.5% sucrose), and media was changed every 24 h. At the end of the 3rd day, each disc was rinsed with phosphate-buffered saline (PBS) to remove loosely attached bacteria and planktonic bacteria. 200 µl of overnight trypticase soy broth culture was added to wells and incubated at 37°C for 24 h.

Preparation of materials

For Group 1, 2, 3, the materials (MTA, MTA Plus, and Biodentine) were mixed according to manufacturer's instructions. For Group 5, 6, 7 conjugates were formed with each material by mixing with chitosan in 1:1 ratio.

A serial two-fold dilutions of the combinations were prepared in PBS (PBS; 10 mM Na2HPO4, 154 mM NaCl, pH = 7.4) and incubated for 2 h at room temperature (RT), to check for minimal inhibitory concentration (MIC) of the materials.

Exposing the materials to biofilm

After 3 days of incubation, wells were aspirated and 100 µl of material and their conjugates were added and incubated at 37°C for 24 h. Each sample was added in triplicate and un-inoculated broth was used as control.

Staining the biofilm

After incubation, the plates were washed with sterile PBS, air dried and stained with 125 µL of 0.1% solution of crystal violet stain. After staining microtiter plate was incubated at RT for 10–15 min and excess stain was removed with distilled water.

Quantifying the biofilm

Plates were dried and 125 μL of 30% acetic acid was added to each well of the microtiter plate to solubilize the crystal violet stain. Optical density of adherent stained biofilm was read at 590 nm using ELISA auto reader.

The readings were recorded and analyzed statistically. Viable counts were transformed to their log10 values. Data was confirmed to be normally distributed using Kruskal–Wallis ANOVA followed by Mann–Whitney U-tests to evaluate whether the conjugates of MTA, MTA Plus, and Biodentine with Chitosan had greater antibiofilm properties than their individual counterparts by reading the optical density of adherent stained biofilm at 590 nm using ELISA auto reader.

RESULTS

E. faecalis was not recovered from any of the negative controls. There was no evidence of carryover of the antibacterial effect from the materials to the bacterial cultures. There was a mean clinical reduction in the biofilms of the conjugates as compared to their individual counter parts. Combining data for all groups, the mean (standard deviation) counts for MTA-Chitosan Conjugate (0.12 ± 0.01), MTA Plus-Chitosan Conjugate (0.18 ± 0.10), and Biodentine-Chitosan Conjugate (0.20 ± 0.10) were lower than the groups MTA (0.17 ± 0.09), MTA Plus (0.22 ± 0.09), Biodentine (0.28 ± 0.21). But when analyzed statistically, there was no significant difference seen ($P = 0.5930$) [Table 1].

Further, Pair wise comparison was done using Mann- Whitney U-test. There was a statistically significant difference seen between the groups (MTA Plus – Chitosan Conjugate) and (MTA – Chitosan Conjugate) with $P = 0.0495$ [Table 2].

DISCUSSION

Endodontic disease is a biofilm-mediated infection, and primary aim in the management of it is the elimination of bacterial biofilm from the root canal system. The microbial population is more conspicuous with the progression of infections. Furthermore, clinical investigations have shown that the complete disinfection of the root canal system is very difficult to achieve because microorganisms are found to persist in the root canal system complexities such as apical portions, deltas, isthmuses, and lateral canals. During biomechanical preparation, apical biofilm plays key rule clinically because they are inherently resistant to antimicrobial agents and cannot be removed by biomechanical preparation alone. This may cause failure of endodontic treatment because of persistent infection.[4]

Table 1: Comparison of seven groups with optical density scores by Kruskal-Wallis ANOVA

Groups	Mean	SD	SE	Mean rank
I	0.17	0.09	0.05	8.00
II	0.22	0.09	0.05	12.67
III	0.28	0.21	0.12	11.83
IV	0.28	0.18	0.11	13.00
V	0.12	0.01	0.01	5.83
VI	0.18	0.10	0.06	10.00
VII	0.20	0.10	0.06	10.67
H			4.6220	
P			0.5930	

SD: Standard deviation; SE: Standard error

Among different clinical bacterial isolates recovered from endodontic infections, *E. faecalis* is the common species that has been widely studied for its capacity to form biofilms.[5,6] *E. faecalis* is a Gram-positive, facultative anaerobic cocci that is strongly associated with endodontic infections. Being an opportunistic pathogen, it causes nosocomial infections and is frequently isolated from the failed root canals undergoing retreatment.[7,8] These virulence traits have also been identified in the clinical isolates of from asymptomatic, persistent endodontic infections of the root canals and the oral cavity.[9-11]

Endodontic repair materials are used for various procedures that include pulp capping, apexification, root-end fillings, and perforation repairs. Various repair materials have been introduced into the market. The most common material used is MTA. A potential disadvantage of MTA is the handling properties and longer setting time of 140 ± 2.6 min.[12] These shortcomings of MTA have led to the continuous efforts in developing the newer materials. In the present study, two newer materials MTA Plus (Prevest Denpro, Jammu, India) and a calcium silicate-based material Biodentine (Septodont) were used to evaluate their antibiofilm activity. The materials were mixed with chitosan gel. It has interested many researchers around the world, particularly in relation to its ability to be a delivery vehicle.[13] Therefore, in this study, the root end materials were mixed with chitosan gel and their conjugates were obtained.

Chitosan is also known as soluble chitin. It is a biopolymer isolated from shellfish, crab, and shrimp. It is a widely used material due to its biodegradable, nontoxic, nonantigenic, and biocompatible properties. It exhibits numerous health-related beneficial effects, including strong antimicrobial property, accelerates wound healing, inhibits bacterial growth, and alleviates pain.[14,15]

The most widely used in microbial assay is serial dilution of the extract in a number of test tubes followed by the

Table 2: Pair-wise comparisons of seven groups with optical density scores by Mann-Whitney *U*-test

Groups	I	II	III	IV	V	VI	VII
Mean±SD	0.17±0.09	0.22±0.09	0.28±0.21	0.28±0.18	0.12±0.01	0.27±0.10	0.20±0.10
I	-						
II	*P*=0.2752	-					
III	*P*=0.5127	*P*=0.8273	-				
IV	*P*=0.5127	*P*=0.8273	*P*=0.8273	-			
V	*P*=0.8273	*P*=0.1266	*P*=0.5127	*P*=0.2752	-		
VI	*P*=0.2752	*P*=0.5127	*P*=0.8273	*P*=0.8273	*P*=0.0495*	-	
VII	*P*=0.5127	*P*=0.8273	*P*=0.8273	*P*=0.5127	*P*=0.2752	*P*=0.2752	-

**P*<0.05 statistically significant. SD: Standard deviation

addition of the test organism to determine the MIC for the test organism using turbidity as an indication of growth. Although microtiter plate assay suffers from major drawback of biofilm detection on the bottom of well only, this assay is considered most frequently used method in biofilm quantification. The optical density measurement obtained from microtiter plate assay eliminates the subjectivity of tube test in interpreting the obtained results and predicts clinical relevance more reliably than tube test. Besides, microtiter plate assay is easy to conduct when only application of different dyes such as crystal violet used in this study, resazurin or dimethyl methylene blue enables the quantitative biofilm measurement.[16] In this study, scaling down of the serial dilution technique using of 96-well microplates to assay extracts was used for investigation.

The results of this *in vitro* investigation showed that the conjugates of MTA, MTA Plus, and Biodentine with Chitosan had better antibiofilm properties when compared to their individual counterparts. There was a statistically significant difference seen only between the groups (MTA Plus – Chitosan Conjugate) and (MTA – Chitosan Conjugate) with $P = 0.0495$. Other conjugates performed better than their counter parts but were not statistically significant. The reason might be; all the three materials possess their antimicrobial properties by release of calcium hydroxide ions produced from the tricalcium silicate hydration,[17] i.e., the pH value of the freshly mixed MTA is 10.2, which increases upto 12.5 after 3 h,[18] whereas pH of Biodentine and MTA Plus is 12. This pH level remains stable over time at a value of around 11–12 for all the three materials.[19] *E. faecalis* can survive in extreme alkaline environments up to pH of 11.1.[3] Perhaps the inherent, persistent alkalinity of these materials are just enough to overwhelm the *E. faecalis*. Mixing chitosan with these materials might decrease their pH, as the pH of chitosan is <6.[20] Although pH of materials was not measured in this study, it is feasible that the pH of the material during its setting reaction contributed to the antibacterial activity seen in the present study.

Within the limitations of the study, root end materials conjugated well with chitosan and performed better than the individual counter parts. Further studies need to be done in relation to its pH stability and cell adherence on their surface which would definitely be indicative of osteoinductive property of the material.

CONCLUSION

Within the limitations of the present study, all the materials proved to have antibiofilm action against *E. faecalis*. Chitosan can be used as a novel material in dentistry and its various properties can be used to enhance the properties of present materials. Only one bacterial strain was used in the study, suggesting that the use of more than one strain in antimicrobial assays is advisable. These findings opened new opportunities for the use of Chitosan alone or in combination to improve bioactivity of dental materials and beyond.

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Conflicts of interest

There are no conflicts of interest.

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