

Bacterial invasion of dentinal tubules from the external root surface with and without an intact cemental layer- a confocal laser scanning microscopic study

Abstract

Aim: To evaluate the invasion of two bacterial species; *Streptococcus sanguinis* (S.s) and *Aggregatibacter actinomycetemcomitans* (A.a) in radicular dentinal tubules with and without an intact cemental layer using confocal laser scanning microscopy.

Methodology: 10 intact freshly extracted human teeth were prepared and divided into 2 groups, Group 1: S.s (1a: S.s without cementum, 1b: S.s with intact cementum) and Group 2: A.a (2a: A.a without cementum, 2b: with intact cementum). The cemental layer on each root was removed from one side and kept intact on the other side. The specimens were incubated in the bacterial suspension for 15 days at 37°C. The specimens were sectioned, mounted, stained with a fluorescent dye and viewed under the Confocal Scanning Electron Microscope at 40X magnification. The penetration of bacteria and presence of live bacteria in dentinal tubules in all the groups was analyzed.

Results: In both the groups there was a significantly higher penetration of bacteria through the exposed dentin as compared to sections with intact cementum. However, there was a significantly higher penetration of *Streptococcus sanguinis* as compared to *Aggregatibacter actinomycetemcomitans*.

Conclusion: An intact cemental layer protects the dentin from invasion of bacterial species. These bacteria can lead to reinfection of treated periodontal pockets, pulpal inflammation or persistence of periapical infections. Therefore, management of endodontic-periodontic lesions and periodontal therapies should aim at preserving or regenerating a healthy cemental layer.

Keywords: bacterial invasion, confocal scanning electron microscope, cementum, radicular dentin

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Introduction

Periodontitis is a complex multifactorial inflammatory condition characterized by the destruction of connective tissue and alveolar bone support following an inflammatory host response secondary to infection by periodontal bacteria.^{1,2}

Streptococci are the primary bacterial colonizers of the oral cavity, and adhesion of streptococci to the acquired pellicle is an essential first step in colonization of the tooth surfaces.³

Periodontopathogenic bacteria are gram-negative, anaerobic or facultative organisms that exhibit various virulent factors. *Aggregatibacter actinomycetemcomitans* and organisms belonging to the 'red complex' that are *P. gingivalis*, *T. denticola*, *T. forsythia* are secondary colonisers and appear later in biofilm formation.⁴ *Aggregatibacter actinomycetemcomitans* is generally associated with localized aggressive periodontitis (LAP).⁵

Invasion of dentinal tubules by bacteria from supra or sub gingival plaque occurs whenever dentin is exposed in the oral cavity. This can be through carious lesions, restorative or periodontal procedures, tooth wear, enamel or dentin cracks, or dental trauma.⁶⁻⁹

Bacterial invasion of radicular dentin of periodontally diseased teeth

has been demonstrated by light microscopic¹⁰⁻¹² and microbiological studies.^{13,14} It has been suggested that the dentinal tubule microflora associated with a periodontal pocket could act as a reservoir for re-colonization of the pocket after debridement.^{13,14}

Numerous studies have been done to review bacterial invasion through dentinal tubules from the pulp space¹⁵⁻¹⁸ but there is less literature on bacterial penetration through cementum.

Cemental matrix is porous and in healthy situation only enables permeability of water and inorganic ions. But in diseased state plaque bacterial invasion has been detected in 10-12micron deep surface layer.¹⁹

Invasion of radicular dentinal tubules by pure cultures of *Streptococcus* has been demonstrated in various studies,¹⁷ but the invasion of gram negative anaerobic bacteria is less clear. While it is known that bacteria are able to invade radicular dentine from the periodontal pocket, a contentious issue whether bacteria invade healthy cementum or gain access to dentinal tubules only from breaches in the cemental layer still exists. The majority of species recovered from radicular dentin are Gram-positive bacteria, with lower numbers of Gram-negative organisms.¹⁴ The inability to detect fastidious anaerobes within the invading dentin may have been due to difficulties in cultivating these bacteria.

In this article we evaluated the penetration of two bacterial species, *Streptococcus sanguinis* and *Aggregatibacter actinomycetemcomitans* into the dentinal tubules with and without an intact cemental layer using a confocal laser scanning microscopy. A fluorescent dye was used to stain the nucleic acid and differentiate between live and dead bacteria.

Materials and methods

Ten intact freshly extracted single rooted human teeth were used for the study. Teeth were scaled and planed with the help of ultrasonic scaler. The cemental layer was removed from one surface and kept intact on the other surface. The teeth were decoronated with diamond disc at 700rpm and dentin blocks of 5mm length were prepared. The root canal opening was sealed with gutta-percha and dentinal tubule sealant was applied on all the surfaces except the test surface. EDTA was applied on the rest of the surface with the help of microbrush and the teeth were washed with normal saline after 30seconds. Teeth were then autoclaved in autoclavable pouches to remove any residual bacteria. The teeth were then randomly divided into 2 groups of 5 samples each, Group 1: *Streptococcus sanguinis* (S.s), Sub group a: S.s without cementum, Sub group b: S.s with intact cementum and Group 2: *Aggregatibacter actinomycetemcomitans* (A.a) Sub group a: A.a without cementum, Sub group b: with intact cementum.

Separate inoculums of *Streptococcus sanguinis* and *Aggregatibacter actinomycetemcomitans* were prepared and adjusted to 0.5 McFarland standards. This ensured uniform concentration of both the bacterial species. Teeth were then incubated in the respective bacterial suspensions for 15 days at 37°C. After the incubation time the teeth were then washed in normal saline. The teeth were then split longitudinally and sectioned with the help of diamond disc, chisel and mallet. After the sectioning, the samples were again washed with 100µl PBS using a micropipette to remove any debris.

For the examination of tooth specimens under the confocal laser scanning microscope (CLSM) 100µl of prepared fluorescent stain (LIVE/DEAD Baclight stain, Molecular Probes, Invitrogen Detection Technologies) was applied on the sectioned dentin blocks according to manufacturer's instruction and the blocks were incubated with the stain at room temperature for 15 minutes in a dark environment to enable bacteria to take up the stain. After 15minutes the samples were washed with Phosphate Buffer Solution PBS to remove any residual fluorescent stain. Following this an antifade mountant (Dakocytomation) was applied over the sample and the specimen was then mounted on a slide with glycerine and a cover slip was placed. The samples were then subjected to CLSM imaging at 40X Magnification. The confocal images were analysed and number of live bacteria (Green stained) was estimated (Figure 1-4).

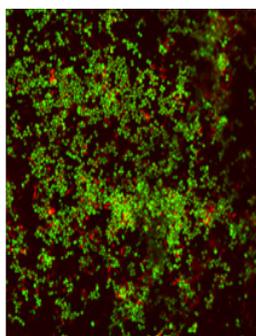


Figure 1 Group 1a: *Streptococcus sanguinis* without intact cemental layer.

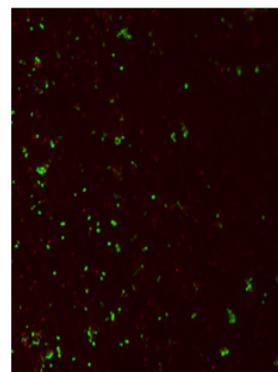


Figure 2 Group 1b: *Streptococcus sanguinis* with intact cemental layer

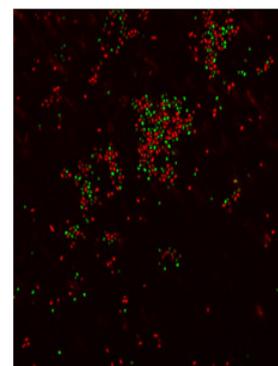


Figure 3 Group 2a: *Aggregatibacter actinomycetemcomitans* without intact cemental layer.

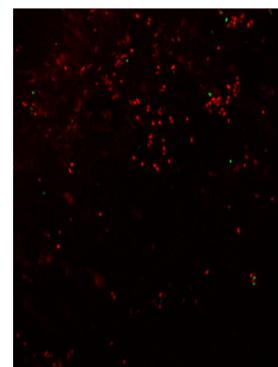


Figure 4 Group 2b: *Aggregatibacter actinomycetemcomitans* with intact cemental layer.

Statistical analysis

A statistical analysis was carried out using the SPSS software version 7.1.1. The paired t-test was used to compare the number of live bacteria that penetrated into the radicular dentinal tubules from a surface with and without an intact cemental layer. A p-value of less than or equal to 0.05 was considered statistically significant.

Results

In the *Streptococcus sanguinis* group there was significant difference in the number of bacteria that invaded the dentinal tubules without cemental layer (Mean=1072, SD=60.23) and with intact cemental layer (Mean=244.6, SD=7.76); p-value=0.000 (Table 1) (Table 2).

In the *Aggregatibacter actinomycetemcomitans* group, there was

a significant difference in the number of bacteria that invaded the dentinal tubules without cemental layer (Mean=172.4, SD=6.5) and with cemental layer (Mean=13.6, SD=3.04), p-value= 0.000.

These results suggest that significantly higher number of bacteria penetrated the radicular dentinal tubules without an intact cemental layer. There were relatively fewer bacteria that invaded the dentinal tubules that had an intact cemental layer.

An intergroup analysis was done to evaluate the number of S.s and A.a that invaded the radicular dentinal tubules without cemental layer. The number of S.s that penetrated in the radicular dentin was significantly higher as compared to A.a.

Table 1 Tabular form for mean and SD for S.s & A.a

Groups	Median	SD
1a	1072.4	60.23
1b	244.6	7.76
2a	172.4	6.54
2b	13.6	3.04

1a, S.s without intact cementum; 1b, S.s with intact cementum; 2a, A.a without intact cementum; 2b, A.a with intact cementum

Table 2 Significant difference in the number of bacteria that invaded the dentinal tubules without cemental layer and with intact cemental layer

Groups	Mean	SD	95% Confidence interval of the difference		t	df	p-value
			Lower	Upper			
1a	827.8	61.19	751.81	903.78	30.2	4	0
1b							
2a	158.8	9.49	147.01	170.59	37.4	4	0
2b							
1a	900	61.95	823.07	976.92	32.5	4	0
2a							

1a, S.s without intact cementum; 1b, S.s with intact cementum; 2a, A.a without intact cementum; 2b, A.a with intact cementum

Discussion

The results in this study point towards the protective nature of an intact cemental layer. Large numbers of *Streptococcus sanguinis* penetrated into the dentinal tubules when the tooth surface was devoid of cemental layer. This is in accordance with the findings of Meyron et al.,¹⁷ where they found rapid penetration of *Streptococcus sanguinis* into exposed dentinal tubules. Fewer number of A.a was observed in the dentinal tubules with and without an intact cemental layer. This is in accordance with Giuliana G et al.,¹⁴ where they recovered lower levels of gram negative organisms from the radicular dentin.

Siqueira et al.²⁰ evaluated *in vitro* root canal dentinal tubule invasion by selected anaerobic bacteria and the scanning electron microscopy (SEM) results indicated that all bacterial strains tested were able to penetrate into dentinal tubules, but to different extents.

During periodontal disease the cementum surface shows loss of collagen, cross banding, break down of dentogingival fibres and dissolution of mineral component. These features are generally perceived by electron microscopy.²¹

Several studies have described invasion of the cementum of periodontally diseased teeth.^{13,14,19} However, it was not evident from any of these studies if the invaded cementum was intact, healthy, or diseased. Exposed cementum is a thin, often discontinuous layer,²² and commonly shows surface defects, e.g., at sites where Sharpey's fibers attach to the cementum matrix.¹² Exposure of cementum to crevicular fluid, bacterial enzymes, or acidic metabolites may induce physicochemical and structural alterations, such as localized resorptive lacunae or demineralization.^{12,19}

Periodontal procedures like scaling and root planning can also cause alterations in the cemental layer. Therefore; it seems likely that bacterial invasion associated with periodontal disease occurs after the cementum has been altered by physiological, bacterial, or environmental factors.

Streptococci express multiple surface protein adhesions²³ that allow cells to bind to a wide range of substrates found in the oral cavity, including other microbial cells, salivary components, host cells, or extracellular matrix or serum components.²⁴ However, while there are considerable data on the mechanisms involved in the formation and development of dental plaque,²⁵ relatively little is known about the mechanisms by which oral bacteria penetrate or invade dentin, and cause pulpitis, root canal infection, periapical and periodontal diseases.

Collagen Type I a major organic component of dentin, is recognized by oral streptococci and when absorbed into hydroxyapatite surfaces, it serves as an adhesion substrate.²⁶ The ability of oral streptococci to bind to collagen may facilitate bacterial adhesion to exposed dentin or cementum, and subsequently tissue penetration. The antigen I/II polypeptides, expressed on the surfaces of most indigenous species of oral streptococci,²⁴ play a major role in mediating Streptococci to collagen.²⁷

A. actinomycetemcomitans is small, nonmotile, gram negative, saccharolytic, capnophilic, round-ended rod⁴ *A. actinomycetemcomitans* binds to collagen I,II,III and V but not IV. It has the ability to invade host cells, penetrate gingival epithelium and survive within eukaryotic cells. These organisms occur in locations like the epithelium wall, epithelial side of basal lamina, connective tissue and alveolar bone.²⁸

Few reports have shown the presence of *A. actinomycetemcomitans* in periapical lesions as well. *A. actinomycetemcomitans* is a resident component of the oral microbiota and is commonly found in periodontal infections, but its presence in periapical and root end samples must be considered as one of the causes in persistent endodontic infections.²⁹

Although pocket debridement suppresses components of the subgingival microflora associated with periodontitis,³⁰ periodontal pathogens may return to baseline levels within days or months.³¹ The return of pathogens to pre-treatment levels generally occurs in approximately 9 to 11 weeks, but can vary dramatically among patients.³²

Failure to eliminate residual bacteria in the periodontal pockets, root surfaces and dentinal tubules can cause re-colonization of bacteria and reinfection. Various treatment modalities such as systemic and local antibiotics,^{33,34} laser therapy,^{35,36} photodynamic therapy³⁷ have been used as adjuncts to non-surgical periodontal therapy to eliminate residual bacteria and have shown promising results in further reducing the bacterial counts.

High-resolution confocal microscopic images can be made of either the surface of a sample or beneath the surface. With microscopes running under normal conditions, the optical section thickness is $>1\mu\text{m}$ and the effective penetration into enamel and dentin a maximum of $100\mu\text{m}$.³⁸ Confocal Laser scanning microscopy was used in this study since it allows us to determine the levels of Live (green) and dead (red) bacteria with the help of the fluorescent dye. Also, optical sections of about $1\mu\text{m}$ can be obtained. Therefore, appropriate images from the inner sections of the specimen can be recorded. Specimen processing techniques can cause disruption of bacterial cells on the surface of the specimen.

The drawback of this study is that it was performed on extracted teeth using single organisms. Future studies can be done using an *ex vivo* model where freshly extracted teeth can be analyzed immediately after extraction. Also, various treatment modalities can be assessed wherein pre and post treatment levels of live and dead bacteria can be evaluated.

Conclusion

An intact cemental layer is an essential barrier for invasion of bacterial species into the dentinal tubules. Presence of bacterial species in the dentinal tubules can cause re-colonization of bacteria in the periodontal pockets, persistence of periapical infections or cause pulpal inflammation.

Therefore, maintaining the integrity of the cemental layer and attempting to regenerate lost cemental tissues through various regenerative procedures should be of prime importance.

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Conflict of interest

The author declares no conflict of interest.

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