SEM Study on the Anaerobic Bacterial Adhesion to the Dentin of Root Canal

Sung-Eun Yang, Kwang-Shik Bae
Dept. of Conservative Dentistry, College of Dentistry, Seoul National University

국문초록

혐기성 미생물의 근관내 상아질 부착에 대한 주사전자현미경적 연구

양성은 · 배광식
서울대학교 치과대학 치과보존학교실

목 적

근관형성시 근관내 상아질벽에는 항상 도말층(smear layer)이 형성되는데, 이는 상아질, 치수조직 잔사, 조상아세포 돌기, 때로는 미생물 등으로 구성되며, 주사전자현미경상으로는 비규칙적이며, 무정형의 구상(granular)구조물로 관찰된다. 본 연구에서는 도말층의 유무에 따른 혐기성 미생물, Prevotella nigrescens의 근관내 상아질 부착정도를 주사전자현미경으로 평가하고자 한다. 지금까지 사용되었던 실험방법에 비하여 보다 임상적 환경에 가까운 실험방법을 고안, 신빙성 있는 연구결과를 기대할 수 있도록 하였다.

방 법

치주질환이나 외상 등의 원인으로 발거된 상, 하악 전치 18개를 사용하였다. 각 치아의 치관부를 백악법랑경계부위에서 절단하고, 1군(5개치아)은 10ml의 생리식염수를, 2군(5개치아)과 3군(5개치아)은 10ml의 3.5% NaOCl을 근관관주용액으로 사용하여 근관형성을 하였다. 근관형성 완료후 1군과 2군은 10ml의 생리식염수로, 3군은 10ml의 0.5M EDTA용액으로 final flush를 시행하여, 3군의 도말층을 제거하였다. 치근수직절단과 ethylene oxide(EO) gas 소독 후 1, 2, 3군의 시편(각군10개시편)을 Prevotella nigrescens가 부유된 Brain Heart Infusion with Yeast estract, Hemin and Menadione(BHIYHM) broth내에 37℃에서 3시간 동안 incubation했다. 4, 5, 6군은 실험과정을 검증하기 위한 대조군으로써, 4군(1개치아)과 5군(1개치아)은 1군, 2군과 같이 각각 생리식염수와 NaOCl만을 이용한 근관형성으로 도말층을 잔존시키고, 6군(1개치아)은 3군과 같이 NaOCl과 EDTA를 적용하여 도말층을 제거한 후, 치근수직절단과 EO gas 소독을 시행했다.

모든 시편(1, 2, 3, 4, 5, 6군)을 통상의 방법에 따라 처리한 후 주사전자현미경을 통하여 관찰, 근관내면에 부착되어 있는 Prevotella nigrescens의 개수, 모양, 상아세관 및 도말층과의 관계 등을 관찰, 비교, 분석하였다. 결과는 t-test와 one-way ANOVA를 통하여 통계처리 하였다.

결 론

1. 근관형성 후 근관내 상아질 표면 전체는 도말층으로 덮여있는 양상을 보였다.
2. 3.5% NaOCl과 0.5M EDTA를 적용하여 근관내 도말층을 효과적으로 제거할 수 있었으며, 상아세관 개구부가 확연히 노출되어 있는 소견을 관찰할 수 있었다.
3. 도말층이 덮인군에서 미생물의 부착이 유의성 있게 높았다($P<0.05$).
4. 근관 형성중 형성되어 근관 상아질을 덮고 있는 도말층이 미생물의 부착을 증가시켜, 근관 재감염의 기회를 증가시킴을 알 수 있었다.

주요어 : 도말층, 미생물부착, 근관상아질, 주사전자현미경, Prevotella nigrescens, 혐기성미생물

※이 연구는 1999년도 서울대학병원 일반연구비(04-1999-076-0)지원에 의한 결과임.

Ⅰ. Introduction

The aim of instrumentation and irrigation is to prepare a clean, debris-free canal for obturation. However, current technique may not cleanse the entire root canal system, especially in irregular and/or curved canals. In addition to superficial debris, it has been shown, using the scanning electon microscope, that a layer of sludge material was always formed over the surface of dentinal walls whenever dentine was cut[20,21].

McComb & Smith(1975)[21] observed this layer on the walls of instrumented root canals and reported that it was similar in appearance to coronal smear layer[18]. They suggested that the smear layer associated with root canal treatment consisted of not only dentin as in coronal smear layer, but also remnants of odontoblastic processes, pulp tissue and bacteria. Hence, it may contain organic and inorganic material.

Whether smear layer should be removed has been the subject of several investigations[15,29,34].

One suggestion is that the removal of the smear layer may contribute to successful intracanal disinfection procedures[15]. Removal of the occluding layer may allow intracanal antimicrobial agents to penetrate dentinal tubules. Conversely, if the canals were inadequately disinfected, or if bacterial contamination occurred after canal preparation, the presence of smear layer might block bacterial entry into the dentinal tubules[34].

Another argument in favor of removal is that the smear layer, while composed primarily of inorganic material[13,18], may have a significant organic component, including viable bacteria and their by-product[3,21]. Pashley(1981)[29] proposed that smear layer containing bacteria or bacterial products might provide a reservoir of irritants. Thus, complete removal of the smear layer would be consistent with elimination of irritants from the root canal system.

On the other hand, the benefit of smear layer may be blockage of dentinal tubules from bacterial penetration and/or fluids by altering dentin permeability. Bacteria remaining in dentinal tubules after canal preparation may then be sealed into tubules by the smear layer and subsequent obturating materials.

Bacterial adherence is a prerequisite for colonization and infection of a susceptible host and the presence of a smear layer may affect the ability of bacteria to adhere to dentin[19]. Therapy will be dictated by the way the bacteria behave on this smear layer. If they adhere to and colonize the dentin more easily when it is covered with smear layer, it must be removed in this case. Recently, root canal dentin has been used to investigate bacterial adherence. Calas et al.(1994)[7] reported that higher numbers of *Streptococcus sanguis* adhered to smeared dentine than to dentin treated so as to remove the smear layer, while Drake et al.(1994)[10] found that significantly more cells of *Sterptococcus anginosus* were recovered from non-smeared root canals than from smeared root canls. At the present time, the use of chelating agents to remove this coating is only recommended for the end of treatment before filling[35].

The purpose of this study is thus to try and establish whether removal of the smear layer can affect the attachment and colonization by a strict anaerobic bacterial strain with a high pathogenic potential such as *Prevotella nigrescens*, on root canal dentin(human teeth).

The study is conducted in vitro with the cell count being effected by direct examination using a scanning electron microscope.

Ⅱ. Materials & Methods

Preparation of samples

Eighteen freshly extracted human teeth with single, straight canals were used. Bone, calculus or soft tissue on the root surface were removed with periodontal currettes. Teeth were stored in saline prior to experimental precedures.

All teeth were sectioned at the cemento-enamel junction and each canal was instrumented to size 60 master apical file(MAF) by modified crown-down technique using profile(Maillefer, Ballaigues, Switzerland) & Gates-Glidden drill. During canal preparation, irrigation was done with a physiologic saline solution(group 1&4) or 3.5% NaOCl(group 2, 3, 5, 6) between each file. A total of 10ml irrigation solution was used during each canal preparation. 10ml physiologic saline solution(group 1, 2, 4, 5) or

Table 1. Classification of samples

	Group	Number	Irrigation solution	Inoculation
Experimental	1	10	physiologic saline	yes
	2	10	3.5% NaOCl	yes
	3	10	3.5% NaOCl + 0.5M EDTA	yes
Control	4	2	physiologic saline	no
	5	2	3.5% NaOCl	no
	6	2	3.5% NaOCl + 0.5M EDTA	no

10ml 0.5M EDTA(group 3&6) was applicated for final flush (Table 1).

Each of samples was then cut in half along the major axis of the root canal. After vertical sectioning, all of samples were cleaned in 10ml rinses with a sterile saline solution and sterilized by ethylene oxide(EO) gas. All samples were stored in sterile saline solution to prevent re-contamination and desiccation.

Inoculation of samples

Prevotella nigrescens(ATCC 33563), which were stored at -70℃ deep-freezer, were thawed out and inoculated in rabbit columbia agar plate for 4days. Samples(group1, 2, 3) were immersed into brain heart infusion with yeast extract, hemin and menadione (BHIYHM) broth inoculated with *Prevotella nigrescens* and incubated for 3hrs at 37℃. All of these operations were conducted in a strict anaerobic atmosphere.

Samples preparation for SEM

After 3hrs of incubation, the samples were prepared for examination using a scanning electron microscope. They were first rinsed with gentle stirring in sterile saline solution to remove the non-attached bacteria and fixed in a 2.5% glutaraldehyde solution for 30min. After rinsing with 0.1M phophate buffer, they were dehydrated in increasingly concentrated alcohol solution (30%, 50%, 70%, and 100%) and left for 30min in each bath. Samples were critical point-dried to preserve the bacterial structure. The preparation was completed by sputter coating with carbon-gold.

SEM observation

With scanning electron microscope (JSM-840S, JEOL, Tokyo, Japan) canal surface was examined for presence/abscence of smear layer and the number of *Prevotella nigrescens* which had attached to the canal surface was determined in 6000-fold magnification.

Each sample of experimental groups was divided into 5 areas from coronal portion to apical portion and 2 spots per one area were chosen at random. 10 observations were made per sample thus enabling the number of cells per surface area analyzed to be determined. The variance analysis method was used to process results.

Ⅲ. Results

Control groups

Overall, controls behaved as expected. In group 4&5, smear layer covered entire root canal surface (Fig. 1, 2). In group 6, most of the smear layer was removed(Fig. 3) The majority of the peritubular dentin had disappeared and the entrances to the tubules were widened. The control groups demonstrated no growth of bacteria throughout the incubation period.

Table 2. Comparison of mean number of cells counted per observation spots of 100 images (magnification of ×6,000)

Group	Mean(/327μm^2)	Standard deviation
1	13.2	10.4
2	15.3	10.6
3	0.4	0.02

symptoms. Bae et al.(1998)[9] reported that *P. nigrescens* was the BPB most often isolated from infections of endodontic origins. Because of this reason, type strain of *Prevotella nigrescens* was used in this study.

The purpose of irrigation is twofold: to remove gross debris originating from pulp tissue, and possible bacteria; the organic component, and to remove smear layer; the mostly inorganic component. Because there is no single solution which has the ability to dissolve organic tissues and to dimineralize the smear layer, a sequential use of organic and inorganic solvents have been recommended[2,35].

The organic tissue-dissolving activity of NaOCl is well known[13] and increases with rising temperature[25]. However, the capacity to remove smear layer from the instrumented root canal walls has been found to be insufficient. Many authors have concluded that the use of NaOCl during or after instrumentation produces superficially clean canal walls with the smear layer present[2,13]. In this study, there was no significant difference between saline irrigation(group 1) and NaOCl irrigation(group 2) ($p > 0.05$). The most common chelating solutions are based on ethylene diamine tetraacetic acid(EDTA) which reacts with calcium ions in dentine and forms soluble calcium chelates. Fehr and Nygaard-Ostby(1963)[11] found that EDTA decalcified dentin to a depth of 20 to 30μm in 5min. Numerous authors have agreed that the removal of smear layer as well as soft tissue and debris can be expedited by the alternate use of EDTA and NaOCl[2,8,35]. In this study, most of the smear layer was removed and the entrances to the dentinal tubules were widened by the alternate use of EDTA and NaOCl (Fig. 3).

The ultrastructural observations supported and may explain the findings of greater recovery of bacteria when smear layer was present. Removal of the smear layer inhibited the bacterial adhesion to the dentinal surface, as observed in scanning electron microscopic micrographs(Table 2). These results suggest that the smear layer produced during root canal therapy may indeed increase bacterial colonization of root canal. Although these findings corroborate many earlier in vitro and in vivo studies[6,7,29], these are in disagreement with others[10,23,34]. Drake et al.(1994)[10] found that significantly more cells of *Streptococcus anginosus* were recovered from non-smeared root canals than from smeared root canals and Love(1996)[19] suggested that dentinal smear layer do not enhance or impede bacterial adherence to the dentinal matrix.

One explanation for these difference may reside in the methodologies used in producing a smear layer. The actual chemical composition of dentinal smear layers produced by different instruments has not been elucidated. But, Drake et al.(1994)[10] preparated root canal with similar method using endodontic files. Then, differences in methodologies may make little effect on results.

In addition to differences in the methodologies followed in producing smear layer, a diverse spectrum of microorganisms has been used. Drake et al. (1994)[10] used *Streptococcus anginosus* in their studies whereas Michelich et al.(1979)[23] used *Streptococcus mutans* and *Streptococcus sanguis*. Other bacteria such as *Escherichia coli*[14], *Pseudomonas aeruginosa*[22], and *Sterptococcus faecalis*[22] have also been used. It is not particularly surprising that different results have been obtained considering the different growth rates, physiological characteristics, and motility status of all of these test organisms.

The mechanisms whereby bacteria adhere to dental hard tissues are not fully understood. Adhesion is dependent on complex interactions between bacteria and the surface on to which it is inoculated. These interactions may be non-specific, e.g. hydrophobic or charge interactions or specific, involving cell-surface polypeptide adhesins and receptors which can identify specific ligands[16].

Other factors such as differences in growth media, incubation conditions, inoculum size, and incubation time could also lead to the differences observed between various studies. Once the smear layer has been removed, when the root canal surface is clean, the number of cells adhering to the dentin decreases. The chelating agents in irrigation at the end of treatment before filling, as recommended by Yamada et al.(1983)[35] and Baumgartner and Mader(1987)[2], is thus essential even in the intermediate stages of the treatment when temporary medication is used. However, if the root canal had the smear layer

removed as part of the initial treatment, it is possible that patent tubules would be exposed when leakage occurs, thereby allowing the accumulation and penetration of bacteria into dentinal tubules. This may complicate disinfection of the dentin during treatment.

Once smear layer is removed, there is always a risk of reinfecting dentinal tubules if the seal fails. Further studies are certainly needed to establish a correlation between endodontic smear layer and clinical performance of root canal fillings.

Ⅴ. Conclusion

The conclusions are as follows :

1. Smear layer covers entire root canal surface after root canal preparation.
2. Smear layer has been removed away and the entrances of dentinal tubules has opened widely, applying 3.5% NaOCl and 0.5M EDTA.
3. A significantly higher number of bacteria were adhered to the root canal dentin with smear layer ($p<0.05$).
4. Smear layer produced during root canal preparation allows adhesion and colonization of *Prevotella nigrescens* to dentin matrix, therefore seems to enhance canal reinfection.

References

1. Abou-Rass M, Bogen G. Microorganisms in closed periapical lesions. *Int Endo J* 1998;31:39-47.
2. Baumgartner JC, Mader CL. A scanning electron microscopic evaluation of four root canal irrigation regimens. *J Endodon* 1987;13:147-57.
3. Brannström M, Nordenvall KJ, Glantz PO. The effect of EDTA-containing surface-active solutions on the morphology of prepared dentin: an in vivo study. *J dental Res* 1980;59:1127-31.
4. Byström A, Sundqvist G. Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. *Scand J Dent Res* 1981;89:321-8.
5. Byström A, Sundqvist G. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. *Int Endod J* 1985;18:35-40.
6. Calas P, Rochd T, Druilhet P, Azais JM. In vitro adhesion of two strains of *Prevotella nigrescens* to the dentin of the root canal. *J Endodon* 1998;24:112-115.
7. Calas P, Rochd T, Michel G. In vitro attachment of *Streptococcus sanguis* to the dentin of the root canal. *J Endodon* 1994;20:71-74.
8. Cengiz T, Aktener BO, Piskin B. The effect of dentinal tubule orientation on the removal of smear layer by root canal irrigants. A scanning electron microscopic study. *Int Endo J* 1990:23:163-71.
9. Dougherty WJ, Bae KS, Watkins BJ, Baugartner JC. Black-pigmented bacteria in coronal and apical segments of infected root canals. *J Endodon* 1998;24:356-358.
10. Drake DR, Wiemann AH, Rivera EM, Walton RE. Bacterial retention in canal walls in vitro: effect of smear layer. *J Endodon* 1994;20:78-82.
11. Fehr FR, Nygaard-Ostby. Effect of EDTAC and sulphuric acid on root canal dentin. *Oral Surg Oral Med Oral Path* 1963;16:199-205.
12. Gharbia SE, Haapasalo M, Shah HN, Kotiranta A, Lounatmaa K, Pearce MA, Devine DA. Characterization of *Prevotella intermedia* and *Prevotella nigrescens* isolates from periodontic and endodontic infections. *J Periodontol* 1994;65:56-61.
13. Goldman LB, Goldman M, Kronman JH, Lin PS. The efficacy of several irrigating solutions for endodontics: A scanning electron microscopic study. *Oral Surg Oral Med Oral Path* 1981;52:197-204.
14. Gutierrez JH, Jofre A, Vollena F. Scanning electron microscope study on the action of endodontic irrigants on bacteria invading the dentinal tubules. *Oral Surg Oral Med Oral Path* 1990;69:491-501.
15. Haapasalo M, Orstavik D. Invitro infection and disinfection of dentinal tubules. *J Dent Res* 1987;66:1375-9.
16. Jenkinson HF. Adherence and accumulation of oral streptococci. *Trends in Mcrobiology* 1994;2:209-12.
17. Karagoz-Kucukay I, Bayirli G. An apical leakage study in the presence and absence of smear layer. *Int Endo J* 1994;27:87-93.
18. Lester KS, Boyde A. Scanning electron microscopy of instrumented, irrigated and filled root canals, *British Dent J* 1997;143:359-67.
19. Love RM. Adherence of Streptococcus gordonii to smeared and nonsmeared dentin. *Int endodon J* 1996;29:108-112.
20. Mader CL, Baumgartner JC, Peters DD. Scanning electron microscopic investigation of the smeared layer on root canal walls. *J Endodon* 1984;10:477-83
21. McComb D, Smith DC. A preliminary scanning electron microscopic study of root canals after endodontic procedures. *J Endodon* 1975;1:238-42.
22. Meryon SD, Jakeman KH, Browne RM. Penetration in vitro of human and ferret dentin by three bacterial species in relation to their potential role in pulpal inflammation. *Int Endod J* 1986;19:213-20.
23. Michelich VJ, Schuster GS, Pashley DH. Bacterial penetration of human dentin in vitro. *J Dent Res* 1979;59:1398-1403.
24. Molven O, Olsen I, Kerekes K. Scanning electron microscopy of bacteria in the apical part of root canals in permanent teeth with periapical lesions. *Endod Dent Traumatol* 1991;7:226-229.
25. Moorer WR, Wesselink PR. Factors promoting the tissue dissolving capability of sodium hypochlorite. *International Endodontic Journal* 1982;15:187-96.
26. Nair NPR. Light and electron microscopic studies of root canal flora and periapical lesions. *J Endodon* 1987;13:29-39.
27. Oksan T, Aktener BO, Sen BH, Tezel H. The penetration of root canal sealers into dentinal tubules. A SEM study. *Int Endo J* 1993;26:301-5.