Effect of Ethylenediaminetetraacetic Acid and Sodium Hypochlorite Irrigation on *Enterococcus faecalis* Biofilm Colonization in Young and Old Human Root Canal Dentin: *In Vitro* Study

Hüseyin Ozgur Ozdemir, DDS, PhD, [*] *Hatice Dogan Buzoglu, DDS, PhD,* [*] *Semra Calt, DDS, PhD,* [*] *Adam Stabholz, DMD,* [†] *and Doron Steinberg, MD, PhD* [‡]

Abstract

Introduction: The alterations in dentin tissue depending on increasing age might cause different adhesion capability of bacteria, yielding differences in clinical approaches regarding root canal irrigation. This study, therefore, aimed to evaluate the effects of ethylenediaminetetraacetic acid (EDTA) and sodium hypochlorite (NaOCl) on *Enterococcus faecalis* biofilm growth in root canal dentin of young and old individuals. **Methods:** The root canals of extracted young (<30 years) and old (>60 years) single-rooted human teeth were sectioned at the crown and the apical parts. The root canals of the mid-root sections were enlarged with #2 Gates-Glidden burs. After treatment with 17% EDTA + 2.5% NaOCl, 17% EDTA alone, 2.5% NaOCl alone, or saline, the samples were incubated in *E. faecalis* suspension for 24 hours. Thereafter, root canal samples were enlarged again with #3 Gates-Glidden burs, and the removed dentin chips were collected. Bacteria were dispersed by using sonication, serially diluted, and then plated for counting on agar plates as colony-forming units. Scanning electron microscopy and confocal laser scanning microscopy investigations were also carried out to examine the biofilm formation on the dentin. Data were analyzed with Kruskal-Wallis test and Mann-Whitney *U* test with Bonferroni adjustment. **Results:** Combination of EDTA and NaOCl significantly reduced the amount of intracanal biofilm in both age groups ($P < .01$). However, the bacterial counts of *E. faecalis* in the old group were still higher ($P < .05$). **Conclusions:** It might be suggested that root canals from elderly population are more susceptible to canal infection. However, combined application of EDTA and NaOCl significantly reduces the amount of intracanal biofilm. *(J Endod 2010;36:842–846)*

Key Words

Aging, biofilm, dentin, EDTA, *Enterococcus faecalis*

Bacteria are the primary etiologic agents of periradicular diseases (1). Root canal treatment aims to eliminate the bacteria from the infected canal and to prevent reinfection. Although chemomechanical preparation and the use of antimicrobial medicaments are effective in reducing bacterial colonization in root canal systems, some bacteria might survive despite the treatment, leading to reinfection of the root canal (2).

Enterococcus faecalis, a facultative anaerobic, gram-positive coccus, is frequently isolated from endodontically treated teeth with persistent periradicular disease (3, 4). It is commonly found in monoinfections, but it is also observed in mixed infections of the root canal system and has the potential of forming a biofilm structure on root canal walls (5, 6). Several stages are critical in biofilm formation. One of the important stages is the initial adhesion of the bacteria onto the tooth surfaces. The primary adhesion of bacteria depends on surface characteristics of dentin as well as specific adhesion characteristics of the bacteria (7, 8). A smear layer forms on the dentin surface during root canal instrumentation, which might affect the adhesion of bacteria to the root canal wall (9–13). It has been reported that removing the smear layer decreased the adhesion of *E. faecalis* (12, 13). On the other hand, bacterial invasion of dentinal tubules might be responsible for persistent root canal infection (2). The exposure time of dentin to bacteria and tubule diameter might play an important role in bacterial penetration into the tubuli. Tubules that are sclerotic or obliterated can physically impede bacterial invasion. Recently, Kakoli et al (14) revealed that the depth of *E. faecalis* penetration into the dentin tubules was lower in aged dentin samples.

With increasing age, several changes occur in the dentin-pulp complex. Dentin sclerosis occurs as a result of an increase in peritubular dentin. Dentinal tubules become obliterated, resulting in narrowing of the tubule to approximately 2.5 μm in diameter near the pulp and 0.9 μm in diameter near the enamel/cement. Thus, a tubule is normally larger in diameter than the average *E. faecalis* cell diameter of approximately 0.8–1 μm (15–19). Recent studies showed that collagen, which forms the organic matrix of dentin, plays a key role in the adhesion capability of *E. faecalis* to the dentin surface (2, 6). However, Yang et al (12) suggested that *E. faecalis* adhesion might be related to nonspecific interaction on the basis of surface properties rather than specific binding to collagen.

From the *Department of Endodontics, Faculty of Dentistry, Hacettepe University, Ankara, Turkey; and †Department of Endodontics and ‡Institute of Dental Sciences, Faculty of Dental Medicine, Hebrew University-Hadassah, Jerusalem, Israel.

Address requests for reprints to Dr Hatice Dogan Buzoglu, Department of Endodontics, Faculty of Dentistry, Hacettepe University, 06100 Sihhiye, Ankara, Turkey. E-mail address: hdogan67@hotmail.com.

0099-2399/$0 - see front matter

doi:10.1016/j.joen.2010.01.008

TABLE 1. Level of Adhered *E. faecalis* to the Root Canal Dentin from Young and Old Human Subjects

Groups	Age (y)	Mean (CFU)	SD	Minimum (CFU)	Maximum (CFU)	Median (CFU)
Control[1,a,b]	<30	289.13	98.31	175.33	444.67	275.84
EDTA[1,a,c]	<30	127.75	64.59	30.33	246.67	119.67
NaOCl[1,d]	<30	149.04	100.11	36.67	328	132.17
EDTA + NaOCl[1,b,c,d,e]	<30	24.79	12.28	7.33	40.33	27
Control[2,f,g,h]	>60	435.38	184.24	186.33	506.67	436
EDTA[2,f]	>60	122.38	77.26	19.67	237.33	117.5
NaOCl[2,g,k]	>60	159.63	104	47.33	377.33	133.5
EDTA + NaOCl[2,b,k,e]	>60	53.62	21.21	17.33	74.33	57

CFU, colony-forming units; SD, standard deviation.

Superscript numbers 1 and 2 ($P < .001$) show the statistical differences according to the Kruskal-Wallis test. Superscript letters *a*, *b*, *c*, *d*, *f*, *g*, *h*, and *k* ($P < .008$) show the statistical differences according to the Mann-Whitney *U* test with Bonferroni adjustment. Superscript letter *e* ($P = .021$) shows the statistical difference according to only the Mann-Whitney *U* test.

Alterations in dentin tissue depending on increasing age might result in different adhesion capability of bacteria. Hence, differences in clinical approaches regarding the use of irrigating solutions during root canal treatment should be considered. Therefore, the aim of this study was to evaluate the adhesion capability of *E. faecalis* on smeared or nonsmeared root canal dentin surfaces in the teeth of young and old individuals.

Materials and Methods

The ethics committee of Hacettepe University approved the collection and use of extracted teeth for this study. Eighty noncarious, unrestored freshly extracted single-rooted human teeth, stored in saline solution at 4°C, were used. Teeth were divided equally into 2 groups according to the age of the patients, young (<30 years) and old (>60 years). The coronal and apical parts of the teeth were cut with a high-speed diamond disk, resulting in a 4-mm-long mid-part of the root samples. Standardization of each root canal was performed by enlarging the canal with #2 Gates-Glidden burs (0.7 mm diameter) (VDW, Munich, Germany). Samples were washed thoroughly, sterilized by autoclave, and preincubated at 37°C in brain-heart infusion (BHI) (Difco; BD Diagnostics, Sparks, MD) to ensure no bacterial contamination. The specimens from each age group were randomly divided into the following 4 subgroups ($n = 8$). Group 1 specimens were treated with 10 mL 17% ethylenediaminetetraacetic acid (EDTA) (pH 7.4) (Merck Co, Darmstadt, Germany) for 10 minutes and 10 mL 2.5% sodium hypochlorite (NaOCl) (Sultan Chemists, Inc, Englewood, NJ) for an additional 10 minutes, groups 2 and 3 were treated with EDTA or NaOCl alone for 10 minutes, and group 4 samples were treated with 10 mL sterile phosphate-buffered saline (PBS) for 10 minutes as control.

Microbiology Procedures

Evaluation of bacteria in the root canal was conducted in a procedure similar to that described by Heling et al (20) and Basrani et al (21). The samples were incubated for 24 hours with *E. faecalis* (ATCC 29212) in BHI at 37°C in atmosphere enriched with 5% CO_2. After incubation, the samples were rinsed 3 times with 10 mL of sterile PBS. The root canal of each tooth sample was again enlarged with sterile #3 Gates-Glidden burs (0.9 mm diameter), and dentin chips were collected into 3 mL of sterile PBS. The Gates-Glidden burs were also placed into the test tube to collect dentin chips that adhered to the bur. All the tubes were sonicated in an ultrasonic water bath (Elma, Singen, Germany) for 10 minutes to dislodge bacteria from the burs and dentin chips and to disperse bacterial aggregation. The bacterial suspension was serially diluted at 1/10 ratio. Fifty microliters was taken from each sample and plated on BHI agar (HY Laboratories Ltd, Rehovot, Israel). Each sample was plated in triplicate. The agar plates were incubated at 37°C in air enriched with 5% CO_2 for 24 hours. After incubation, the colony-forming units (CFU) were counted by using a visual counter (New Brunswick Scientific Co, Inc, Edison, NJ). Statistical comparison of the means was calculated by using the Kruskal-Wallis test and Mann-Whitney *U* test with Bonferroni adjustment.

Scanning Electron Microscopy and Confocal Laser Scanning Microscopy Procedures

Four similarly prepared tooth samples from each age group were used for scanning electron microscopy (SEM) evaluation. The teeth were bisected longitudinally, irrigated with EDTA + NaOCl or PBS as control, and incubated as described above. After fixation, dehydration, and gold coating, the samples were examined under SEM (Quanta 200; FEI, Eindhoven, The Netherlands).

Four additional tooth samples from each age group were prepared as described above, bisected longitudinally, and then flattened with sandpaper. After applying the same irrigation and infection procedures as described above, the samples were washed and stained to detect live or dead bacteria by using a bacterial viability kit (Molecular Probes, Inc, Eugene, OR), according to the manufacturer's instructions. Thereafter, the samples were immediately analyzed by using confocal laser scanning microscopy (CLSM) (Zeiss, Oberkochen, Germany) at different biofilm depths (22).

Results

Table 1 summarizes the levels of adhered *E. faecalis* to the root canal dentin in young and old groups with descriptive statistics (mean, standard deviation, and minimum, maximum, and median values) and also includes the analysis of the Kruskal-Wallis test and Mann-Whitney *U* test with Bonferroni adjustment. Comparison of the 4 tested groups for <30 years and >60 years age groups with the Kruskal-Wallis test revealed statistically significant differences ($P < .001$). The adhesion capability of *E. faecalis* to the root dentin after exposure to EDTA and NaOCl solutions was compared with the nontreated control groups. Both the old and young control groups demonstrated the highest amount of *E. faecalis* adhesion in the root canal. Application of the combined EDTA and NaOCl solutions resulted in a significant reduction of *E. faecalis* adhesion to the root canal dentin compared with control or with the single use of solutions in both age groups ($P < .008$). However, the biofilm-forming capability of *E. faecalis* to the root canal dentin was still significantly higher in the old group compared with the young group ($P < .05$). Applying each irrigation solution separately also reduced the amount of adhered bacteria compared with control ($P < .008$) but less than the effect of the combined solution. SEM evaluation showed that biofilm formation in the smeared samples of the old group was greater than in the young

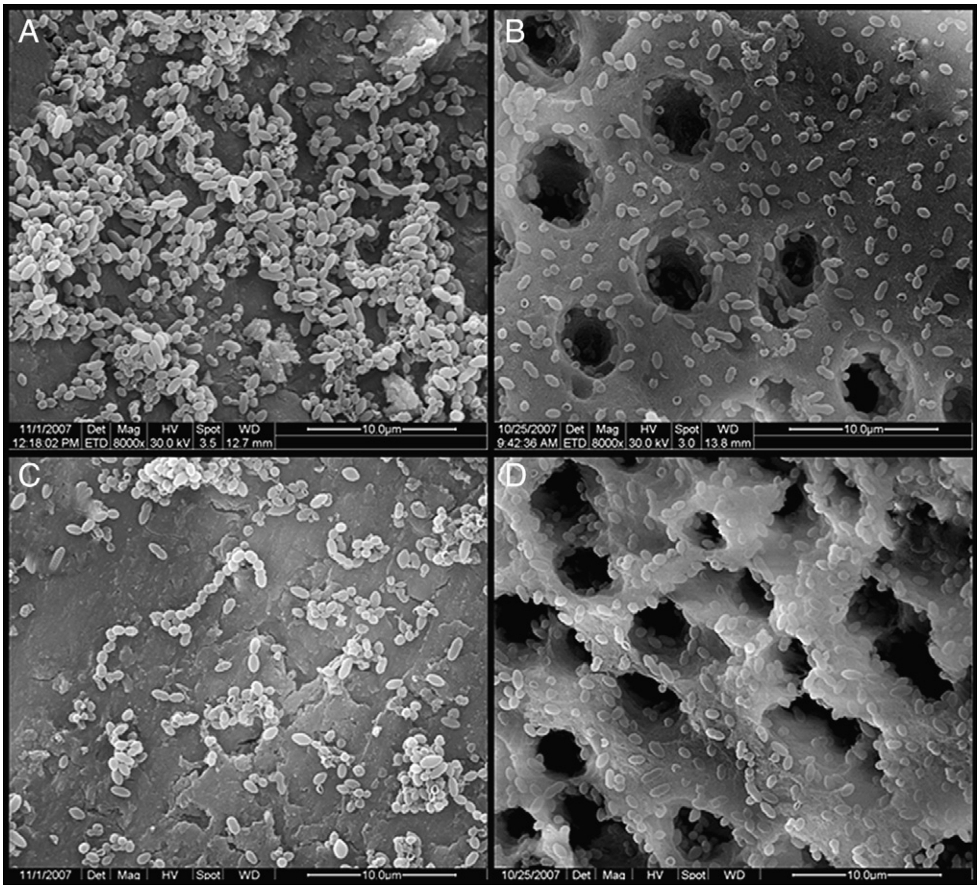

Figure 1. Adhesion of *E. faecalis* to the root canals of old (*A, B*) and young (*C, D*) human subjects (SEM; original magnification, ×3000). (*A, C*) Smeared control group; (*B, D*) nonsmeared: combined use of 17% EDTA and 2.5% NaOCl irrigation.

group (Fig. 1*A, C*). After using the combined EDTA and NaOCl solutions, in addition to reduction in biofilm, no smear layer could be found, and the openings of the dentin tubules were observed clearly (Fig. 1*B, D*). CLSM evaluation revealed that live bacteria were found in each tested group (Fig. 2). However, biofilm formation was more apparent and thicker in the old control group compared with the young group (Fig. 2*A, C*). Fewer bacteria and much reduced biofilm depth were observed by using the combination of EDTA and NaOCl solutions in both young and old samples (Fig. 2*B, D*).

Discussion

In the present study, the biofilm-forming ability of *E. faecalis* on root canal dentin of teeth originating from young and old human subjects was evaluated by using 3 different techniques: CFU, SEM, and CLSM methods. CFU is a primary microbial technique, allowing determination of the number of viable bacteria per sample. SEM evaluation shows the presence of total bacteria on intratubular and intertubular dentin but fails to determine the viability of the immobilized organisms. CLSM analysis determines the viable and dead bacteria immobilized in the dentin tubules and the biomass (13, 22). The CFU counting results showed that applying a combination of EDTA and NaOCl solutions significantly reduced the *E. faecalis* adhesion in the root canals of both young and old groups. This reduction in number of *E. faecalis* was significantly lower in the old group compared with the young group. Smeared control surfaces indicated the highest amount of bacterial adhesion in both age groups. Application of each irrigation solution separately also significantly reduced the adhered bacteria in the root canal but to a lesser extent than the combined application.

The mechanisms whereby oral bacteria adhere to solid surfaces are influenced by the properties of the outer hard surface as well as the unique adhesive properties of the bacteria (7, 8). Bacterial adhesion has been suggested to occur in 2 main phases. Phase 1 is a physicochemical process and occurs within seconds to minutes, whereas phase 2 is considered as a biologic cellular-molecular process of a mature biofilm, occurring in a time frame of hours to days (23). In the present study dentin samples were inoculated with bacteria for 24 hours to allow maturation of the biofilm structure on dentin.

Dentin represents the primary substratum for bacterial adhesion and biofilm formation in both primary and secondary infections of root canals (24). Basically, dentin consists of an inorganic phase of apatite crystals and an organic matrix primarily of collagen. Dentinal tubules contain appreciable amounts of unmineralized collagen (25). It has been demonstrated that *E. faecalis* adheres to collagen and maintains the capability to invade dentinal tubules (2, 6). There is limited understanding of the changes in the collagen matrix in dentin with aging. Ager et al (26) reported that the amid 1 peak intensity of dentin collagen increased, whereas Nazari et al (27) noted that collagen fibrils lose their extensibility or the collagen content is decreased depending on patient age. These alterations in dentin collagen with aging might be one of the reasons for the differences of *E. faecalis* adhesion capability to the root canal dentin observed in our study.

It has been reported that EDTA reduces the hydrophobicity and surface free energy of root dentin (28) and thereby influences the nature of bacterial adhesion, adhesion forces, and biofilm formation

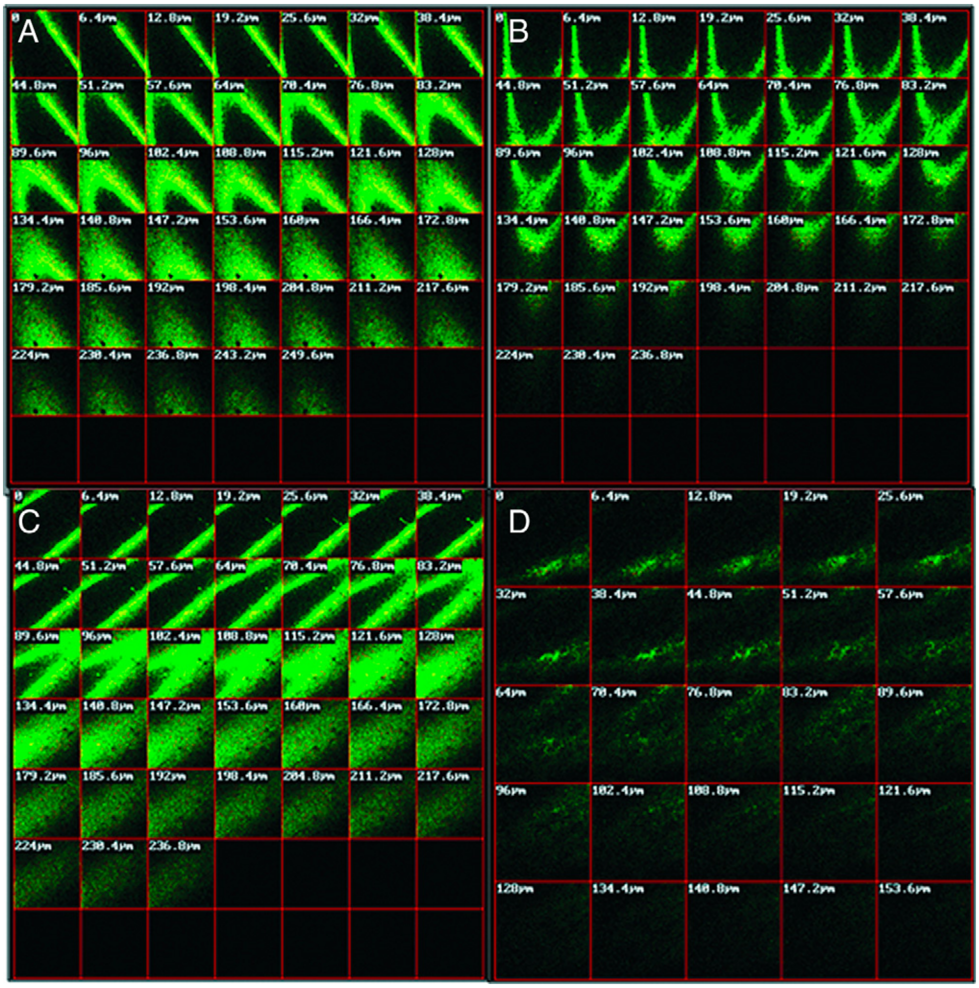

Figure 2. CLSM images of the biofilm surface in root canals of old (*A, B*) and young (*C, D*) human subjects at different biofilm depths. (*A, C*) Smeared control group; (*B, D*) nonsmeared: combined use of 17% EDTA and 2.5% NaOCl irrigation.

of *E. faecalis* to dentin (23). Kishen et al (23) also demonstrated that the last irrigation used on root canal dentin significantly influences bacterial adherence to dentin. When EDTA was used as the last irrigant, adhesion of bacteria on dentin was higher than when NaOCl was used as the last irrigant. Use of chelating agents or acids alone results in selective removal of inorganic dentin components, exposing collagen fibers and creating an ideal substrate for adherence by *E. faecalis* (29, 30). However, in the present study, the use of EDTA or NaOCl alone had a similar effect on *E. faecalis* adhesion to the root canal dentin, but with a lower effect than the combined use of these agents. This result seems to support the notion that bacterial adhesion of *E. faecalis* might also be the result of a nonspecific physical interaction based on surface properties rather than specific binding to collagen (12). Although NaOCl is an effective solution capable of both physically removing biofilm and killing bacteria (31, 32), in the present study, when NaOCl was used alone, it was not effective in the elimination of *E. faecalis* adhesion to the root canal dentin as expected. It has been suggested that the buffering capacity of dentin against some antimicrobials (29) or tissue debris on the dentin surface might reduce the efficacy of NaOCl or EDTA on the smear layer and thereby limit the effect on bacterial attachment.

The SEM pictures of the present study demonstrated that *E. faecalis* adheres to the intertubular dentin as well as to the intratubular dentin (Fig. 1), similarly as demonstrated in the study of Chivatxaranukul et al (33). On the other hand, Kishen et al (34) showed that *E. faecalis* is capable of forming a distinct calcified biofilm in a calcium carbonate and calcium phosphate rich microenvironment. Venegas et al (35) reported that the adhesion of several types of bacteria to hydroxyapatite was enhanced with increasing Ca^{2+} concentration apart from the dentin surface. The higher mineral content in age-induced sclerotic dentin as well as the collagen of the dentin surface might affect bacterial adhesion in old root dentin. There is only scant knowledge on the effect of dentin aging on bacterial adhesion, although such information is clinically important. Love (11) demonstrated no differences in the degree of adhesion of *Streptococcus gordonii* in sclerotic dentin with or without smear layer. However, Kakoli et al (14) showed that the depth of *E. faecalis* invasion into the dentin tubules and the number of invaded tubules were lower in the teeth of an old group compared with a young group, suggesting that sclerotic or obliterated tubules could physically impede bacterial invasion of dentin. It is known that age-induced sclerotic dentin not only shows higher mineral content but consistently shows closure of the dentinal tubule lumens (17, 18). It is not clear whether the differences in bacterial adhesion are due to age-induced changes in the dentin or the formation of more smear layer. However, it is reasonable to assume that the increase in adherence of *E. faecalis* in older teeth observed in the present study is due to the dentin material. Further studies are needed to evaluate the efficacy of inorganic components in the adhesion of *E. faecalis* to the root canal dentin.

Our microbial findings, supported also by the SEM and CLSM analyses, corroborate with results in the literature (12, 13) showing that the combined application of EDTA and NaOCl significantly decreased the biofilm of *E. faecalis*, but it did not totally eliminate all bacteria in the root canals. This residue of alive or dead bacteria attached on the surface might result in future reinfection of the root canal after chemomechanical preparation. Because higher amounts of bacteria were found in old root dentin, it might suggest that in old patients, the volume or contact time of irrigation solutions during root canal treatment should be much longer than in young patients to prevent reinfection.

Acknowledgments

This study was supported by the D. Walter Cohen Center for Middle East Dental Education and Hacettepe University Research Center Office (06.D05.205.002).

References

1. Kakehashi S, Stanley HR, Fitzgerald RJ. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral Surg Oral Med Oral Pathol 1965;20:340–9.
2. Love RM. *Enterococcus faecalis*: a mechanism for its role in endodontic failure. Int Endod J 2001;34:399–405.
3. Murray BE. The life and times of the Enterococcus. Clin Microbiol Rev 1990;3:46–65.
4. Sundqvist G, Figdor D, Persson S, Sjögren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998;85:86–93.
5. George S, Kishen A, Song KP. The role of environmental changes on monospecies biofilm formation on root canal wall by *Enterococcus faecalis*. J Endod 2005;31:867–72.
6. Kowalski WJ, Kasper EL, Hatton JF, Murray BE, Nallapareddy SR, Gillespie MJ. *Enterococcus faecalis* adhesin, Ace, mediates attachment to particulate dentin. J Endod 2006;32:634–7.
7. Steinberg D. Studying plaque biofilms on various dental surfaces. In: An YH, Friedman RJ, eds. Handbook of bacterial adhesion: principles, methods and applications. Totowa, NJ: Humana Press; 2000:353–70.
8. Kolenbrander PE, Andersen RN, Blehert DS, Egland PG, Foster JS, Palmer RJ Jr. Communication among oral bacteria. Microbiol Mol Biol Rev 2002;66:486–505.
9. Calas P, Rochd T, Michel G. In vitro attachment of *Streptococcus sanguis* to the dentin of the root canal. J Endod 1994;20:71–4.
10. Drake DR, Wiemann AH, Rivera EM, Walton RE. Bacterial retention in canal walls in vitro: effect of smear layer. J Endod 1994;20:78–82.
11. Love RM. Adherence of *Streptococcus gordonii* to smeared and nonsmeared dentine. Int Endod J 1996;29:108–12.
12. Yang SE, Cha JH, Kim ES, Kum KY, Lee CY, Jung IY. Effect of smear layer and chlorhexidine treatment on the adhesion of *Enterococcus faecalis* to bovine dentin. J Endod 2006;32:663–7.
13. Peters LB, Wesselink PR, Moorer WR. Penetration of bacteria in bovine root dentine in vitro. Int Endod J 2000;33:28–36.
14. Kakoli P, Nandakumar R, Romberg E, Arola D, Fouad AF. The effect of age on bacterial penetration of radicular dentin. J Endod 2009;35:78–81.
15. Ketterl W. Age-induced changes in the teeth and their attachment apparatus. Int Dent J 1983;33:262–71.
16. Morse DR. Age-related changes of the dental pulp complex and their relationship to systemic aging. Oral Surg Oral Med Oral Pathol 1991;72:721–45.
17. Love RM, Jenkinson HF. Invasion of dentinal tubules by oral bacteria. Crit Rev Oral Biol Med 2002;13:171–83.
18. Kinney JH, Nalla RK, Pople JA, Breunig TM, Ritchie RO. Age-related transparent root dentin: mineral concentration, crystallite size, and mechanical properties. Biomaterials 2005;26:3363–76.
19. Rigden MD, Baier C, Ramirez-Arcos S, Liao M, Wang M, Dillon JA. Identification of the coiled-coil domains of *Enterococcus faecalis* Div IVA that mediate oligomerization and their importance for biological function. J Biochem 2008;144:63–76.
20. Heling I, Irani E, Karni S, Steinberg D. In vitro antimicrobial effect of RC-Prep within dentinal tubules. J Endod 1999;25:782–5.
21. Basrani B, Tjäderhane L, Santos JM, et al. Efficacy of chlorhexidine- and calcium hydroxide-containing medicaments against *Enterococcus faecalis* in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003;96:618–24.
22. Steinberg D, Moreinos D, Featherstone J, Shemesh M, Feuerstein O. Genetic and physiological effects of noncoherent visible light combined with hydrogen peroxide on *Streptococcus mutans* in biofilm. Antimicrob Agents Chemother 2008;52:2626–31.
23. Kishen A, Sum CP, Mathew S, Lim CT. Influence of irrigation regimens on the adherence of *Enterococcus faecalis* to root canal dentin. J Endod 2008;34:850–4.
24. Portenier I, Waltimo TM, Haapasalo M. Enterococcus faecalis: the root canal survivor and star in post-treatment disease. Endodontic Topics 2003;6:135–59.
25. Marshall GW Jr. Dentin: microstructure and characterization. Quintessence Int 1993;24:606–17.
26. Ager JW 3rd, Nalla RK, Balooch G, et al. On the increasing fragility of human teeth with age: a deep-UV resonance Raman study. J Bone Miner Res 2006;21:1879–87.
27. Nazari A, Bajaj D, Zhang D, Romberg E, Arola D. Aging and the reduction in fracture toughness of human dentin. J Mech Behav Biomed Mater 2009;2:550–9.
28. Dogan Buzoglu H, Calt S, Gümüsderelioglu M. Evaluation of the surface free energy on root canal dentine walls treated with chelating agents and NaOCl. Int Endod J 2007;40:18–24.
29. Haapasalo M, Qian W, Portenier I, Waltimo T. Effects of dentin on the antimicrobial properties of endodontic medicaments. J Endod 2007;33:917–25.
30. Nallapareddy SR, Qin X, Weinstock GM, Höök M, Murray BE. *Enterococcus faecalis* adhesin, ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. Infect Immun 2000;68:5218–24.
31. Heling I, Rotstein I, Dinur T, Szwec-Levine Y, Steinberg D. Bactericidal and cytotoxic effects of sodium hypochlorite and sodium dichloroisocyanurate solutions in vitro. J Endod 2001;27:278–80.
32. Clegg MS, Vertucci FJ, Walker C, Belanger M, Britto LR. The effect of exposure to irrigant solutions on apical dentin biofilms in vitro. J Endod 2006;32:434–7.
33. Chivatxaranukul P, Dashper SG, Messer HH. Dentinal tubule invasion and adherence by *Enterococcus faecalis*. Int Endod J 2008;41:873–82.
34. Kishen A, George S, Kumar R. Enterococcus faecalis-mediated biomineralized biofilm formation on root canal dentine in vitro. J Biomed Mater Res A 2006;77:406–15.
35. Venegas SC, Palacios JM, Apella MC, Morando PJ, Blesa MA. Calcium modulates interactions between bacteria and hydroxyapatite. J Dent Res 2006;85:1124–8.