Basic Research—Technology

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

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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 medications 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.
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) (Mercck, 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% CO2. 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 were 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% CO2 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.

**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, 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 non-treated 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

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Age (y)</th>
<th>Mean (CFU)</th>
<th>SD</th>
<th>Minimum (CFU)</th>
<th>Maximum (CFU)</th>
<th>Median (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;30</td>
<td>289.13</td>
<td>98.31</td>
<td>175.33</td>
<td>444.67</td>
<td>275.84</td>
</tr>
<tr>
<td>EDTA</td>
<td>&lt;30</td>
<td>127.75</td>
<td>64.59</td>
<td>30.33</td>
<td>246.67</td>
<td>119.67</td>
</tr>
<tr>
<td>NaOCl</td>
<td>&gt;60</td>
<td>149.04</td>
<td>100.11</td>
<td>36.67</td>
<td>328</td>
<td>132.17</td>
</tr>
<tr>
<td>EDTA + NaOCl</td>
<td>&lt;30</td>
<td>24.79</td>
<td>12.28</td>
<td>7.33</td>
<td>40.33</td>
<td>27</td>
</tr>
<tr>
<td>Control</td>
<td>&gt;60</td>
<td>435.38</td>
<td>184.24</td>
<td>186.33</td>
<td>506.67</td>
<td>436</td>
</tr>
<tr>
<td>EDTA + NaOCl</td>
<td>&gt;60</td>
<td>122.38</td>
<td>77.26</td>
<td>19.67</td>
<td>237.33</td>
<td>117.5</td>
</tr>
<tr>
<td>NaOCl</td>
<td>&gt;60</td>
<td>159.63</td>
<td>104</td>
<td>47.33</td>
<td>377.33</td>
<td>133.5</td>
</tr>
<tr>
<td>EDTA + NaOCl</td>
<td>&gt;60</td>
<td>53.62</td>
<td>21.21</td>
<td>17.33</td>
<td>74.33</td>
<td>57</td>
</tr>
</tbody>
</table>

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.
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. 1B, 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. 2A, 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. 2B, 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.
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 Ca2+ 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 re-infection 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 re-infection.

### Acknowledgments

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### References

11. Love RM. Adherence of *Streptococcus gordonii* to surface might result in future re-infection 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 re-infection.

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