Evaluation of the Antibacterial Efficacy of Silver Nanoparticles against 

Enterococcus faecalis

Biofilm

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Abstract

Introduction: The purpose of this study was to evaluate the antibacterial efficacy of silver nanoparticles (AgNPs) as an irrigant or medicament against Enterococcus faecalis biofilms formed on root dentin. Methods: Dentin sections were inoculated with E. faecalis for 4 weeks to establish a standard monospecies biofilm model. These biofilms were tested in 2 stages. In stage 1, the biofilms were irrigated with 0.1% AgNP solution, 2% sodium hypochlorite, and sterile saline for 2 minutes, respectively. In stage 2, the biofilms were treated with AgNP gel (0.02% and 0.01%) and calcium hydroxide for 7 days. The ultrastructure of one half of the specimens from each group was evaluated by using scanning electron microscopy, whereas the structure and distribution of viable bacteria of the other half of the specimens were assessed with confocal laser scanning microscopy combined with viability staining. Results: Syringe irrigation with 0.1% AgNP solution did not disrupt the biofilm structure, and the proportion of viable bacteria in the biofilm structures was not different from that of the saline group (P > .05) but was less than that of the control group (P < .05). The biofilms treated with 0.02% AgNP gel as medicament significantly disrupted the structural integrity of the biofilm and resulted in the least number of post-treatment residual viable E. faecalis cells compared with 0.01% AgNP gel and calcium hydroxide groups (P < .05). Conclusions: The findings from this study suggested that the antibiofilm efficacy of AgNPs depends on the mode of application. AgNPs as a medicament and not as an irrigant showed potential to eliminate residual bacterial biofilms during root canal disinfection. (J Endod 2014;40:285–290)

Key Words

Antibacterial, biofilms, Enterococcus faecalis, silver nanoparticles

A mature bacterial biofilm consists of surface-adherent multilayer of bacteria embedded in an extracellular polymeric matrix. It is understood that no single mechanism may account for the general resistance of biofilm bacteria to antimicrobials, and different mechanisms act in concert within the biofilm to present the biofilm bacteria with considerably high resistance to antimicrobials (1, 2). Importantly, the current concept in endodontic microbiology emphasizes endodontic disease as a biofilm-mediated infection (3). Consequently, elimination or significant reduction of bacterial biofilms is an essential element for the successful outcomes of endodontic treatment. However, clinical studies have shown that even after meticulous chemomechanical disinfection and obturation of the root canals, bacterial biofilm may still persist in the root canal system (2). Thus, it is vital to develop advanced endodontic disinfection strategies that are effective in eliminating biofilm bacteria within the root canals (1).

Enterococcus faecalis is the predominant microorganism and occasionally the only species detected in root canals of teeth associated with persistent periodontal lesions (4). It is a hardy microbe that possesses certain virulence factors including lytic enzymes, cytolsin, aggregation substance, pheromones, and lipoteichoic acid (5). E. faecalis is able to invade dentinal tubules and remain viable within the tubules for prolonged period of time (6), adhere and form biofilm on dentin under different environmental conditions (7), resist intracanal disinfectants, and survive harsh conditions within root-filled teeth (5). Historically, efforts to eliminate E. faecalis and its concomitant biofilm have been somewhat limited while using commonly used root canal irrigants. Furthermore, biofilm models that use this bacterium have been used to test the efficacy of different disinfectants (8).

Sodium hypochlorite in concentrations from 0.5%–6% is the most commonly recommended root canal irrigant (9). Aqueous solution of sodium hypochlorite is a dynamic balance of sodium hydroxide and hypochlorous acid, which on interaction with microorganisms and organic tissue causes chloramination, amino acid neutralization, and saponification reactions leading to strong antibacterial and tissue-dissolving effects (10). Calcium hydroxide is the most commonly used intracanal medication during root canal procedures (11). Its antibacterial property is generally related to the release of hydroxyl ions, which produces the lethal effects on bacterial cells including protein denaturation and damage to the bacterial cytoplasmic membranes and DNA (12). However, the antimicrobial activity of sodium hypochlorite and calcium hydroxide can be inactivated by dentin, exudate from the periapical area, and microbial biomass (13). In addition, both sodium hypochlorite and calcium hydroxide do not always eliminate E. faecalis biofilms from the root canal system (4, 14).
Basic Research—Technology

Recently, silver nanoparticles (AgNPs) have been applied in many health care fields because of their broad-spectrum bactericidal and virucidal properties. AgNPs have high surface-area-to-volume ratio and unique chemical and physical properties, which result in increased reactivity (15). AgNPs show multiple antibacterial mechanisms such as adherence and penetration into the bacterial cell wall, leading to the loss of integrity of bacterial cell membrane and cell wall permeability (16). Previous studies suggested that AgNPs with size in the range of 10–100 nm showed powerful bactericidal potential against both gram-positive and gram-negative bacteria (17, 18), including the multidrug resistant bacteria (16). AgNP solution has been recommended as an alternative to root canal irrigating solution not only for its strong bactericidal potential but also for its biocompatibility, especially in lower concentrations (19). A previous study showed that 0.1% AgNP solution has a strong bactericidal effect against *E. faecalis* biofilm formed on dentin after 24 hours of exposure (20). However, no studies have characterized the application of AgNPs in the disinfection of root canal system. The objectives of this study were 2-fold: to evaluate the antibiofilm efficacy of (1) AgNP solution as an irrigant and (2) AgNP gel as a medicament against *E. faecalis* biofilm on root dentin.

**Materials and Methods**

**Preparation of Dentin Specimens**

Human single-rooted mandibular premolars with mature apices were collected for this study under a protocol approved by the Ethics Committee of the School and Hospital of Stomatology, Wuhan University. The crown and the apical portion of the teeth were sectioned off by using a diamond bur. The teeth were then vertically sectioned along the mid-sagittal plane into 2 halves, and the cementum was removed from the root surface by using a diamond bur. One hundred eighty dentin sections were prepared to the size of 4 \( \times \) 4 \( \times \) 1 mm (width \( \times \) length \( \times \) height). The smear layer was removed by placing the dentin sections in an ultrasonic bath of 5.25% sodium hypochlorite and 17% EDTA for 4 minutes each. Finally, all the dentin sections were rinsed in sterile distilled water for 1 minute and autoclave sterilized for 20 minutes at 121°C.

**Bacterial Inoculation of Specimens**

*E. faecalis* (ATCC 29212) was plated on BHI broth supplemented with 1.5% (wt/vol) agar (Biosharp, Hirono, Japan) and incubated anaerobically at 37°C for 24 hours. A single colony of *E. faecalis* from a BHI agar plate was collected and suspended in sterile BHI broth at 37°C. Sterilized dentin specimens were placed in sterile centrifuge tubes containing 3 mL *E. faecalis* suspension (1 \( \times \) 10^8 CFU/mL). The specimens were incubated under anaerobic conditions at 37°C for 4 weeks. Fresh BHI broth was replaced every second day to remove dead cells and to ensure bacterial viability.

After incubation, the specimens were removed from the tubes aseptically and gently rinsed with sterile phosphate-buffered saline (PBS) to remove the culture medium and nonadherent bacteria. Four dentin sections randomly selected were observed by a field emission scanning electron microscope (Hitachi S-4800, Ibaraki, Japan) to verify the presence of *E. faecalis* biofilms on the dentin surfaces.

**Stage 1: Antibacterial Activity of AgNP Solution**

The irrigants tested for antibiofilm activity were as follows: 0.1% AgNP solution (Huzheng Nano Technology Co, Ltd, Shanghai, China), 2% sodium hypochlorite (Baishi Chemical Co, Ltd, Tianjin, China), and sterile saline.

Ninety-six dentin sections were divided randomly into 4 groups of 24 specimens each. Control group received no irrigation. In experimental groups, the dentin sections were irrigated by using 6 mL of the irrigant listed above for 2 minutes. Each solution was delivered by using a syringe with a 27-gauge needle. After irrigation, the specimens treated by 2% sodium hypochlorite were neutralized with 5% sodium thiosulfate solution. All irrigation procedures were performed at room temperature under aseptic conditions by the same operator.

Twelve sections from each group were immersed in 4% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, dehydrated through ascending grades of ethanol, dried by critical point dryer, and sputter-coated with gold in a vacuum evaporator (Hitachi E-1045). The samples were examined with a field emission scanning electron microscope (Hitachi S-4800) or a scanning electron microscope (Hitachi S-3000N) for assessing the before and after irrigated biofilm structures.

The remaining 12 dentin sections from each group were stained with fluorescent LIVE/DEAD BacLight Bacterial Viability stain (Molecular Probes, Eugene, OR) and were viewed by using a confocal laser scanning microscope (CLSM) (Nikon A1Si, Tokyo, Japan). Two to 3 areas of the biofilm on each dentin section were scanned with a 2-µm step size by the CLSM. Simultaneous dual-channel imaging was used to display the green fluorescence (live cells) and red fluorescence (dead cells). CLSM images of the biofilms were analyzed and quantitated by using the software Nis-Elements AR (Nikon). The volume ratio of green fluorescence to total fluorescence indicated the proportion of live cells in the biofilm. Statistical analyses were performed by using one-way analysis of variance and Student-Newman-Keuls tests (SPSS 13.0; SPSS Inc, Chicago, IL) at a significance level of *P* < .05.

**Stage 2: Antibacterial Activity of AgNP Gel**

The 4 medicaments tested for this analysis were as follows: 0.02% and 0.01% AgNP gel (Huzheng Nano Technology Co, Ltd), calcium hydroxide (Sigma-Aldrich, Munich, Germany) (mixed with sterile distilled water at a ratio of 1:1.5), and sterile saline (negative control). Eighty dentin sections were divided randomly into 4 groups of 20 specimens each. The medicaments mentioned above were placed on the dentin surfaces from different groups and laid in sterile centrifuge tubes. All specimens were incubated anaerobically at 37°C for 7 days in a 100% humid environment (21). Then each specimen was washed with 5 mL sterile PBS to remove the tested medication. The specimens treated by calcium hydroxide were neutralized with 0.5% citric acid. Finally, 10 specimens of each group were evaluated by using the scanning electron microscope (Hitachi S-4800 or Hitachi S-3000N), and the other 10 specimens of each group were observed by the CLSM (Nikon A1Si) by using viability staining as described for stage 1. Statistical analyses were performed by using the nonparametric Kruskal-Wallis and Mann-Whitney *U* test by means of the SPSS 13.0 software.

**Results**

**Stage 1: Antibacterial Activity of AgNP Solution**

**Assessment of the Residual Biofilm Structure.** Figure 1A shows the typical 4-week-old *E. faecalis* biofilm model used as control group. Some biofilm structure and dentin surfaces without adherent biofilm structure were observed in the 2% sodium hypochlorite group (Fig. 1B). The integrity of the biofilm structure was not destroyed after irrigation with 0.1% AgNP solution (Fig. 1C) or saline (Fig. 1D).

**CLSM Analysis.** A homogenous and dense *E. faecalis* biofilm on dentin surface was observed in control group (Fig. 2A). After
2-minute irrigation, the *E. faecalis* biofilms were destroyed in 2% sodium hypochlorite group, with very little residual biofilm structure left on dentin sections (Fig. 2B). Most of the *E. faecalis* biofilms were intact in 0.1% AgNP solution (Fig. 2C) and saline groups (Fig. 2D).

The proportion of live bacteria in the biofilms of 2% sodium hypochlorite group could not be determined by viability staining and CLSM. No significant differences in the proportion of live bacteria in the *E. faecalis* biofilm were observed between 0.1% AgNP solution and saline groups (*P* > .05), but both of these groups showed less live bacteria than that of control group (*P* < .05) (Table 1).

**Stage 2: Antibacterial Activity of AgNP Gel Scanning Electron Microscopy Observations.** The biofilms treated with 0.02% and 0.01% AgNP gel exhibited different degrees of structural damages, with many bacterial cells remaining on the dentin surfaces (Fig. 1E and F). The biofilm structures treated with calcium

**Figure 1.** Representative *E. faecalis* biofilms on dentin surface were scanned by field emission scanning electron microscope (×3000 magnification). (A) Control group (4-week-old biofilm). (B) Two percent sodium hypochlorite–irrigated group (arrows: residual biofilms on dentin surface and isolated cells in dentinal tubule). (C) The 0.1% AgNP solution–irrigated group. (D) Saline-irrigated group. (E) Biofilm treated with 0.02% AgNP gel for 7 days. (F) Biofilm treated with 0.01% AgNP gel for 7 days. (G) Biofilm treated with calcium hydroxide for 7 days. (H) Biofilm treated with saline for 7 days.
Figure 2. CLSM 3-dimensional reconstructions of *E. faecalis* biofilms after irrigation and medication. (A) Control group (4-week-old biofilm). (B) Two percent sodium hypochlorite–irrigated group. (C) The 0.1% AgNP solution–irrigated group. (D) Saline-irrigated group. (E) Biofilm treated with 0.02% AgNP gel for 7 days. (F) Biofilm treated with 0.01% AgNP gel for 7 days. (G) Biofilm treated with calcium hydroxide for 7 days. (H) Biofilm treated with saline for 7 days (*green*, live cells; *red*, dead cells).
Hydroxide were disrupted, whereas some bacteria were still observed adhering to the dentin surfaces and in dentinal tubules (Fig. 1G). The treatment with saline did not influence the structure of E. faecalis biofilm (Fig. 1H).

**CLSM Analysis.** CLSM and 3-dimensional reconstructions of images showed that the biofilm structure in 0.02% AgNP gel (Fig. 2F) and 0.01% AgNP gel groups (Fig. 2F) was destroyed, and the biofilm bacteria were found to be aggregated as clusters of dead cells, with few live bacterial cells in the biofilm. Most of the E. faecalis biofilms were destroyed in calcium hydroxide group, whereas residual biofilm structure was still covering the dentin with a high proportion of live bacteria cells (Fig. 2G). The E. faecalis biofilms were intact in the saline group, and numerous live bacterial cells were observed along with a few dead cells (Fig. 2H).

The tested medications reduced the number of E. faecalis significantly when compared with saline (P < .05). The proportion of live E. faecalis in the residual biofilm structure in 0.02% AgNP gel groups was significantly less than that of 0.01% AgNP gel and calcium hydroxide groups (P < .05) (Table 2).

**Discussion**

Pulpal and periapical diseases are biofilm-mediated infections, and the elimination of bacterial biofilm from the root canal system remains the primary goal of root canal treatment (1). The nature of the biofilm structure and physiological characteristics of resident microorganisms render the biofilm bacteria protection or resistance against harmful exogenous influences including antimicrobial agents (22). The anatomic complexities of the root canal system, structure and composition of dentin, and factors associated with the chemical disinfectants also contribute to the current limitations in endodontic disinfection (1). Moreover, long-term exposure of bacteria to the disinfectants might induce resistance to subsequent exposure at levels that might normally be lethal (23). Therefore, the effective elimination of the biofilm structure and destruction of the resident bacteria remain important challenges in root canal disinfection.

Antibacterial nanoparticles such as chitosan nanoparticles exhibit significant antibacterial activity in biofilm disinfection of root canal (24). Incorporating chitosan nanoparticles into the zinc oxide–eugenol sealer could inhibit biofilm formation within the sealer-dentin interface (25), and these nanoparticles also exhibited the antibacterial property even after aging in saliva (26). Antibacterial nanoparticles did not provide the bacteria any ability to gain resistance against the antimicrobial (16). The pronounced antibacterial efficacy of cationic nanoparticles might be due to the fact that positively charged nanoparticles electrostatically interact with the negatively charged bacterial cells, resulting in altered cell permeability, leakage of intracellular components, and killing of bacteria (27). However, chitosan nanoparticles require sufficient interaction time to display significant antibiofilm efficacy, and tissue inhibitors existing within the root canal, such as the pulp tissues and bovine serum albumin, inhibit the antibacterial efficacy of chitosan nanoparticles (28).

**TABLE 1.** Proportion of Live Bacteria in the E. faecalis Biofilms after Irrigation (green)

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Scans</th>
<th>Green (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>33</td>
<td>96.67 ± 2.03a</td>
</tr>
<tr>
<td>2% sodium hypochlorite</td>
<td>12</td>
<td>30</td>
<td>Not measured</td>
</tr>
<tr>
<td>0.1% AgNP solution</td>
<td>12</td>
<td>32</td>
<td>92.33 ± 6.78b</td>
</tr>
<tr>
<td>Saline</td>
<td>12</td>
<td>32</td>
<td>93.34 ± 5.33b</td>
</tr>
</tbody>
</table>

aData labeled with different superscript letters are significantly different from each other (P < .05).

**TABLE 2.** Median and Range Values of the Proportion of Live Bacteria in the E. faecalis Biofilm after Treatment (green)

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Scans</th>
<th>Green (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02% AgNP gel</td>
<td>10</td>
<td>24</td>
<td>4.72 (0.05–33.06)a</td>
</tr>
<tr>
<td>0.01% AgNP gel</td>
<td>10</td>
<td>25</td>
<td>28.71 (6.13–54.58)b</td>
</tr>
<tr>
<td>Calcium hydroxide</td>
<td>10</td>
<td>24</td>
<td>32.55 (4.29–75.63)b</td>
</tr>
<tr>
<td>Saline</td>
<td>10</td>
<td>25</td>
<td>85.39 (64.36–98.2)c</td>
</tr>
</tbody>
</table>

aData labeled with different superscript letters are significantly different from each other (P < .05).
Optimum duration of interaction between AgNPs and biofilm bacteria was important to achieve significant antibiofilm efficacy with AgNPs.

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The authors deny any conflicts of interest related to this study.

References


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