Effectiveness of Nanoparticles Solutions and Conventional Endodontic Irrigants against Enterococcus faecalis Biofilm

Abstract

Context: To overcome the challenge imposed by the presence of biofilm and reach significant bacterial reduction of the root canals, many irrigants have been indicated during endodontic treatment, among them nanoparticles solutions. Aims: This study aims to evaluate the effectiveness of experimental solutions containing silver and zinc oxide nanoparticles (ZnO Np) and conventional endodontic irrigants against Enterococcus faecalis biofilm, in root canals. Methods: Seventy-six extracted human teeth were biomechanically prepared and sterilized. The root canal surface was exposed to E. faecalis suspension to form a 7-day-old biofilm. Four teeth were analyzed by scanning electron microscopy (SEM) to confirm the presence of biofilm. The remaining teeth were randomly divided into 6 groups (n = 12) and treated with passive ultrasonic irrigation and different solutions: G1 – 0.85% saline (control); G2 – 2% chlorhexidine gluconate (CHX); G3 – 5% sodium hypochlorite (NaOCl); G4 – 1% NaOCl; G5 – 1% silver nanoparticles (Ag Np) solution; and G6 – 26% ZnO Np solution. The susceptibility of E. faecalis biofilms to disinfecting solutions (n = 10) was determined by quantification of colony-forming units. SEM analysis was also carried out to examine the biofilm structure after treatments (n = 2). Data were analyzed by Kruskal–Wallis and Dunn post hoc tests (P < 0.05). Results: All tested solutions showed superior effectiveness compared to 0.85% saline (P < 0.05). Overall, 2% CHX presented the most effective action against E. faecalis biofilm, followed by 5% NaOCl, 1% Ag Np, 26% ZnO Np, and 1% NaOCl. Conclusions: 1% Ag Np and 26% ZnO Np were effective against E. faecalis biofilm similarly to conventional endodontic irrigants.

Keywords: Biofilms, Enterococcus faecalis, nanoparticles, silver, zinc oxide

Introduction

Endodontic infection has been recognized as a polymicrobial infection.[1] Among Gram-positive and facultative anaerobes frequently detected in persistent intraradicular infections, Enterococcus faecalis is the most prevalent.[2] The difficulty to eliminate this microorganism is not only due to the complex anatomy of the root canal system, which makes specific areas inaccessible to conventional instrumentation techniques,[3] but also due to its ability to penetrate the dentinal tubules[4] and organize as a biofilm.[5]

Trying to overcome the challenge imposed by the presence of biofilm and reach significant bacterial reduction or complete disinfection of the root canals, many irrigants have been indicated during endodontic treatment. The most commonly used are sodium hypochlorite (NaOCl) from 1% to 6%[6,7] and chlorhexidine gluconate (CHX) at 2%.[7,8] Ultrasonic devices have also been introduced in endodontics to achieve effective cleaning of the root canal walls, with emphasis to passive ultrasonic irrigation (PUI).[9]

Due to the limited disinfection action promoted by commonly used root canal irrigants, alternative solutions have been tested.[10] Positive antimicrobial and antibiofilm results were observed with the addition of silver nanoparticles (Ag Np)[11,12] and zinc oxide nanoparticles (ZnO Np)[13] to different dental materials. This encouraged the use of nanoparticles, such as Ag Np, to disinfect root canals during endodontic therapy as intracanal dressing or irrigant.[10,12] Similarly, ZnO Np solutions were used to prevent E. faecalis adherence to dentinal walls,[13] as well as to eliminate and disaggregate the biofilm structure.[14]

Considering the promising results concerning nanoparticles, the objective of...
the present investigation was to evaluate and compare the effectiveness of experimental solutions containing silver and ZnO Np and conventional endodontic irrigants against *E. faecalis* biofilm, in root canals.

**Methods**

**Specimen preparation**

Seventy-six noncarious, extracted single-rooted premolars were used. The teeth presented straight root canals, fully formed apices, and similar size. The similarity of root canals was confirmed by radiographs. The study was approved by the Institutional Review Board of the University (protocol number 359.070).

To standardize the root canal length, teeth were decoronated at 12 mm from the root end, and apical patency was established with a #10-K file (Dentsply Maillefer, Ballaigues, Switzerland). The canals were cleaned and shaped using #3 and #2 Gates Glidden drills by the crown-down technique, followed by Flexofile (Dentsply Maillefer) up to #35, at 1 mm from the apex (working length [WL]). A 3-mm deep reservoir, in the coronal aspect of the root canal, was created using a #4 Gates Glidden drill. Intermittent 1% NaOCl irrigation was carried out between each instrument. The final canal rinse was performed with 3 mL of 17% ethylenediaminetetraacetic acid followed by 3 mL of 1% NaOCl to remove debris.

The external surfaces of all specimens were made impermeable with two layers of epoxy adhesive (Araldite, Ciba-Geigy AS, Taboão da Serra, SP, Brazil) including the apical foramen. All root segments were individually mounted and fixated in a particular container, and the apparatus was sterilized with ethylene oxide gas (ACECIL, Central de Esterilização Com. Índ. Ltda., Campinas, SP, Brazil).

**Biofilm formation**

A standard strain of *E. faecalis* (ATCC 29212) was used. Previous to testing, the *E. faecalis* counts in brain heart infusion broth (BHI) (Difco Laboratories, Becton Dickinson and Company, Franklin Lakes, NJ) were determined by decimal dilutions. Aliquot portions (OD$_600$ ≈ 0.5) were plated on the surface of BHI agar and incubated at 37°C for 48 h. After the incubation period, the number of colony-forming units (CFUS)/mL was determined (≈10$^6$ CFUs/mL).

Under aseptic conditions, 0.1-mL aliquots of *E. faecalis* culture supplemented with 0.4% sucrose were inoculated into the canals with a sterile needle. The medium was replaced every 24 h, for seven days, by replacing BHI inoculated with *E. faecalis* with a new 100 µL aliquot of sterile BHI. The aliquot removed was tested to confirm bacterial viability. The apparatus was incubated at 37°C during the experimental period. To confirm biofilm formation, four roots were prepared and analyzed by scanning electron microscopy (SEM). Briefly, the roots were fixated in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), longitudinally sectioned, dehydrated through an ascending series of ethanol, mounted on aluminum stubs, sputter-coated with a 300-Å gold layer, and evaluated by SEM (Philips, SEM XL 30), operating at 10 or 15 kW and using magnifications between $\times$1000 and $\times$5000.

**Groups and disinfecting solutions**

The remaining root segments were randomly divided in six groups ($n = 12$) and treated with PUI and different solutions, as follows: G1 = 0.85% saline (control); G2 = 2% CHX; G3 = 5% NaOCl; G4 = 1% NaOCl; G5 = 1% Ag Np (TNS Nanotecnologia Ltda, Florianópolis, SC, Brazil) solution; and G6 = 26% ZnO Np (Nanum Nanotecnologia S. A., Belo Horizonte, MG, Brazil) solution. First, conventional irrigation with syringe was performed. A sterile needle was introduced into the root canal until 2 mm short of the WL and 2.5 mL of the specific solution was injected, for 2 min. After that, the solution was ultrasonically activated for 1 min using an endodontic tip E1 Irrisonic (Helse, Capelli e Fabris, São Paulo, SP, Brazil) mounted in a piezoelectric ultrasonic device (Gnatus Equipamentos Médico-Odontológicos Ltda, Ribeirão Preto, SP, Brazil). The ultrasonic instrument was used at 1 mm short of the WL. Finally, additional 2-min syringe irrigation with 2.5 mL was performed, totaling 5 mL of each solution. Thereafter, the root canals were washed with 2 mL of sterile saline solution.

Similar to the procedures previously described, two root segments of each group were prepared and evaluated by SEM. Attention was given to examine similar sites along the entire length of the canals using magnifications between $\times$1000 and $\times$5000.

**Colony counting**

Immediately after each irrigation protocol, three #35 sterile absorbent paper points (Tanari Industrial Ltda, Manacapuru, AM, Brazil) were consecutively inserted into the root canals until the WL. After 1 min, they were transferred to sterile Eppendorf tubes containing 1 mL of fresh saline. All tubes were vortex mixed for 1 min to dislodge bacteria from paper points. The bacterial suspension was serially diluted. Aliquot portions (100 µL) of 1000-fold and 10,000-fold dilution were taken from each sample and pipetted in triplicate onto the surface of BHI agar plates (Difco Laboratories), which were incubated at 37°C for 48 h. Then, the number of CFUs per plate was determined.

**Statistical analysis**

Assumption of normality was violated as shown by the Shapiro–Wilk test. Nonparametric data were analyzed using Kruskal–Wallis test, and the post hoc Dunn test was performed for multiple comparisons ($P < 0.05$). Analyses were carried out using SPSS software version 21.0 (SPSS
de Almeida, et al.: Intracanal solutions against E. faecalis biofilms

Inc, Chicago, IL, USA), and $P < 0.05$ was considered statistically significant.

**Results**

SEM examination confirmed the presence of a dense biofilm over the root canal surface after the 7-day period of biofilm formation.

After 0.85% saline treatment, the remaining biofilms contained $1.91 \times 10^7$ CFUs $\pm 1.84 \times 10^6$ CFUs [Figure 1]. Compared to the control group, all conventional and experimental solutions showed superior effectiveness against *E. faecalis* biofilm ($P < 0.05$) [Figure 1]. Nanoparticles solutions showed similar antimicrobial activity compared to conventional endodontic irrigants ($P > 0.05$), and none of them was able to reliably reduce counts to zero. Overall, 2% CHX showed the most effective action, reducing 76.81% of the CFUs compared to the control, followed by 5% NaOCl (70.02%), 1% Ag Np (57.28%), 26% ZnO Np (56.65%), and 1% NaOCl (55.97%).

The SEM images allowed visualization of the root canal surface and biofilm architecture [Figure 2a-f].

**Discussion**

The present study demonstrated that none of the tested solutions was able to completely eliminate *E. faecalis* biofilm. Previous studies also showed difficulty to eradicate biofilm from the root canal.[10,15] Not only the complexity of the root canal system and the presence of biofilm itself could hamper reaching a total disinfection but it is also possible that the 5-min period of biofilm exposure to the solutions in this study has not allowed the adequate biofilm elimination.[16] Nevertheless, all conventional and experimental solutions eliminated significantly more *E. faecalis* biofilm than the saline control.

Overall, 2% CHX was the most effective solution, followed by 5% NaOCl although there was no significant difference. The literature has reported inconclusive results when these two endodontic irrigants are used aiming at antimicrobial activity. Some studies have demonstrated that CHX is less effective at killing microorganisms organized as biofilm than NaOCl in high concentrations.[7,16] Despite the recognized antimicrobial action,[17] the limited chemical effect of CHX, in the present study, may have occurred due to its limited penetration into *E. faecalis* biofilm, with higher antimicrobial effect only on the outer layer of the biofilm.[19] On the other hand, several *in vitro* works showed that both solutions, 2% CHX and 5% or 5.25% NaOCl, have comparable antimicrobial effects,[17,19] being effective against several bacteria, such as *E. faecalis*.[17] NaOCl in high concentrations has a great amount of undissociated hypochlorous acid (HClO) in solution, which is directly related to the NaOCl disinfecting efficiency.[19] In the present investigation, possibly, the antibacterial effectiveness of 5% NaOCl was partially inhibited by the chemical environment of the root canal, more specifically by the dentin,[20] preventing the complete biofilm elimination.

Taking into consideration the mechanism of action of NaOCl, the results obtained using 1% NaOCl were probably due to the low concentration of HClO. Chemical
agents in lower concentrations allow the resistance of some microorganisms such as *E. faecalis*, mainly when organized as a structured biofilm.

A current tendency in dentistry, which has presented positive results with regard to antimicrobial and antibiofilm activities, is the use of nanoparticles, such as Ag Np[10‑12] and ZnO Np.[13,14] In the present study, Ag Np with size ranges from 5 to 20 nm provided capacity to significantly decrease the number of viable CFUs present in the biofilm. Other studies also demonstrated disinfection action promoted by Ag NP as solution or gel[10] or associated with intracanal medicament.[12] It is suggested that Ag Np with size range of 1–100 nm have a great bactericidal potential against both Gram-positive and negative bacteria, which could be linked to the high-contact, surface-dependent silver antimicrobial action.[21] Silver nanoparticles bind to the cell membrane leading to altered cell permeability, thus affecting the transport system through the plasmatic membrane. Hence, silver ions, released due to the oxidation of nanoparticles after binding to the cell membrane, are able to penetrate inside the bacteria and react with specific proteins. This alters the bacterial metabolism and inhibits vital enzymatic systems, such as respiratory process and cellular division, resulting in cell death.[21] Recently, a different result from the present study was published. The antibiofilm efficacy of 0.1% Ag Np solution used for 2 min against a 4-week-old *E. faecalis* biofilm was almost entirely limited (92.33% viable CFUs after irrigation), and there was no difference when compared to saline solution.[10] According to previous investigations, the rate of bacterial killing by nanoparticles depends on the concentration and duration of interaction.[13,14] Therefore, aside from the inferior period for biofilm formation used in the present study, Ag Np at 1% and 5-min irrigation protocol could positively influence the results compared to the saline control.

Similar to 1% Np Ag solution, reduction in viable CFUs was obtained after the irrigation protocol using 26% ZnO Np solution. Previous investigations also reported antibacterial efficacy of ZnO Np against *E. faecalis*.[13,14] Recently, a study showed significant reduction on the structure of a 7-day-old biofilm treated with ZnO Np.[14] Although the time of biofilm exposure to the ZnO Np was superior (24 h), the result matches the present research findings. A possible explanation for the antimicrobial action is similar to that previously described for Ag Np and might be related to the damage and disorganization of the bacterial cell wall caused by the smaller particles of ZnO Np used in this study (45 nm). Binding of ZnO Np to the bacterial cell might have altered the cell wall permeability, which leads to leakage of proteins and other components, and consequently, to cell death.[22,23] A possible explanation for the incomplete biofilm elimination after irrigation with 1% Ag Np and 26% ZnO Np solutions might be the excellent resistance of *E. faecalis* biofilm matrix. Organized as a biofilm, microorganisms are phenotypically and physiologically different from planktonics[24] and require higher concentration of antimicrobials.[25] In addition, the extracellular matrix produced by microbial cells during the biofilm maturation process may have served as a chemical barrier[26] and hampered nanoparticles diffusion through the bacterial cells.[27] An inadequate interaction between nanoparticles and bacterial cells during the 5-min period of root canal irrigation may also have affected the efficiency of nanoparticles.[10] Even after 24 h of *E. faecalis* biofilm treatment with Np ZnO, 10⁴ to 10⁵ live bacteria could be microbiologically detected.[14]

Although several studies have been performed to investigate the cytotoxic effects of nanoparticles,[28] controversies are still present due to their physical and chemical properties, such as particle size and surface characteristics.[29] Moreover, the type of human cell used and the amount of metal released inside the cells also influence the results about cytotoxicity.[30] Therefore, caution is necessary, and further experiments are required to evaluate if the effectiveness of 1% Ag Np and 26% ZnO Np against a bacterial biofilm can be improved after a longer period of irrigation or as intracanal dressing.

**Conclusion**

Within the parameters of the present study and regardless its limitations, 1% Ag Np and 26% ZnO Np were able to reduce *E. faecalis* biofilm in root canals, similar to conventional endodontic irrigants.

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**Conflicts of interest**

There are no conflicts of interest.

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