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Evaluation of Antibacterial Effects by Atmospheric Pressure Nonequilibrium Plasmas against *Enterococcus faecalis* Biofilms *In Vitro*

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Abstract

Introduction: The aim of this study was to evaluate the *in vitro* antibacterial activity by atmospheric pressure nonequilibrium plasmas (APNPs) as an effective approach against bacterial biofilms in root canal systems during endodontic therapy. **Methods:** Sterile cover slips were placed into the wells of tissue culture plates to permit the formation of *Enterococcus faecalis* biofilms. Biofilms were treated for 5 minutes with APNPs or 2% chlorhexidine digluconate (CHX). The viability of biofilm bacteria was analyzed by staining and confocal laser scanning microscopy. In addition, infected single-rooted teeth were exposed to APNPs or 2% CHX for 5, 10, and 15 minutes. After treatment, the root canals were flushed, and the resulting suspensions were inoculated onto brain-heart infusion agar to assess bacterial survival. Finally, micro–computed tomography scanning was used to observe and verify the root canal systems relative to the antibacterial effects obtained. **Results:** Treatment for 5 minutes with APNPs or 2% CHX killed the majority of bacteria in the *E. faecalis* biofilms. Moreover, APNP treatment was as effective as 2% CHX for inactivating bacteria in infected root canals ($P > .05$). Bacterial survival after treatment with APNPs or 2% CHX remarkably reduced with increasing exposure times ($P < .05$). There was no significant difference between bacterial survival in complex root canal systems and simple straight canals ($P > .05$). **Conclusions:** APNPs can be an effective adjunct to standard endodontic antimicrobial treatment. *(J Endod 2012;38:545–549)*

Key Words

Atmospheric pressure nonequilibrium plasmas, bacterial biofilms, endodontic disinfection, *Enterococcus faecalis*, root canal systems

Apical periodontitis is an inflammatory reaction of the periradicular tissues that is caused by a microbial infection in the root canal (1). Because bacteria in necrotic root canal systems grow mostly as sessile biofilms, the success of any endodontic treatment depends on the effective elimination of these biofilms and the preservation of the root canal in a disinfected state (2). Chemomechanical instrumentation is a key element of present endodontic treatment. Importantly, mechanical canal preparation can aid in disinfection because this process disturbs or detaches biofilms that adhere to canal surfaces, and this procedure can also remove a layer of infected dentin. However, several studies using advanced techniques, such as micro–computed tomographic (micro-CT) scanning, have shown that proportionally large areas of the main root canal wall can remain untouched by these mechanical instruments (3). In addition to mechanical preparation, irrigation solutions with strong antibacterial effects are necessary. However, none of the available irrigants used alone is capable of eliminating all of the biofilm bacteria from the root canal (4). The protective mechanisms underlying the antimicrobial resistance of biofilms are not fully understood although several mechanisms have been proposed (5, 6). These mechanisms include physical or chemical diffusion barriers that prevent antimicrobial penetration into the biofilm, the slow growth of bacteria in the biofilm due to nutrient limitation, the activation of the general stress response, and also the emergence of a biofilm-specific phenotype (7).

Some physical approaches have been used as assistant tools to enhance the effectiveness of traditional antimicrobial intracanal disinfection (8–12). However, the antibacterial activity of these methods can be limited in complex root canal systems. Recently, atmospheric pressure nonequilibrium plasmas (APNPs) have been shown to be useful for disinfection, and several studies have considered APNPs for root canal disinfection (13–16). Laroussi (17) was the first to show that APNPs are effective after sufficient exposure times for causing the complete destruction of living microorganisms. The plasma, which is called the fourth state of matter, is generated between 2 insulated metal plate electrodes that are powered by a low-frequency radiofrequency supply (18). The compelling advantage of APNPs is their ability to eliminate diverse microorganisms including bacteria, fungi, and viruses (19–23). APNPs kill microorganisms and deactivate viruses caused by the constant bombardment of short-living reactive species and charged particles (electrons and ions), especially free radicals (24, 25). A novel installation of the Model RC-1, a plasma biomedical application that our group specially designed and installed for root canal disinfection, has been developed, and it is certified safe to use. However, the capacity of new types of

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APNPs to disinfect root canals has not yet been established. Therefore, this study aimed to evaluate the antibacterial effects by APNPs against *Enterococcus faecalis* biofilms in root canal systems.

Materials and Methods

Bacteria Preparation

E. faecalis (American Type Culture Collection 29212) was inoculated onto Mueller-Hinton agar (Oxoid; Basingstoke, Hampshire, UK) and grown overnight at 37°C in an atmosphere of 5% CO_2 and 10% H_2 in nitrogen. Then, a colony of the pure-cultured strain was collected and suspended in sterile brain-heart infusion (BHI) broth (Becton, Dickinson and Co, Sparks, MD). Cell numbers were adjusted with sterile broth to an optical density (OD_{600}) of 0.1 to give a suspension at approximately 3.0×10^7 CFU/mL.

Tooth Specimens Preparation

Ninety-six extracted teeth containing a single-canal (confirmed by radiography) but lacking radicular pathology or deformity were collected and kept in 0.85% physiologic saline for 2 weeks at 4°C. Samples were decoronated at or below the cementoenamel junction with a low-speed diamond saw to give a uniform specimen with a root length of 12 to 13 mm. The patency of the apical foramina was completed by inserting a size 15 K-file (Dentsply Maillefer, Ballaigues, Switzerland), and then the working length was measured at the point where the file was visible at the apical foramen before 0.5 mm was subtracted to give the final length (26). Root canals were shaped with a ProTaper file (Dentsply Maillefer) to an apical size of F3 according to the manufacturer's instructions. After each instrumental procedure, canals were irrigated with 1 mL 5.25% sodium hypochlorite dispensed using a 30-G Maxiprobe needle (Dentsply Tulsa Dental, Tulsa, OK). After the shaping process was completed, the teeth were irrigated with a 17% EDTA solution for 5 minutes to remove the smear. Then, each specimen was submerged in 1 mL 0.85% saline in a 2-mL microcentrifuge tube for sterilization by autoclaving at 121°C for 20 minutes. The saline was discarded before any subsequent experimentation.

Set up of Model RC-1 for Generating APNPs

The main body of the Model RC-1 was composed of a medical syringe and a needle (Fig. 1*A*). The needle also acted as the electrode, and this was connected to a high-voltage submicrosecond pulsed direct-current power supply. For all following experimentation, the amplitude of it was set to 8 kV, the repetition rate was 8 kHz, and the pulse width was 1,600 ns. The APNPs generated by the Model RC-1 can be ejected from the needle of the syringe for root canal disinfection (Fig. 1*B*). The APNP's working gas was He/O_2, the flow rate of which was controlled by a mass-flow controller set at 1:0.01 L/min.

Antimicrobial Activity by APNPs against *E. faecalis* Biofilms on Cover Slips

A sterile cover slip was placed into each well of a 12-well tissue culture plate (Costar, Corning, NY). Two hundred microliters of *E. faecalis* suspension and 2.8 mL sterile BHI broth were transferred to each well. After incubation at 37°C for 1 week in anaerobic conditions, the broth was aspirated aseptically from each well. Then, 0.85% saline was added, and this was left for 3 minutes in order to remove the unattached bacteria and culture broth (27, 28). The *E. faecalis* biofilms on the cover slips were treated for 5 minutes with (1) APNPs, (2) the working gas only (with the plasma jet closed), (3) 3 mL 2% chlorhexidine digluconate (CHX; prepared freshly from 20% stock solution; Sigma Chemical Co, St Louis, MO), or (4) 3 mL 0.85% saline. The working conditions of the APNPs were 8 kV, 8 kHz, and 1600 ns, and the flow rate of He/O_2 was 1:0.01 L/minute as described previously. The needle tip of the Model RC-1 was placed 5 mm above the top of the biofilm. After the exposure time, each sample was washed gently with saline.

After the various treatments, the LIVE/DEAD BacLight Bacterial Viability kit L-7012 (Molecular Probes, Eugene, OR) containing SYTO 9 and propidium iodide (PI) was used to stain live and dead bacteria in the biofilms according to the manufacturer's instructions. Bacteria with intact cell membranes stain fluorescent green by SYTO 9, whereas bacteria with damaged membranes stain red by PI. The excitation/emission maxima for these dyes are approximately 480/500 nm for SYTO 9 and 490/635 nm for PI. The specimens were observed immediately using a confocal laser scanning microscopy (OLYMPUS FV500, Olympus, Japan) with the 40× lens. Images were captured using the Fluoview version 4.3 software (Olympus, Melville, NY).

Antimicrobial Activity by APNPs against *E. faecalis* Biofilms in Root Canal

Infection of Root Canal. The apical access of each specimen was sealed aseptically with Parafilm (Pechiney Plastic Packaging, Chicago, IL) to allow the formation of infected root canals. The 96 teeth were transferred into separate 2-mL sterile microcentrifuge tubes, and 20 μL of bacterial suspension (3.0×10^7 CFU/mL) was injected into the root canal system of each tooth using a needle. After injection, each sample was submerged in BHI broth and grown anaerobically at 37°C for 48 hours. After this incubation, the broth was removed entirely from the tubes.

Treatment with APNPs for Different Exposure Times. Infected teeth were randomly treated for 5, 10, and 15 minutes with APNPs or working gas only (with the plasma jet closed). The APNPs were generated according to the conditions described previously. For comparisons, groups of infected teeth were also irrigated with 2% CHX or sterile saline for corresponding time periods. Actually, 8 specimens were used per treatment group, and specimens were processed in sterile conditions. The needle generating the APNPs was inserted into the coronal patency of each root canal, which meant that residual bacteria in the main root canal, accessory canal, isthmuses, lateral canals, and dentinal tubules would be exposed to the plasma jet. To simulate the *in vitro* continuous chemical irrigation during nonsurgical endodontic therapy, tooth specimens in the irrigation treatment groups were injected with the agents using an irrigation needle and then submerged in the solutions for the indicated times. After irrigation, all solutions were removed completely. After canal irrigation, the remaining intracanal CHX was neutralized with 5 mL 3% Tween 80 and 0.3% a-lecithin.

After all treatments, the Parafilm in the patency of each apical foramen was discarded, and 1 mL of BHI broth was used to flush each root canal using an irrigating needle inserted into the coronal patency of the canal. During the flush, the contents of the root canal and bacterial solution were collected in a 2-mL sterile microcentrifuge tube, which was put below the access of apical foramen of each specimen. The solution was vortexed for 20 seconds, serially diluted, and 100-μL aliquots were inoculated onto BHI agar plates and incubated anaerobically for 24 hours.

Micro-CT Examination. A total of 16 infected teeth treated with APNPs for 10 and 15 minutes were examined using a SCANCO micro-CT scanner (SCANCO μ-CT 80; Medical AG Co, Bassersdorf, Switzerland). Each tooth was mounted in the sample holder on the scanning platform, and the root was oriented vertically. The micro-CT scanner was set at 60 kV, 40 μA, and 180° of rotary transmission. The distance

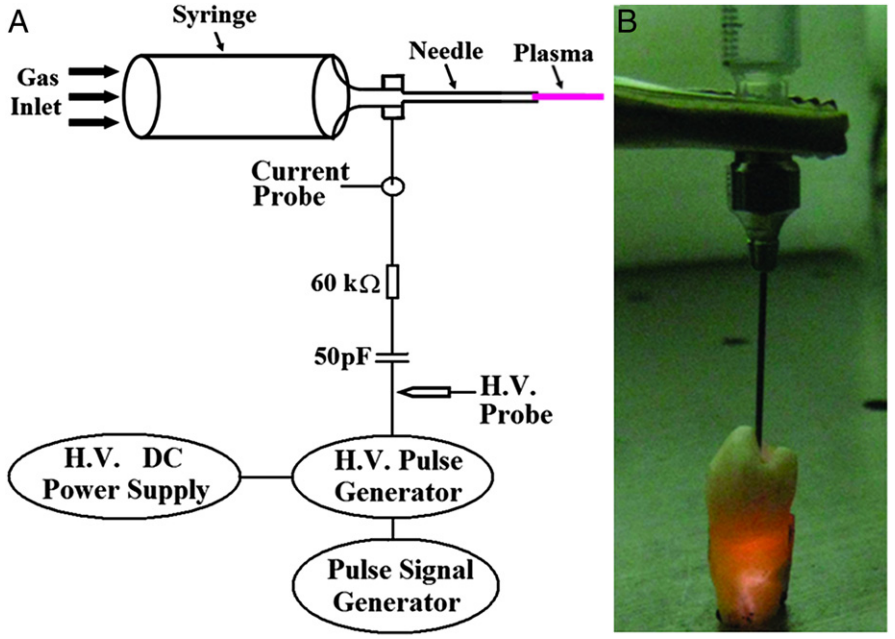

Figure 1. A photograph of the APNPs. (*A*) A schematic of the Model RC-1 experiment setup. (*B*) A photograph of the plasma jet acting in an infected root canal.

between each observed section was 25 μm. A series of cross-sectional images was obtained and reconstructed using the DICOM software package (OsiriX, Atlanta, GA). During reconstruction, the 3-dimensional images of the external surface of the root and the internal root canal were viewed for each specimen so that the external and internal morphology of each root could be measured.

Statistical Analysis

For the disinfection experiments, each treatment group contained 8 specimens, and CFU counts were transformed by $\log_{10}$ to reduce the variance. CFU values are presented as mean $\pm$ one standard deviation. Differences between groups were evaluated by one-way analysis of variance tests, with post hoc multiple comparisons using the least significant difference procedure or the Student-Newman-Keuls q test. All tests were performed using the SPSS statistics software (version 16.0; SPSS Inc, Chicago, IL), and the results were considered statistically significant when $P < .05$.

Results

Antimicrobial Activity by APNPs against *E. faecalis* Biofilms on Cover Slips

In the working gas only and saline groups, the biofilm structures in the confocal laser scanning microscopic images consisted of both live (green) and dead (red) bacterial cells in a multilayer architecture. The proportion of live bacteria was greater than the proportion of dead cells. Treatment with APNPs or 2% CHX reduced the abundance of viable bacteria in the biofilms (Fig. 2*A*).

Antimicrobial Activity by APNPs against *E. faecalis* Biofilms in Root Canals

Treatment with APNPs for Different Exposure Times. The mean $\log_{10}$ CFU data in the different treatment groups (Fig. 2*B*) revealed that for the same exposure times the APNPs and 2% CHX treatment groups were equally effective at reducing bacterial numbers ($P > .05$), and these treatments were remarkably more effective than treatment with working gas only or saline ($P < .05$). Although similar to the 2% CHX treatment, treatment with APNPs was more effective for killing microorganisms as the exposure time increased ($P < .05$).

Micro-CT Analysis. The root canal system of each specimen was scanned using micro-CT scanning. Five teeth (31.25%) were found to have accessory canals and lateral canals located in the apical third of the root, which were regarded as complex root canal systems (Fig. 2*C*). Figure 2*D* shows that mean $\log_{10}$ CFU levels were not significantly different ($P > .05$) in 10- and 15-minute APNP treatments between teeth having a complex root canal system and those that only had a simple straight root.

Discussion

The use of a new type of APNP device is a novel approach to eradicate microorganisms from root canal systems. The most attractive features of nonequilibrium plasma for biomedical disinfection application are the following: the gas temperature stays close to or at room temperature and the chemically reactive species generated by the plasmas have a relatively short lifetime. These advantages mean that the plasma is delivered into the root canals of teeth without any heating or painful irritation to living tissues (25). In an *in vitro* study, Sladek et al (24) also found that plasma disinfectants diffused toward irregular surfaces, such as cracks and fissures, in order to kill living microorganisms. These characteristics are necessary for disinfecting root canals *in vivo*. The latest portable Model RC-1 used in this study is specially designed for root canal disinfection, and it can conduct plasma jets via the needle of a medical syringe directly into the root canals. It is worth mentioning that the plasma jet generated by this equipment has the ability to touch complex root canal systems because the short-living reactive species, such as charged particles and free radicals, can spread into these areas. In this present study, APNPs and 2% CHX had similar effects with respect to endodontic antimicrobial treatment.

In this present study, samples were divided into 4 treatment groups: APNPs, working gas, 2% CHX, and saline. To evaluate the antibacterial efficacy of APNPs generated by the new Model RC-1 device, a dual-fluorescent method consisting of SYTO 9 and PI was used. The

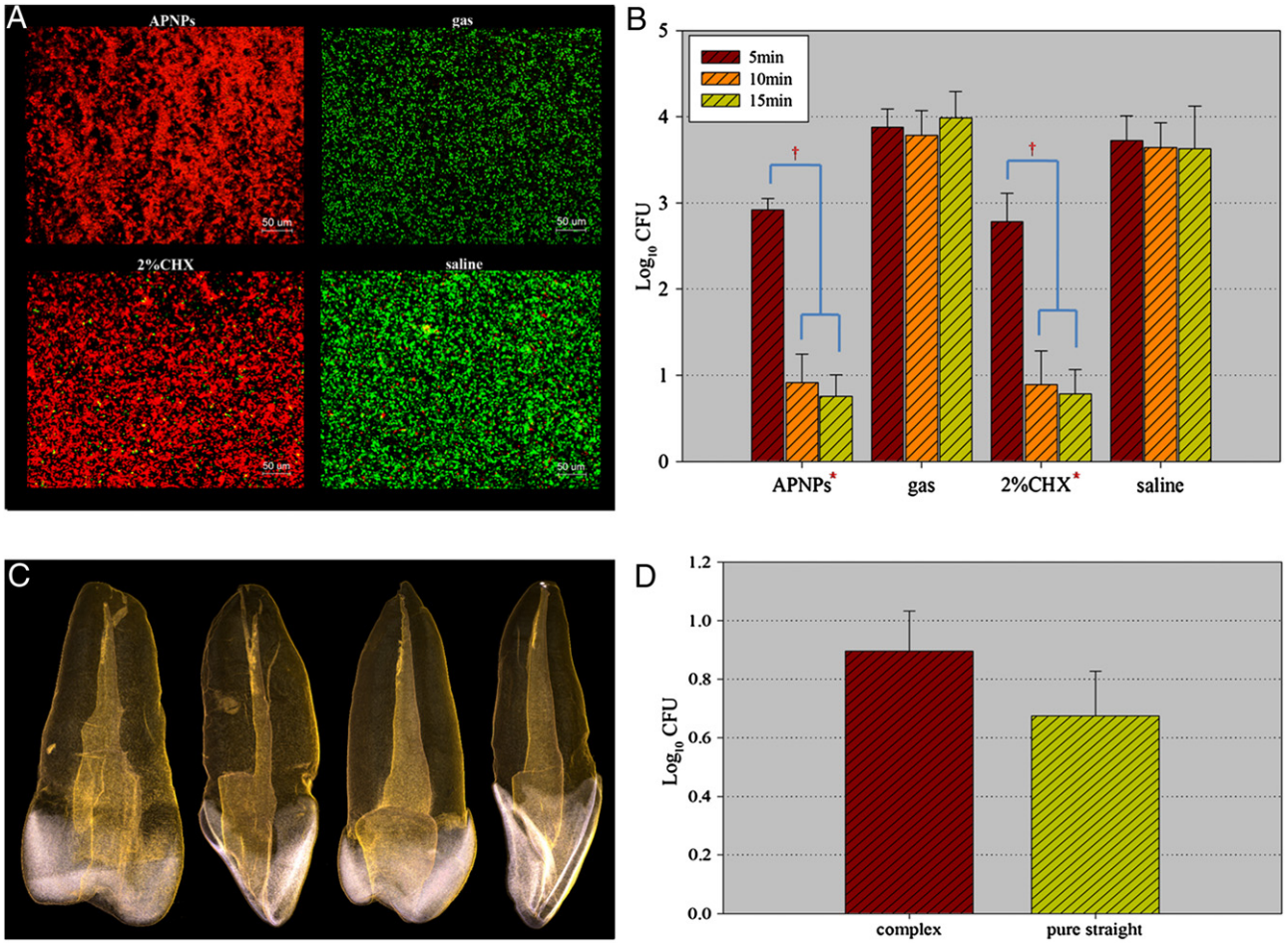

Figure 2. An evaluation of the antibacterial activity by APNPs against *E. faecalis* biofilms. (*A*) Confocal laser scanning microscopic images of biofilms after exposure to APNPs, working gas, 2% CHX, and saline for 5 minutes followed by viability staining (red [dead cells] and green [live cells]). (*B*) Mean $\log_{10}$ CFU levels in infected root canals after disinfection treatment for 5, 10, and 15 minutes. *A significant difference between treatments ($P < .05$). †A significant difference between exposure times ($P < .05$). (*C*) The 3-dimensional micro–CT images of infected root canals after treatment for 10 or 15 minutes with APNPs. The single-root teeth had complex root canal systems and pure straight canals. (*D*) The mean $\log_{10}$ CFU levels in the infected complex root canal systems and pure straight canals after APNP treatment.

saline treatment was a negative control because of its antibacterial ineffectiveness. CHX is regarded as the gold standard in antimicrobial endodontic therapy because of its broad-spectrum efficacy against various microorganisms (29), low irritation, and acceptable biocompatibility (30). Thus, 2% CHX was used as a positive control to compare with the APNP treatment. From the confocal laser scanning microscopic images, it was observed that APNPs and 2% CHX disrupted the integrity of the cell membrane and killed the major biofilm bacteria. During APNP treatment, short-living excited OH, O, N_2, N_2^+, and He species are present in the plasma jet, and these generate typical emission spectra between 300 and 800 nm (23) (Fig. 3). It has been reported that OH and reactive oxygen species, including O_3, metastable state O_2, and atoms O, have lifetimes in the millisecond range and play key roles in inactivation processes (25). However, whether APNPs are capable of dissolving tissue, removing dentin, or weakening tooth structure requires further investigation.

To evaluate the antibacterial efficacy by APNPs against bacterial biofilms in infected root canals, *E. faecalis* biofilms were formed in human single-canal teeth. The findings show that APNPs are effective at decreasing bacterial populations at levels similar to 2% CHX. Moreover, the results showed that a longer duration of APNP treatment improved the antibacterial efficacy, presumably because short-living reactive oxygen species were able to react continuously with molecules in bacteria to accomplish the disinfection, which is consistent with previous studies (13, 14, 25). Thus, APNPs killed more biofilm

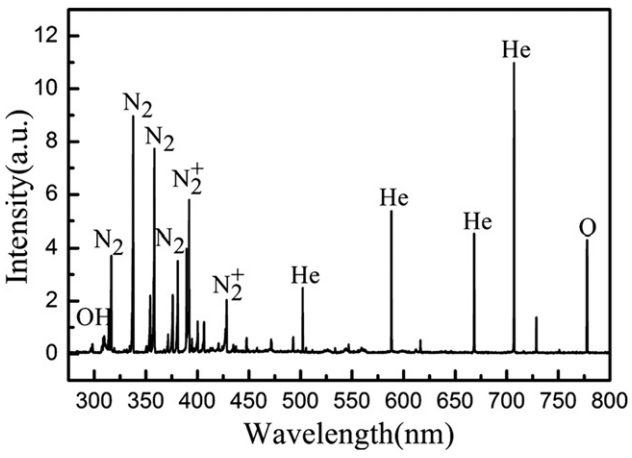

Figure 3. The optical emission spectra of the plasma jet. Working conditions were 8 kV, 8 kHz, and 1,600 ns, and the flow rate of He/O_2 was 1:0.01 L/min.

bacteria in infected root canals with an increasing exposure time. However, a longer exposure time increases the difficulty of root canal disinfection procedures. Flushing with broth led to the detachment of biofilm bacteria from the surface of the root canal and the openings of the dentinal tubules (26), which may have introduced some errors during the collection of bacteria for quantification. Notably, when paper points were used, fewer bacteria were obtained compared with flushing. A recent study showed that a sterile paper point could be placed in the lumen of the root canal for 60 seconds, and then this was transferred into tubes containing saline, which was vortexed for subsequent bacterial quantification by plating across agar (31). This sampling technique is an alternative method for collecting bacteria. Crushing of the root samples can also be used to assay resultant fragments for bacterial numbers, but this can give unreliable data because the files can destroy bacteria (26). Thus, of these options, we considered the flushing method to be most preferable compared with paper point and file procedures. The *in vivo* root canal is a closed-end channel, which often causes gas entrapment at the apical end, producing a vapor lock effect during the delivery of irrigants. Because of this limitation and the *in vivo* environment, caution is necessary when drawing conclusions from the results of the present *in vitro* study.

It has been reported that *E. faecalis* is detected in up to 38% of cases of failed root canal therapy (32). One important reason for treatment failure may be the complexity of root canal systems that may contain residual bacteria in the isthmuses, lateral canals, and dentinal tubules, which will reproduce and metabolize after the reintroduction of nutrition. From the 3-dimensional reconstructed images of single-root teeth treated with APNPs, root canal systems were found to be so complicated that more than 30% of the main root canals had several apical ramifications and lateral branches. Nevertheless, there was no significant difference between the reduction of bacterial populations in the pure straight root canals and in the complex collateral pulp canals. This finding confirmed that the excited species generated by APNPs could reach isthmuses, lateral canals, dentinal tubules, and even inactive parts of the root canal systems. Actually, CHX cannot be delivered to the most apical parts of the main root canal and fins by traditional approaches (4), and the resulting insufficient exposure time of the disinfecting compound will result in ineffective irrigation. Unfortunately, no direct evidence is yet available to confirm that reactive oxygen species can kill bacteria in these parts of the root canal systems. Although APNPs have been applied to inactivate microorganisms in nonsurgical endodontic treatments (15, 16, 24, 25, 33), this is the first *in vitro* study to evaluate the antimicrobial effectiveness by APNPs on complex infected root canal systems during endodontic therapy.

In conclusion, APNPs had antimicrobial activity against *E. faecalis* biofilms in infected root canals *in vitro*. The use of APNPs is an exciting novel product that is attractive for clinical use and may assist in antimicrobial endodontic therapy. Our future studies will focus on the treatment parameters and mechanisms of the APNP equipment, the distance of penetration of the short-living reactive oxygen species in the dentinal tubules, and the synergistic antimicrobial effects between APNPs and other bactericidal agents.

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

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