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

Tianfeng Du, PhD, Jingzhi Ma, PhD,* Ping Yang, MDS,* Zilan Xiong, PhD,† Xinpei Lu, PhD,† and Yingguang Cao, PhD**

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

From the *Department of Stomatology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, and †State Key Laboratory of Advanced Electromagnetic Engineering and Technology, Huazhong University of Science and Technology, Wuhan, China.

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Address requests for reprints to Dr Yingguang Cao, Chair, Department of Stomatology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan, China, 430030. E-mail address: cyg0729@tjh.tjmu.edu.cn

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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

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