Cold Plasma Therapy of a Tooth Root Canal Infected with Enterococcus faecalis Biofilms In Vitro

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Abstract

Introduction: Complete sterilization of an infected root canal is an important challenge in endodontic treatment. Traditional methods often cannot achieve high-efficiency sterilization because of the complexity of the root canal system. The objective of the study was to investigate in vitro the feasibility of using a cold plasma treatment of a root canal infected with Enterococcus faecalis biofilms.

Methods: Seventy single-root teeth infected with E. faecalis biofilms were divided into 7 groups. Group 1 served as the negative control group (no treatment), and group 7 was the positive control group with teeth treated with calcium hydroxide intracanal medication for 7 days. Groups 2 to 6 included teeth treated by cold plasma for 2, 4, 6, 8, and 10 minutes, respectively. The disinfection of the E. faecalis biofilm was evaluated by colony-forming unit (CFU) counting. Scanning electron microscopy was used to evaluate the structural changes of the E. faecalis biofilm before and after plasma treatment. Confocal scanning laser microscopy was used to investigate the vitality of the microorganisms in the biofilm before and after plasma treatment. Results: A significant decrease in the number of CFUs was observed after prolonged cold plasma treatment (based on the statistical analysis of the teeth in groups 2–6). Compared with the positive control group, cold plasma treatment of 8 or 10 minutes (groups 5 and 6) had a significantly higher antimicrobial efficacy (P < .05). The scanning electron microscopic analysis showed that the bacteria membrane was ruptured, and the structure of the biofilm was fully destroyed by the plasma. Confocal scanning laser microscopic studies indicated that the plasma treatment induced E. faecalis death and destruction of the biofilm.

Conclusions: The cold plasma had a high efficiency in disinfecting the E. faecalis biofilms in vitro dental root canal treatment. (J Endod 2013;39:105–110)

Key Words

Biofilm, cold plasma, disinfection, Enterococcus faecalis, root canal

Bacterial infection has long been recognized as the primary etiologic factor in the development of pulp and periapical lesions (1). The purpose of root canal treatment is to eliminate entirely the infection of the root canal system and prevent reinfection. However, traditional treatments such as mechanical debridement, chemical irrigation, laser irradiation, and ultrasound cannot achieve a complete elimination of biofilms from endodontic sites (2), with remaining cultures ranging from 40%–60% in those standard intracanal antisepsis strategies (3, 4). This is attributed to the complexity of the root canal system consisting of lateral canals, apical ramifications, isthmus, and apical deltas.

Many studies have shown that persistent endodontic infections are frequently caused by Enterococcus faecalis (5). E. faecalis can be easily destroyed in an open environment, but it becomes more resistant when growing in the root canal system (6). According to previous studies, proteases such as serine protease, gelatinase, and collagen-binding protein are secreted and easily bind to dentin firmly (7). Because of these biological properties, E. faecalis has the capacity to live in dentinal tubules and endure prolonged times of starvation (8, 9), and E. faecalis biofilms are orders of magnitude more resistant to phagocytosis, antibodies, or antimicrobials than planktonic bacteria (10, 11).

To eliminate bacteria not affected by the traditional treatment, interappointment intracanal medication has been recommended. Calcium hydroxide (Ca[OH]2) is the most commonly used intracanal medication (12, 13). Its antibacterial property stems from its high pH value (14). However, some studies reported that the application of Ca(OH)2 may still show E. faecalis resistance and survival after more than 10 days of treatment (15, 16). Furthermore, it has limited effectiveness in eliminating bacterial biofilms from human root canals (17, 18). In this situation, more effective disinfection methods are needed to eradicate E. faecalis biofilms from persistent root canal infections.

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In recent years, atmospheric pressure plasma technologies have been used in various biological and biomedical applications such as sterilization (19), gene transfer to cells (20), promotion of blood clotting (21), cell detachment (22), and inducing tumor cells apoptosis (23). Plasma-based dental applications have attracted much attention in applications such as dental material modification (24, 25) and tooth whitening (26, 27) because of the ability to operate the plasma at an ambient temperature and the ability of the plasma to generate high concentrations of highly reactive free radicals.

Root canal disinfection using plasma-based approaches has been reported before (28–31). In this study, we investigated the disinfection effectiveness of a novel cold plasma jet for in vitro inactivation of *E. faecalis* biofilms in root canals and compared the results with the effect of traditional Ca(OH)₂ intracanal treatment.

### Materials and Methods

#### Bacteria Culture

The *E. faecalis* (American Type Culture Collection 29212) was cultured in brain-heart infusion (BHI) broth under aerobic condition at 37°C. The bacterial concentration used in the experiments was adjusted to $1.5 \times 10^8$ colony-forming units (CFUs)/mL as measured by a spectrophotometer.

#### Root Canal Samples

Eighty-five single-rooted extracted human teeth were cleaned and stored in 0.1% thymol solution at 4°C before the experiments. The teeth were decoronated at 2–3 mm below the cementoenamel junction to obtain a standard root length of 10 mm, and pulp was extirpated using a barbed broach. The patency of the apical foramina was established by using a size 15 K-file. The working length was set by subtracting 1 mm of the length from the sectional surface to the anatomic apex. All specimens were prepared with nickel-titanium hand files (Mani Inc, Takanezawa, Japan) up to size #40 in a step-back manner. The canals were irrigated with 5.25% sodium hypochlorite (NaOCl). Subsequently, the smear layer was cleaned in an ultrasonic bath of 17% EDTA for 2 minutes and an ultrasonic bath of 5.25% NaOCl for 2 minutes. All root apical foramens of the specimens were sealed with composite resin (Clearfill AP-X; Kuraray Dental, Okayama, Japan). Finally, the samples were sterilized in an autoclave for 20 minutes at 121°C before further treatment (15).

#### Experimental Root Canal Infection

The 85 root canal specimens were placed in sterile microcentrifuge tubes with 1 mL BHI broth containing $10^8$ CFUs/mL of *E. faecalis*. Each specimen was incubated anaerobically for 7 days, and 1 mL sterile BHI broth was refreshed every 2 days to ensure viability of the bacteria (32). Seventy teeth were used in the disinfection experiments. Nine teeth and 6 teeth, respectively, were separated and prepared for scanning electron microscopy (SEM) and confocal scanning laser microscopic (CSLM) analysis.

#### Cold Plasma Device

A single electrode nonthermal atmospheric pressure plasma jet was used to treat the *E. faecalis* biofilms. Figure 1 shows a schematic diagram of the experimental arrangement (Fig. 1A) and a photograph of the in vitro plasma treatment of a single root canal (Fig. 1B). The cold plasma jet device consists of a Teflon tube (Daxiang Inc, Beijing, China), a 1-mΩ resistor, and an outer copper foil that surrounds the Teflon tube. The outer copper foil, serving as a single electrode, is connected to a 10-kHz sinusoidal high-voltage source with an 18-kV peak-to-peak voltage. The Teflon tube has outer and inner diameters of 10 and 7 mm, respectively. The diameter of the Teflon tube is reduced to 1.5 mm at the nozzle. Premixed argon and oxygen (98% Ar and 2% O₂ per volume, referred to as Ar/O₂[2%] from here on) is used as working gas and passed through the Teflon tube at a flow rate of 5 L/min. The plasma is generated inside the Teflon tube near the powered outer electrode and then propagates to generate a continuous plasma jet with a length of 5 cm outside the Teflon tube in the surrounding atmospheric pressure air. During each treatment, the distance between the tip of the plasma jet and the sample was approximately 5 mm, and the gas temperature near the top of the root canal ranged from 25°C to 31°C.

#### Disinfection of the Root Canal and Assessment

Seventy specimens were used to evaluate the effectiveness of the cold plasma disinfection of *E. faecalis* biofilms in root canals. After...
7 days of culturing, the biofilms were formed in the root canals. The teeth were randomly divided into 7 groups of 10 teeth (groups 1–7). The teeth in group 1 (ie, the negative control group) were incubated according to the previously described protocol but not subjected to plasma treatment or any other treatment. The teeth in groups 2–6 were treated by the cold plasma for 2, 4, 6, 8, and 10 minutes, respectively. The teeth in group 7 (ie, the positive control group) were treated by conventional intracanal dressing of Ca(OH)₂. After the canal was completely filled with Ca(OH)₂, the orifices were sealed by temporary restorative cement. The samples were put in a 1.5-mL centrifuge tube each, and 100 μL ultrapure water was added to keep a moist environment. They were placed in an incubator at 37°C and cultured for 7 days.

After plasma treatment of the teeth in groups 2–6, 15 μL ultrapure water was injected into the root canal. A smooth broach was inserted into the root canal and churned for 1 minute, and then the 2 #15 sterile paper points were inserted into the canal to draw the bacteria out. The paper points contaminated with the bacteria were put into a centrifuge tube with 1 mL ultrapure water, and the process described previously was repeated 3 times. The centrifuge tube was then shaken for 1 minute, and 100 μL of the liquid was removed to be cultured on a Petri dish for a count of the CFUs. The same procedure was performed with the samples in group 1. After incubation, the Ca(OH)₂ of the samples in group 7 was removed, and the effectiveness of Ca(OH)₂ was evaluated using the same approach described earlier.

**Scanning Electron Microscopic Analysis**

In an effort to investigate the structural changes of the biofilms after cold plasma treatment, the specimens were examined by using SEM. Nine of the processed specimens were kept with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate (pH = 7.4) for 48 hours at 4°C and then dehydrated sequentially in ethanol. Subsequently, they were grooved and split longitudinally, dried naturally, and mounted on a silicon slice. The mounted samples were sputter coated with gold palladium and examined with SEM.

**CSLM Analysis**

To confirm the viability of the biofilms after plasma treatment, 6 specimens were prepared for CSLM analysis. The specimens were split longitudinally with a diamond disk. Both halves were infected with *E. faecalis* as described previously. Seven days later, one half of every specimen was exposed to plasma treatment for 10 minutes; the other half served as part of a control group. Subsequently, all specimens were incubated with the reagents of the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, Carlsbad, CA) for 15 minutes. A laser scanning microscope (with an LSM 5 Exciter laser module; Zeiss, Oberkochen, Germany) equipped with a ×20 dry objective lens was used to analyze the *E. faecalis* biofilms. An argon ion laser (488 nm and 543 nm) was used as the excitation source of the reagents.

**Statistical Analysis**

Analyses were performed using the Games-Howell multiple-comparison post hoc test, and a *p* value <.05 was considered significant. Pair-wise comparisons were made between each plasma group (G2–G6) and the positive control group (G7) to determine if the plasma treatment was more effective than the Ca(OH)₂ treatment.

**Results**

**Effectiveness of Plasma Disinfection**

The antimicrobial efficacy of the cold plasma was evaluated by CFU counting. In this experiment, after 7 days of incubation, the initial total CFU count in a specimen reached $10^7$. All plasma-treated groups (G2–G7) revealed reduced CFUs of *E. faecalis* compared with the negative control group (G1), and the degree of inactivation increased with increasing plasma exposure time. Comparing the plasma-treated specimens with treatment times of 2, 4, and 6 minutes with those in the positive control (G7) showed that the plasma treatment was less effective than the conventional Ca(OH)₂ treatment. On the other hand, longer plasma exposure times of 8 and 10 minutes showed significantly better results than the positive control group (*p* < .05, Fig. 2). At a plasma exposure time of 10 minutes, there were no detectable residual CFUs in the samples.

**SEM**

The specimens in 3 groups (A–C) were included in this analysis. The specimens in group A were prepared root canals. The specimens in group B were prepared root canals infected by *E. faecalis* biofilms without plasma treatment, and the specimens in group C were prepared root canals infected by *E. faecalis* biofilms and subjected to a 10-minute plasma treatment. Pictures were taken at the apical one third of the root canals.

As shown in Figure 3, the smear layer of the root canal was entirely removed after preparation (group A). After 7 days of culturing, the *E. faecalis* biofilm successfully formed in the root canals, with a thickness of about 100 μm (group B-b). Many layers of bacteria accumulated around the opening of the dentinal tubules with numerous mycelia cross-linking (red arrows in group B-c,d). After 10 minutes of cold plasma treatment, we observed that the regular structure of the biofilm was destroyed and replaced with ruptured bacteria (group C-d). The mycelia and bacteria spheres in the *E. faecalis* biofilm on the surfaces of dentinal tubules disappeared (group C-c,d). It is noteworthy to state that the debris of the biofilm remained on the canal surfaces (group C-b).

**Confocal Scanning Laser Microscopy**

CSLM images were obtained from 2 halves of 1 specimen (Fig. 4a); 1 half was treated with plasma. In the untreated half of
the sample, most areas of the biofilm were green, which is indicative of live bacteria (Fig. 4B). In addition, live bacteria are also clearly observed in the dentine tubes. The plasma-treated part shows the biofilm with a pronounced red area indicative of dead bacteria and that is also true inside the dentine tubes (Fig. 4C). The thickness of the treated *E. faecalis* biofilms was similar to that of the control specimen (as indicated by the distance between the 2 arrows, Fig. 4C).

**Figure 3.** Scanning electron microscopic micrographs of group A (prepared root canal), group B (*E. faecalis* biofilm infected root canal), and group C (*E. faecalis* biofilm infected root canal after 10 minutes of plasma treatment) at magnifications of (A) 50×, (B) 1,000×, (C) 3,000×, and (D) 10,000×. The distance between 2 arrows (eg, group B-b and group C-b) refers to the thickness of the *E. faecalis* biofilm. The arrows in group B-c,d indicate mycelia in the biofilm.

**Figure 4.** (A) CSLM images obtained from the midarea of root canals (box). (B) Bacteria in the untreated *E. faecalis* biofilms, which are all alive (green areas). (C) Bacteria in the treated biofilm, which are all dead after 10 minutes of plasma treatment in the root canal (red areas). The distance between 2 arrows represents the thickness of the biofilm.
**Discussion**

*E. faecalis* is known to have a high resistance to antibacterial substances and is therefore often detected in persistent apical lesions or retreatment cases (5, 6). Eliminating the residual microorganisms within the biofilm in the complex root canal system is a challenging task. The antibacterial activity of different irrigants and intracanal dressings was tested. NaOCl is a widely used irrigant in the treatment of root canal infections. It can cause large zones of inhibition against *E. faecalis* (33). Chlorhexidine (CHX) also has the ability to inactive *E. faecalis*, but the efficacy depends on the concentration and the type (liquid or gel) of the irrigant (34). Ca(OH)₂ is widely used as intracanal dressing (12, 13). Although *E. faecalis* is less sensitive to Ca(OH)₂ (16, 17), there is evidence *in vitro* showing that Ca(OH)₂ has a similar bactericidal effect as CHX and Ca(OH)₂ mixed with CHX (35). Therefore, the intracanal dressing with Ca(OH)₂ for 7 days was set as the positive control (group 7) in this study. Comparisons with other irrigants and intracanal dressings will be the subject of future experiments.

Either irrigation or intracanal antimicrobial medication inactivates most of the endodontic bacteria within the biofilm when in direct contact. However, the anatomic variation of the root canal system makes it difficult for these liquid chemicals to penetrate into all infected sites (2, 4, 30). Atmospheric pressure cold plasmas are a gas-phase alternative consisting of charged particles and chemically reactive species. The overall gas temperature is at or around the room temperature. The biggest advantage is its capability to reach deep into the infected sites in the complex root canal system. Thus, it is a novel method of root canal disinfection.

We used a single-electrode, alternating current–driven, nonthermal atmospheric pressure plasma jet with Ar/O₂(2%) as the working gas to treat *E. faecalis* biofilms in root canals. The CUF counts showed that the plasma-induced inactivation of *E. faecalis* biofilms increased with the exposure time and that complete inactivation was achieved in 10 minutes. It was inferred that a certain dosage of ions and reactive species is needed to achieve a better bactericidal effect. The temperature evaluated near the top of the root canal was only slightly above room temperature, which excludes undesirable thermal effects. Scanning electron microscopic photos showed that untreated biofilms consisted of a compact and homogeneous structure, whereas the structure of the biofilms was destroyed after plasma treatment. In addition, confocal scanning laser microscopy verified that the entire biofilm covering the root canal had been completely inactivated. It is noteworthy to mention that complete inactivation was not observed in the positive control group (Ca(OH)₂ treatment for 7 days) in which approximately 10–100 CFUs remained in the canals. The result is similar to what was reported in former studies (15, 16).

We believe that the effective inactivation of *E. faecalis* biofilm in root canals can be attributed to the excited species, charged particles, and ultraviolet radiation generated in the atmospheric nonthermal plasma jet. Possible mechanisms contributing to the inactivation are (36) direct destruction by ultraviolet irradiation and energetic charged particles; erosion of the microorganisms by atomic oxygen or other radicals emanating from the plasma jet; destruction of the matrix of the extracellular polymeric substance in the biofilm as well as rupture of bacteria membranes by a strong coulombic force caused by charge accumulation; and inactivation by long-lived reactive species dissolved in water on the surface of the root canal system, which subsequently produce reactive radicals (such as -OH and O₂- (37). Further detailed study of the mechanism of *E. faecalis* inactivation is the focus of future work. Under the operating conditions used in the current studies, the use of atmospheric pressure cold plasmas has the distinct advantage compared with the conventional procedure used in endodontic clinical applications of achieving full inactivation in a short period of time.

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**References**