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ORIGINAL ARTICLE

Oral bacterial inactivation using a novel low-temperature atmospheric-pressure plasma device

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novel low-temperature atmospheric-pressure plasma;
sterilization *D*-value

Abstract *Background/purpose:* Atmospheric-pressure plasma is a new technology for biomedical applications. Utilization of an ionized gas (plasma) to achieve disinfection is an alternative sterilization technique that has become popular recently due to its safety, cost effectiveness, and superior performance to traditional methods. The purpose of this study was to evaluate the germicidal effectiveness of a low-temperature atmospheric-pressure plasma device by treating *Enterococcus faecalis* for different durations.

Materials and methods: A novel low-temperature atmospheric-pressure plasma device was developed for this study. A suspension of *E. faecalis* (BCRC 10789) was standardized to 10^7 colony-forming units (CFUs)/mL, as confirmed by an optical spectrophotometer. *E. faecalis* was first transferred and spread on 70 sterile cover glasses measuring 18 mm^2. Each batch of 10 specimens was exposed to the low-temperature plasma device and treated for 1 minute, 2 minutes, 3 minutes, 5 minutes, 10 minutes, and 15 minutes; the specimen treated for 0 minute served as the control. The cover glasses containing plasma-treated bacteria were then immersed into 10 mL deionized distilled water and vibrated with an ultrasonic device to detach the residual fluid. Bacterial colonies were finally inoculated into Luria—Bertani agar plates and cultured at 37°C for 24 hours. The numbers of bacterial colonies were counted to evaluate the germicidal efficacy of the plasma device, and the results were expressed as CFUs. Meanwhile, field emission scanning electron microscopy was performed to observe the cell morphology of *E. faecalis* prior to and after plasma treatment.

Results: Quantitative analysis of sterilization revealed a reduction in the number of bacterial colonies with time duration. When specimens were treated for 10 minutes, colonies of *E.*

Conflicts of interest: The authors declare no conflicts of interest.

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faecalis decreased from 10^5 CFUs to 10^2 CFUs. The sterilization *D*-value (90% cell reduction) of experiments was 2 minutes.
Conclusion: The novel low-temperature atmospheric-pressure device was capable of achieving effective sterilization of *E. faecalis* within a 2-minute interval. Further studies are needed to validate complete inactivation, refine the laboratory-made low-temperature plasma device, and develop a new plasma-jet device, which will be superior to traditional sterilization methods and can be used in root canal environment. This novel sterilization method can also be used as a clinical reference tool.

Introduction

There are many methods for sterilization, which use heat, chemical agents, radiation, pulsed light, and plasma. Each sterilization method has its own advantages and shortcomings. Physical methods, such as thermal sterilization, cannot be used for heat-sensitive materials. The chemical method that uses ethylene oxide is no longer recommended, as ethylene oxide is carcinogenic and requires a long time for evaporation prior to use. Atmospheric-pressure plasma is a new technology for biomedical and environmental applications. In recent years, sterilization by low-temperature plasma has become more common in a variety of fields, such as material science, disinfection medicine, and military medicine. It is generally agreed that low-temperature plasma is able to overcome many of the limitations of traditional sterilization techniques and is regarded as one of the most promising sterilization methods.[1,2] Plasma sterilization has a number of advantages, including its safety and efficiency, shorter sterilization time, low processing temperature, and use in both living and nonliving tissues.[1–3] In recent years, plasma sterilization has been used widely in both living and nonliving materials.[2–4]

Plasma is a partially ionized gas composed of charged electrons, ions, uncharged neutral gas molecules, atoms, and free radicals, as well as UV photons.[5] The term of plasma was introduced in the field of physics by Irving Langmuir in 1928 and is considered to be the fourth state of matter. Different types of energy sources, including heat, electrical current, electromagnetic radiation, laser, and so on, can be used to produce or create various forms of plasma. The most common method of producing plasma is to pass an electrical current through a gas. Power sources can be used at different frequencies, such as alternating current, direct current, radiofrequency, microwave, low frequency, and medium frequency, to generate discharges.[6,7] Many past studies demonstrated that low-temperature plasma is able to overcome many of the limitations of traditional sterilization techniques and is regarded as one of the most promising sterilization methods used in clinical medicine.[1,2,4,6,7]

Bacterial infection has long been recognized as a polymicrobial infection and agreed to be the main cause of pulpal and periradicular diseases. The goal of root canal therapy is to eliminate the pathogenic microorganisms from the root canal system and prevent reinfection. A positive correlation exists between the number and species of bacteria in an infected root canal and treatment failure. Many previous studies have demonstrated that endodontic failures are associated with Gram-negative anaerobic rods and facultative organisms. It is generally believed, as reported in some recent studies, that the major causes of persistent endodontic infections are frequently related to the survival of *Enterococcus faecalis* in root-filled tooth.[8] Proteases such as serine protease, gelatinase, and collagen-binding protein are secreted by *E. faecalis* and bind to dentin. Consequently, *E. faecalis* can survive in the dentinal tubules of the root canal system, as its biofilm structure has synergistic sustenance and becomes more resistant to traditional root canal cleansing.[9,10] *E. faecalis*, therefore, can endure prolonged time of starvation and become a recalcitrant microorganism known to be a causative factor for persistent pulpal and periradicular infections.

The purpose of this study was to evaluate the effectiveness of oral bacterial inactivation of a novel laboratory-made low-temperature atmospheric-pressure plasma device. *E. faecalis* (ATCC 29212) bacteria were treated with the plasma device for different time intervals, from 0 minute to 15 minutes. The germicidal and sterilization efficiencies found in this study can be used as a clinical reference guide and to develop a new plasma-jet device for endodontic usage.

Materials and methods

Low-temperature atmospheric-pressure plasma device

A novel, portable, low-temperature atmospheric-pressure plasma device was designed and developed by the Department of Biomedical Engineering, Chung Yuan Christian University, Taiwan, for this study (Fig. 1). The device was a dielectric barrier discharge (DBD) reactor and used radiofrequency power supply. The main principle of operation of the DBD reactor was to apply an energetic electric field between two electrodes that were separated by a barrier, to generate primary electrons. The reactor of the plasma device was supplied with air, frequency was set at 2.6 kHz, mean energy was 7.6 mJ, and excitation power was 1.1 W.

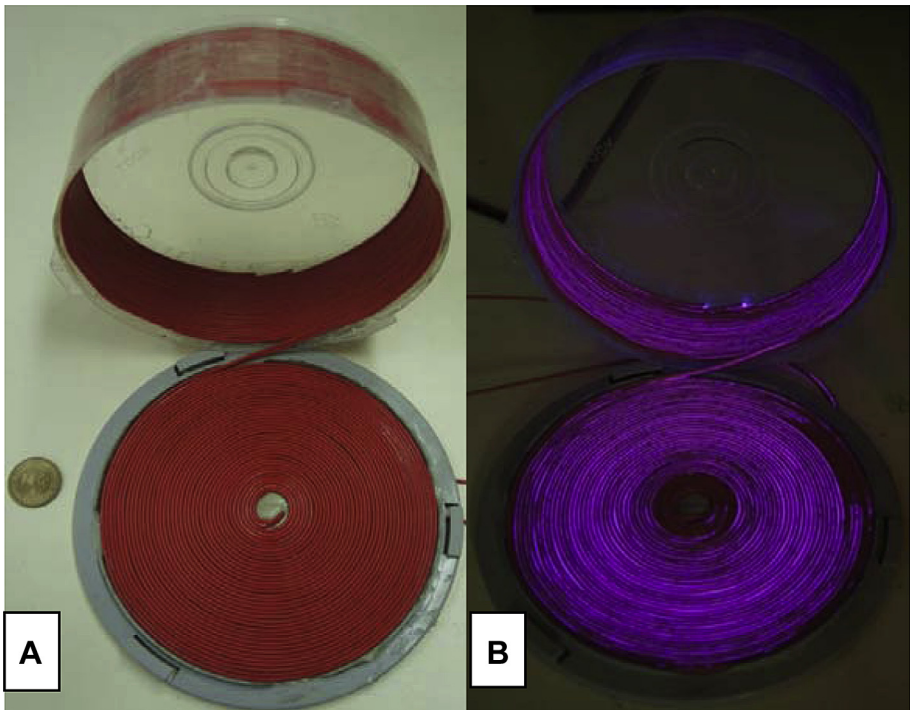

Figure 1 Novel low-temperature atmospheric-pressure plasma device: (A) before emission; (B) after emission. Various parameters of the device were as follows: gas—air, frequency—2.6 kHz; mean energy—7.6 mJ; and excitation power—1.1 W.

Optical emission spectroscopy

Optical emission spectroscopy (OES) was performed to observe and verify the neutral oxygen atom emission lines, neutral helium emission lines, hydroxyl emission lines, and emission lines of many neutral and atomic species of nitrogen. The effectiveness of plasma device was tested if the active species was present in the novel low-temperature atmospheric-pressure plasma. Light irradiated by plasma was captured using an optical emission spectrometer (Ocean Optics Inc., Dunedin, FL, USA).

Experimental procedures

E. faecalis (BCRC 10789) was obtained from the Bioresource Collection and Research Center of Taiwan. Suspensions of *E. faecalis* were diluted 30-fold in broth, and 22 mL of the 1 in 30 dilution [concentration of 10^7 colony-forming units (CFUs) per milliliter] that were verified by an optical spectrophotometer at the Microbiology laboratory of Taichung Veterans General Hospital. The bacteria samples (10 μ/mL) were transferred and spread on 10 sterile cover glasses measuring 18 mm $\times$ 18 mm, and then dried at room temperature. Each batch of 10 samples was then exposed to the low-temperature atmospheric-pressure device for 0 minute (as the control group), 1 minute, 2 minutes, 3 minutes, 5 minutes, 10 minutes, and 15 minutes; experiments were repeated three times to ensure the stability and accuracy of the equipment. After the sterilization treatment, each cover glass was placed in a test tube containing 10 mL deionized distilled water, and then shaken with an ultrasonic device for 5 minutes. A volume of 1 mL was taken from the colonies suspended in water, inoculated into Luria—Bertani agar plates, and cultured at 37°C for 24 hours. The numbers of bacterial colonies were counted to determine the efficacy of bacterial inactivation of the plasma device. The results were expressed as CFUs. The germicidal efficiency was calculated using the following formula:

$$\text{Germicidal efficiency} = (N_0 - N_t)/N_0 \cdot 100\% \quad [1]$$

where N_0 represents the number of colony-forming units of control, whereas N_t represents the number of CFUs after treatment with plasma.

Morphological examination

Ten samples were also used for field emission scanning electron microscopy (FE-SEM; JSM; JEOL, Japan) examination of the cell morphology of *E. faecalis* prior to and after plasma treatment. All the treated samples were coated on the cover glasses that were set prior to and after plasma treated were initially fixed with 4% glutaraldehyde, and stored at 4°C for 12 hours. The specimens were then dehydrated with gradient ethanol (15%, 30%, 50%, 70%, 85%, 95%, and 100%, twice), and stored in a refrigerator at −80°C for 30 minutes. The cover glasses were finally freeze dried and coated with gold for SEM examination. Images were recorded for analysis.

Results

Examination of active species of plasma

During this study, OES was performed to examine the plasma species present in the novel low-temperature

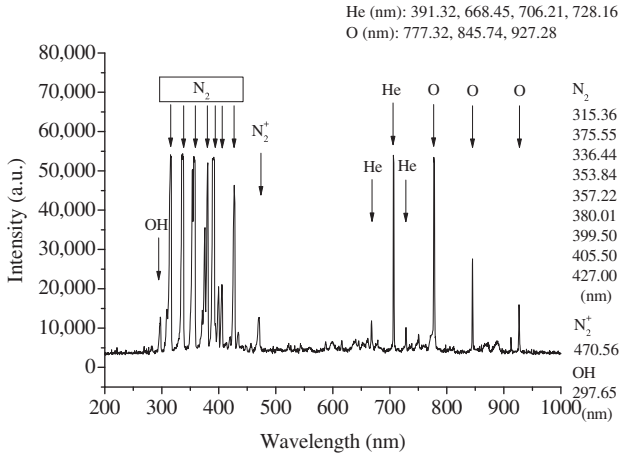

Figure 2 Wavelength of the novel low-temperature atmospheric-pressure plasma examined by optical emission spectroscopy.

atmospheric-pressure plasma device. The results showed that common species of plasma, such as neutral helium radiation lines, appeared at 391.32 nm, 668.45 nm, 706.21 nm, and 728.16 nm, whereas neutral oxygen atom radiation lines were detected at 777.32 nm, 845.74 nm, and 927.28 nm. Furthermore, emission lines of plasma species containing hydroxyl radicals were observed at 297.65 nm; emission lines of neutral and atomic species of nitrogen were also found. The results of OES, therefore, confirmed that the novel low-temperature atmospheric-pressure plasma was capable of destructing microorganisms (Fig. 2).

Analysis of plasma sterilization

The germicidal efficacy of novel low-temperature atmospheric-pressure plasma was evaluated, and the results are shown in Fig. 3. The numbers of bacterial colonies were cultured until it was possible to count for the purpose of verifying the inactivation effects of plasma-treated specimens. Experiments revealed that the numbers of bacterial colonies were obviously reduced in proportion to the duration of plasma exposure time. After oral bacteria were exposed to plasma for 2 minutes, the number of *E. faecalis* colonies reduced to almost 10^3 CFUs. Moreover, if the plasma exposure lasted for 10 minutes, the number of *E. faecalis* colonies reduced markedly from 10^7 CFUs to 10^2 CFUs, as shown in Fig. 4.

Analysis of germicidal efficiency by plasma device

Germicidal efficiency and sterilization *D*-values were determined to demonstrate the sterilization performance of the plasma device. *D*-value was defined as the time required to decrease the count of microorganism population by 90%, which is estimated based on the inverse of the slope of the survival curve.[11] The characteristic *D*-value was calculated from the data of plasma-treated bacteria using the following equation:

$$t = D_T\,[\log_{10}(N_0) - \log_{10}(N_t)] = D_T\,[\log_{10}(N_r)] \qquad [2]$$

where t is the treatment time in minutes and D_T is the *D*-value in minutes for a single decimal reduction of bacteria. The *D*-value of *E. faecalis* species in this experiment was measured at 2 minutes, and the germicidal efficiency of the treatment time is shown in Fig. 5.

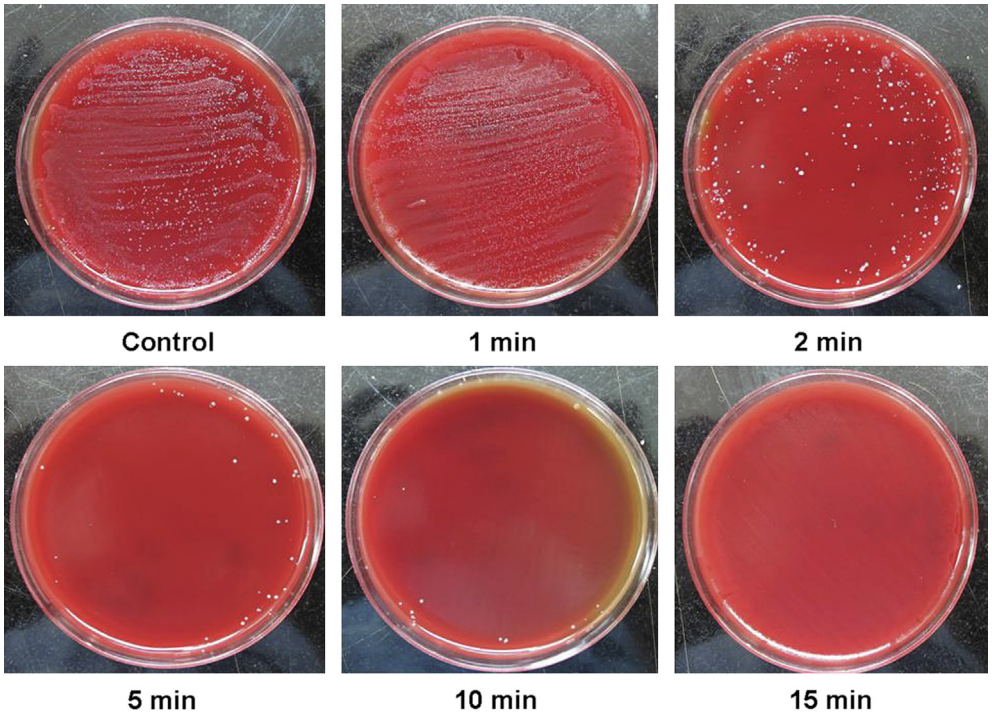

Figure 3 Germicidal efficacy of the novel low-temperature atmospheric-pressure plasma device at different time intervals. *D*-value of this device is 2 minutes.

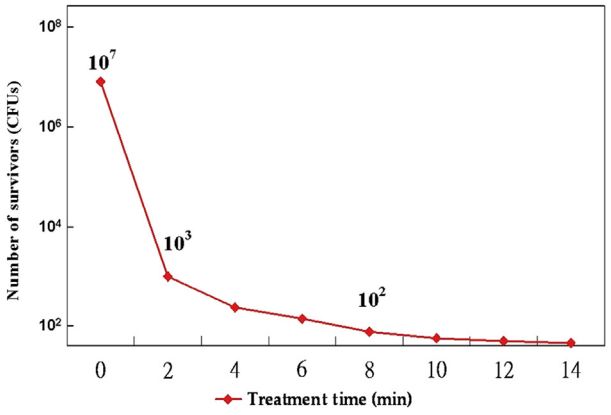

Figure 4 The number of surviving *Enterococcus faecalis* cells after sterilization by novel low-temperature atmospheric-pressure plasma device for different treatment durations. CFU = colony-forming unit.

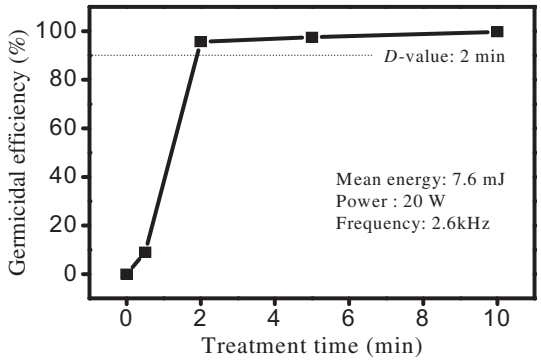

Figure 5 Sterilization *D*-values of *Enterococcus faecalis* (90% of cell reduction) measured at 2 minutes.

Morphological examination

Ten samples were prepared for FE-SEM examination. The results showed an intact surface morphology of *E. faecalis* prior to plasma treatment, but bacteria cell surface and outer wall were found to be ruptured and their integrity was lost after exposure to plasma (Fig. 6). The results proved that integration of bacteria was effectively impaired and destructed by plasma treatment for 2 minutes. Some bacterial fragments remained after 10 minutes of treatment, but severe damage was observed in 90% of *E. faecalis*.

Discussion

In addition to laser as well as photodynamic therapy (PDT) equipment that has been used in the past, different root canal irrigants such as sodium hypochlorite, H_2O_2, and chlorohexidine were reported to assist in root canal disinfection.[9,11,12] Clinically, these are commonly used, but uncertain effectiveness of photodynamic therapy equipment, safety and complication of chemical solutions, and expensive cost of laser are still of concern.

The mechanism of plasma sterilization differs from that of other sterilization methods. Plasma is an ionized gas, which comprises electrons, ions, free radicals, excited atoms, and UV photons that are capable of achieving sterilization. Plasma generates a high concentration of reactive oxygen species and charged particles that interact with microorganisms, which results in impairment of cell structure by oxidation reaction and in etching, and influences the conductivity of the cell surface, leading to the death of microorganisms.[13] Reactive oxygen species are chemically reactive molecules containing superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), and singlet oxygen (O_2). These oxygen species are highly reactive due to the presence of unpaired valence shell electrons. As already mentioned, the mechanism underlying plasma sterilization is different from that of traditional sterilization methods. Three mechanisms are involved in plasma inactivation of microorganisms: (1) direct destruction of the genetic material of microorganisms by UV irradiation, resulting in the death of microorganisms; (2) erosion of the microorganisms, atom by atom, through intrinsic photo-desorption (breaking of chemical bonds in the microorganisms by UV photons), forming volatile compounds by combining atoms intrinsic to the microorganisms; and (3) the etching mechanism, which involves erosion of microorganisms. Etching results from the adsorption of reactive species of plasma, which undergo chemical reactions to form volatile compounds. The reactive species could be molecular or atomic radicals, and excited molecules.[2,6,11]

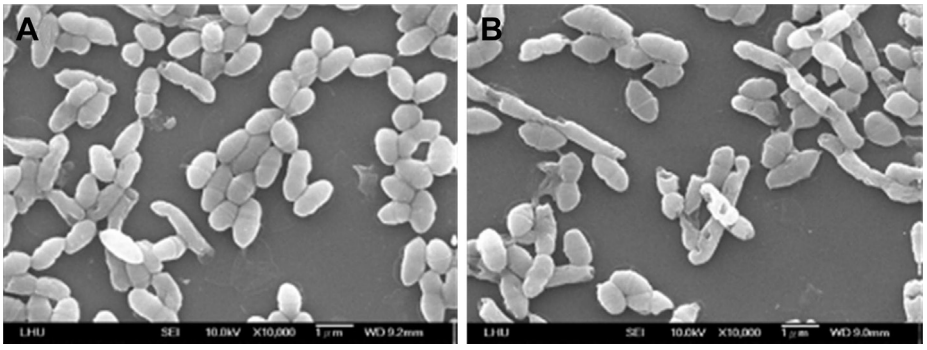

Figure 6 Scanning electron microscopy images of the cell morphology of *Enterococcus faecalis* (A) before plasma treatment and (B) 2 minutes after plasma treatment.

Oral bacterial sterilization and disinfection using low-temperature atmospheric-pressure plasma with different energy sources had been reported earlier. According to SEM evaluation of the sterilization effects of atmospheric-pressure plasma, reported by Choi,[14] DBD atmospheric-pressure plasma caused the death of *Escherichia coli* species after 10 seconds of plasma treatment. If the treatment time was set at 50 seconds, 90% of *E. coli* was destroyed. Choi et al[14] concluded that the amount of residual bacteria decreased with the duration of treatment. Our present study utilized the same DBD reactor plasma device that used and radiofrequency power supply. CFU counting results recognized the plasma-induced inactivation of *E. faecalis* at a time duration of 2 minutes (90% of bacteria reduction). The germicidal efficacy of the plasma treatment was found to be related to plasma exposure time. Furthermore, Ekem et al[15] used atmospheric-pressure plasma to treat *Staphylococcus aureus* and found that the plasma was effective for bacterial sterilization, with a reduction in the number of colonies of *S. aureus* from 2500 to 450 within a 5-minute period. In 2009, Sureshkumar and Sudarsan[16] studied the sterilizing effect of radiofrequency plasma generated using argon gas on inactivation of *S. aureus*. They observed a change in bacterial inactivation from $\log_{10}$ to $\log_7$ after plasma treatment for 5 minutes. A synergistic effect of plasma species such as UV radiation and argon-free radicals is thought to exert a destructive effect on bacterial cells and account for the major sterilization effect observed. Zhao et al[17] evaluated the effects of VUV/UV radiation and oxygen radicals on low-temperature sterilization of *Geobacillus stearothermophilus* using surface-wave-excited O_2 plasma, and they demonstrated that VUV photons act synergistically with the etching of the reactive oxygen atoms, allowing efficient sterilization of the bacteria spores by direct action of pure oxygen plasma. Hong et al[18] used an atmospheric-plasma mixture created by the ionization of helium and oxygen to observe the inactivation of *E. coli* and *Bacillus subtilis*, and confirmed that oxygen radicals generated by radiofrequency plasma were effective for impairing bacterial cells and endospores. Kikuchi et al[19] studied the inactivation of *Bacillus atrophaeus* spores by DBD plasma in atmospheric humid air. The results revealed that the bacteria inactivation was completed within 15 minutes at a relative humidity of 90% and a temperature of 30°C, indicating that the sterilization rate depended not only on the relative humidity but also on the temperature, which affects the generation of reactive species such as hydroxyl radicals. According to our present study and some past studies, low-temperature atmospheric-pressure plasma with different energy sources was proved to have some effectiveness in the inactivation of oral bacteria.

The vast majority of bacteria isolated from endodontic infections are anaerobic. Bacterial cultures and 16S rRNA analyses have given an even more diverse picture of bacteria associated with different species including *Pseudoramibacter*, *Propionibacterium*, *Dialister*, and *Filifactor*.[20] A large number of isolates including *Actinomyces*, *Streptococci*, *Peptostreptococcus*, and *Prevotella*, in addition to *Enterococci*, have also been reported.[21] Significantly higher success rates have been demonstrated clinically in cases of teeth that do not have bacterial growth at the time of root canal filling. Although the bulk of infecting microorganisms is removed during root canal preparation, residual bacteria are readily detectable in more than 50% of teeth at the time of obturation, despite extensive irrigation with 5.25% NaOCl and 2.0% chlorohexidine.[12] Moreover, Nair et al[22] detected bacterial biofilm in 90% of specimens using histological sections of intracanal isthmus, ramifications, and apical accessory canals (3.0 mm). Therefore, the complexity of the root canal system, presence of a smear layer after instrumentation, and the ability of bacteria to penetrate inside dentinal tubules make complete removal of bacteria with instrumentation and irrigation almost impractical. Fortunately, a very limited number of species has been reported in the root canal system and the periradicular region of teeth that have undergone proper, conventional endodontic treatment, even in those with persisting, asymptomatic periradicular lesions. *E. faecalis* species is one of the most frequently isolated and characterized microorganisms associated with unsuccessful endodontic treatment of teeth. Siqueira et al[20] demonstrated that *E. faecalis*, *Propionibacterium acnes*, and *Actinomyces israelii* heavily invaded root canal dentin.

The DBD low-temperature atmospheric-pressure plasma device used for bacterial sterilization in this *in vitro* study was designed at the Department of Biomedical Engineering, Chung Yuan Christian University, Taiwan.[23] A radiofrequency power supply was used. A DBD reactor applied an energetic electric field between two electrodes that were separated by a barrier to generate primary electrons. The reactor of the plasma device was generally supplied with air, frequency was set at 2.6 kHz, mean energy was 7.6 mJ, and excitation power was 1.1 W.

Moreover, in our study, images of the cell morphology of *E. faecalis* of 10 samples prior to and after plasma sterilization were also analyzed using FE-SEM to evaluate the severity of cell damage. As shown in Fig. 6, plasma treatment of *E. faecalis* resulted in a significant alteration in cell size and morphology when compared with untreated samples. After 2 minutes of plasma exposure, distinct damage to the cell structure was observed, which was suspected to have been caused by active oxygen atoms. These observations were similar to those reported by Zhao et al[17] and Yang et al[24] who observed marked erosions of *Geobacillus stearothermophilus*, *Streptococcus mutans*, and *Lactobacillus acidophilus* after low-temperature sterilization under surface-wave-excited O_2 plasma treatment. Kikuchi et al[19] also found that *B. atrophaeus* spores were deformed (SEM images) by DBD plasma treatment. In addition, the observation of severe damage of cell morphology in this study was also similar to that of Hong et al[18], who demonstrated that *E. coli* was obviously destroyed after treatment with an atmospheric-plasma mixture created by the ionization of helium, as shown by transmission electron microscopy scanning. Notable cytoplasmic deformity as well as leakage of bacterial chromosome was observed. This might explain the loss of viability of microorganisms after plasma inactivation. According to the results of a study by Wang et al,[23] a low-temperature atmospheric-pressure plasma jet was capable of inactivating *E. coli*, and a *D*-value of 120 seconds (2 minutes) was demonstrated. The results of the study by Yang et al[24] are consistent with the findings of this study, which demonstrated that argon plasma was very effective

in deactivating oral bacteria and could be a promising technique in various dental clinical applications such as bacterial disinfection and early caries prevention.

According to the experimental conditions and results of this study, a novel laboratory-made low-temperature atmospheric-pressure plasma device was shown to have similar effects on inactivation and sterilization of oral bacteria (*E. faecalis*) within a *D*-value of 2 minutes. We believe that the effective inactivation of *E. faecalis* can be attributed to the excited species, charged particles, and UV radiation generated in the atmospheric nonthermal plasma device. The authors intend to establish the exact treatment time and develop a plasma-jet device to achieve complete inactivation of different oral bacteria in future studies.

In conclusion, the results of our current studies demonstrated that a novel low-temperature atmospheric-pressure plasma device could indeed achieve fast and effective sterilization of *E. faecalis* within a short period of time. Further *in vivo* studies will be needed to confirm its safety and biocompatibility for use in oral tissues.

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