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Effect of Modified Nonequilibrium Plasma with Chlorhexidine Digluconate against Endodontic Biofilms *In Vitro*

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Abstract

Introduction: Nonequilibrium plasma has been reported to effectively kill *Enterococcus faecalis* in endodontic biofilm compared with chlorhexidine digluconate (CHX). The purpose of this study was to evaluate the antimicrobial *in vitro* activity of modified nonequilibrium plasma with CHX against *E. faecalis* and multispecies biofilms on bovine dentin discs. **Methods:** Sterile bovine dentin discs were incubated with *E. faecalis* or a mixture of bacteria from human dental root canal infections to form 1- and 3-week-old biofilms. The specimens were subjected to nonequilibrium plasma, modified nonequilibrium plasma with CHX, and 2% CHX for 2- and 5-minute exposure. After treatment, the biofilms were stained with viability dyes and examined by confocal laser scanning microscopy and 3-dimensional reconstruction analysis. The proportions of bacterial cells killed by the treatments were calculated. **Results:** The 3-dimensional reconstruction images showed that 1- and 3-week-old biofilms adhered to bovine dentin discs. The proportions of dead cells increased significantly with the longer exposure in each treatment group ($P < .05$). Modified nonequilibrium plasma was the most effective in killing bacteria in *E. faecalis* and multispecies biofilms at both 2 and 5 minutes ($P < .05$). No significant difference was detected between nonequilibrium plasma and CHX groups ($P > .05$). Significantly more cells were killed in 1-week-old biofilms than in 3-week-old biofilms in all groups ($P < .05$). **Conclusions:** The modified nonequilibrium plasma killed more bacteria than conventional nonequilibrium plasma and 2% CHX in *E. faecalis* and multispecies endodontic biofilms *in vitro* and thus shows promise as an additional tool in infection control during endodontic treatment. *(J Endod 2013;39:1438–1443)*

Key words

Bacterial biofilm, chlorhexidine digluconate, endodontic treatment, *Enterococcus faecalis*, nonequilibrium plasma

Endodontic infections are caused by microorganisms, usually bacteria, residing in the root canal system of the affected tooth (1–3). Therefore, an ideal endodontic treatment will eliminate microorganisms from infected root canal systems and prevent reinfection (4, 5). A variety of different measures have been used to eradicate root canal bacteria, including chemomechanical instrumentation techniques, irrigation, and intracanal medicaments. However, it has been shown that complete eradication of microorganisms is difficult to achieve (6–8). The extracellular polymeric matrix produced by the microorganisms and their low metabolic activity in endodontic multispecies biofilms can protect individual bacteria against disinfecting agents (9–11). Therefore, new methods are continuously being developed to eradicate all bacteria and secure healing.

Plasma is the fourth state of matter after solid, liquid, and gas and is an ionized gas containing free charge particles (electrons and ions), active radicals, and excited molecules (12). Nonequilibrium plasma exists at atmospheric pressure and room temperature and does not inflict thermal damage to nearby objects. Recently, nonequilibrium plasma has attracted particular interest because it can be used for disinfection when in direct contact with living tissue. In recent years, much activity has been focused on the killing of microorganisms with nonequilibrium plasma, short-lived oxygen reactive species and charged particles (electrons and ions), and especially free radicals (13–15). The preliminary results of nonequilibrium plasma against endodontic bacteria in planktonic culture and biofilms have been promising (16, 17). Du et al (18) recently reported that the killing activity of nonequilibrium plasma against *Enterococcus faecalis* biofilm was similar to 2% chlorhexidine digluconate (CHX). It is of value to hypothesize that a low-frequency radiofrequency supply at a high voltage could ionize chlorhexidine when the plasma jet operates at atmospheric pressure and room temperature although it cannot be confirmed yet with certainty. Kuruvilla and Kamath (19) showed that when mixed with sodium hypochlorite (NaOCl), the chloro groups (Cl^-) might get added on to the guanidine component of the chlorhexidine molecule, thereby forming "chlorhexidine chloride." The authors suggested that the formation of ionized chlorhexidine chloride was a factor in the improved antibacterial effect over NaOCl or CHX alone. Accordingly, both short-lived reactive species charged by

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nonequilibrium plasma and ionized "chlorhexidine chloride" could result in damage to bacterial cells. Hence, the purpose of this study was to evaluate the *in vitro* killing activity of modified nonequilibrium plasma with CHX against *E. faecalis* and multispecies biofilms on bovine dentin discs.

Materials and Methods

This study was approved by the Science and Ethics Commission of Tongji Hospital of HUST, Wuhan, China.

Bovine Dentin Disc Preparation

One hundred twenty freshly extracted, caries-free bovine incisors from animals killed for commercial reasons were used as the biofilm substrate in this study. The teeth were kept in 0.01% NaOCl solution until used (20). The bovine root was horizontally cut from each tooth at the cementoenamel junction with a 0.6-mm-thick diamond saw (IsoMet; Buehler Ltd, Lake Bluff, IL) under water cooling. Using a low-speed handpiece with a bur, a thin groove was prepared in the middle of each crown, which was then coronally fractured with a blade and a hammer into 2 halves (20). To allow each biofilm to grow in the same substrate, the enamel of each specimen was removed, and the dentin surfaces were ground by 600-grit silicon carbide paper to obtain a standard thickness of 1.5 mm, whereas the lingual halves were not used because of their thin and rough surfaces. The dentin discs were then shaped with a fine carbide bur using a low-speed handpiece under water cooling to create a uniform shape with a diameter of 12 mm. The prepared dentin discs were irrigated with 17% EDTA solution for 5 minutes to remove the smear layer and then rinsed in distilled water for 5 minutes. The specimens were placed in a glass vial containing distilled water for sterilization in an autoclave at 121°C for 20 minutes. The water was discarded before any subsequent experiments.

Model RC-2 for Generating Modified Nonequilibrium Plasma with CHX

A schematic presentation of the plasma device Plasma Creator of Model RC-2 is shown in Figure 1*A*, with a detailed description given in the literature (18, 21). However, the current study differed from previous studies because an additional vial containing 2% CHX prepared freshly from 20% stock solution (Sigma-Aldrich, St Louis, MO) was added to the device and connected to the gas tube, so that both gas and medicament would be excited at the same time. The equipment creating the plasma stream was operating at 8 kV, 8 kHz, and a pulse width of 1600 ns. The working gas of plasma was He/O_2, and the flow rate was controlled by a mass-flow controller at a setting of 1:0.01 L/min. The atmospheric pressure–modified nonequilibrium plasma with CHX generated by the Model RC-2 was ejected from the syringe nozzle (Fig. 1*B*).

Biofilm Cultures

The bacteria used in this study were *E. faecalis* (American Type Culture Collection 29212) and mixed bacterial flora from human dental root canal infections. *E. faecalis* stocked in −80°C was streaked on brain-heart infusion (BHI) agar (Becton, Dickinson and Company, Sparks, MD) plates and incubated aerobically (AnaeroGen; Oxoid Ltd, Hampshire, UK) at 37°C for 24 hours. A single colony from a BHI agar plate was then inoculated on another sterile BHI agar plate for an overnight aerobic incubation at 37°C, and after that period, the cultures were harvested, checked for purity, and suspended in BHI broth (Becton, Dickinson, and Company). The cell density was adjusted in a spectrophotometer to a density of approximately 3.0×10^7 colony-forming units per milliliter in BHI broth. The mixed bacterial growth was collected from the infected canals of single-rooted teeth of 5 volunteers with apical periodontitis and mixed in BHI to adjust the bacterial cell concentration of 3.0×10^7 colony-forming units per milliliter as described previously.

Each well of a 24-well tissue culture plate (Costar, Corning, NY) hosted 1 sterile bovine dentin disc. The dentin surface in half of the 120 samples was infected with *E. faecalis* and the dentin surface in the other half with mixed root canal bacteria. Two hundred microliters of bacterial suspension and 1.8 mL sterile BHI broth were transferred to each well for 1- and 3-week anaerobic incubation periods at 37°C. Fresh BHI medium was changed once a week for the 3-week-old specimens.

Antibacterial Treatments of the Endodontic Biofilms

After anaerobic incubation for 1 and 3 weeks, the excess broth was removed from each well. The dentin discs with biofilms were rinsed in sterile saline for 1 minute to remove unattached bacteria and culture broth (22). The 1- and 3-week-old *E. faecalis* or multispecies biofilms on dentin discs were randomly allocated to the following treatments for 2 and 5 minutes with 3 specimens in each group: (1) nonequilibrium plasma, (2) modified nonequilibrium plasma with fresh 2% CHX, (3) working gas only with 2% CHX (when the plasma jet closed), (4) 2 mL 2% CHX, and (5) 2 mL 0.85% saline. The needle tip of the Plasma Creator was placed 5 mm above the top of the biofilm and moved back and forth at the same level. After the exposure, each specimen was gently washed with saline.

Examination by Confocal Laser Scanning Microscopy

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 following the manufacturer's instructions. Bacteria with intact cell membranes stain fluorescent green with SYTO 9, whereas bacteria with damaged membranes stain red with PI. The excitation/emission maxima for the 2 dyes are approximately 480/500 nm for SYTO 9 and 490/635 nm for PI. The mounted specimens were viewed immediately using a confocal laser scanning microscope (Olympus FV500, Olympus, Japan) with the 40× lens. The confocal laser scanning microscopic images of 512 × 512 pixels were captured using Fluoview version 4.3 software (Olympus, Melville, NY).

In general, 5 randomly chosen areas (0.3 mm × 0.3 mm for each area) of each specimen were examined by confocal laser scanning microscopy and 3-dimensional (3D) reconstruction analysis. Thirty scans were observed for each group, 15 for both exposure times. Fifteen-micrometer-deep scans (1-μm step size, 15 slices/scan) of each 1-week-old biofilm were performed to standardize the area and volume of the biofilm scanned, whereas the depth of scanning for the 3-week-old biofilms was set at 50 μm. The proportion of dead cell volume (red fluorescence) in 3D reconstruction images was analyzed by the Imaris 7.2 software (Bitplane Inc, St Paul, MN) (20, 23). Differences between groups were evaluated using 1-way analysis of variance tests, with post hoc multiple comparisons using the SPSS statistics software (version 16.0; SPSS Inc, Chicago, IL). The results were considered statistically significant when P was $<.05$.

Results

The biofilms grew well in both 1- and 3-week-old dentin disc specimens as shown by the confocal laser scanning microscopic scans and 3D reconstructions (Fig. 2*A1–A10* and *B1–B10*). The proportion of dead cells varied from 40%–80% in the experimental groups, whereas

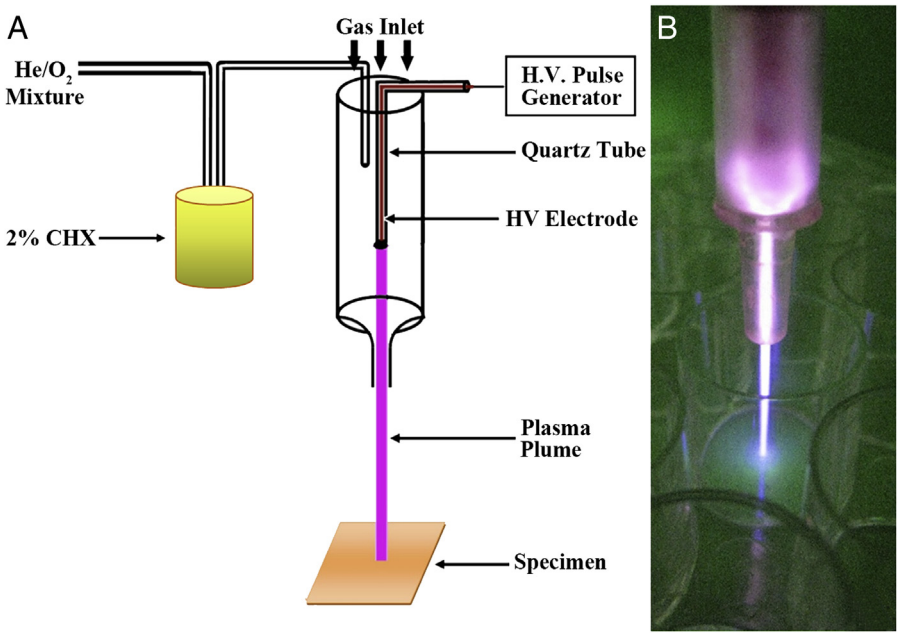

Figure 1. A photograph of the atmospheric pressure nonequilibrium plasma. (*A*) A schematic of the plasma creator of Model RC-2 experiment setup. (*B*) A photograph of the modified nonequilibrium plasma acting on endodontic biofilms.

only 10%–13% of the cells were dead in the 2 control groups (saline and gas only).

Figure 3 showed that significantly more bacteria in endodontic biofilms were dead in each experimental group with the increasing exposure time ($P < .05$). Modified nonequilibrium plasma (54%–80% killing) was the most effective antibacterial treatment at both 2 and 5 minutes ($P < .05$). No significant difference of bactericidal activity was found between the nonequilibrium plasma (41%–68% killing) and CHX (40%–69% killing) groups ($P > .05$). The amount of dead cells was 7%–14% higher in 1-week-old biofilms than in 3-week-old biofilms ($P < .05$).

Discussion

It is well established that microorganisms play a key role in the progression and perpetuation of the different forms of apical periodontitis. A large body of scientific evidence has shown that *E. faecalis* is often isolated from previously treated teeth presenting with persistent disease and can therefore be regarded as a suitable organism to study and test *in vitro* the effectiveness of different disinfection applications designed for endodontic treatment (24, 25). Often, however, a polymicrobial infection has invaded the root canal systems *in vivo* to form multispecies biofilms in teeth with apical periodontitis. Although the development of *in vitro* multispecies biofilm models can be challenging, they are needed to better understand the behavior of true *in vivo* root canal biofilms (26). Currently, both mono- and multispecies bacterial biofilm *in vitro* models are developed to estimate the antimicrobial strategies in endodontics (22, 27, 28). *E. faecalis* and multispecies biofilms on bovine dentin discs were selected for the present study to evaluate the *in vitro* effects of killing bacteria by conventional and modified nonequilibrium plasma.

Bovine dentin was chosen instead of human dentin because of easier availability. The small structural differences between bovine and human dentin are unlikely to have an impact on the results of the present study. Dentin discs were autoclaved in a water bath to eliminate the possible presence of bovine bacteria in the dentin and their growth into the biofilm. Although not sure, autoclaving might affect dentin collagen and thereby modify the initial attachment of bacteria to the dentin surface. However, it is not likely that it affected further biofilm growth and maturation or its susceptibility to the antibacterial methods and substances (29).

Biofilms of only 1 strain of *E. faecalis* and 1 pooled mixed flora from several infected root canals were used for the present study. Stojicic et al (30) recently reported the antibacterial effect of different disinfecting agents against biofilms grown separately from mixed oral bacteria collected from 6 different individuals. The results of that study showed that all 6 biofilms became resistant to the 3 different disinfectants at 3 weeks of growth and remained resistant after that. The study concluded that the development of resistance in biofilms from oral bacteria may be less dependent on the detailed composition of the flora and more related to biofilm maturation by growth (30). Therefore, although several different biofilms were not included, the results of the present study may nevertheless represent biofilm susceptibility to conventional and modified plasma. Interestingly, there were only relatively small differences in the susceptibility between the single-species *E. faecalis* biofilm and the multispecies biofilm (Figs. 2 and 3). This may also be regarded as an indication of the dominant role of the general biofilm features (eg, growth, maturation, ecology, and extracellular polymeric substance) in determining the biofilm susceptibility and resistance.

Three-week-old biofilms were more resistant than the 1-week old biofilms, confirming the results of several earlier studies (31, 32) although the differences were rather small. Extending the time of exposure to the disinfecting methods from 2 to 5 minutes increased the number of bacteria killed. Nonequilibrium plasma is one of the advanced therapeutic strategies used against endodontic biofilms; the strategy uses short-lived reactive species and free radicals generated by plasma jet, such as OH and reactive oxygen species, including O_3, metastable state O_2, and O, which play a major role in the

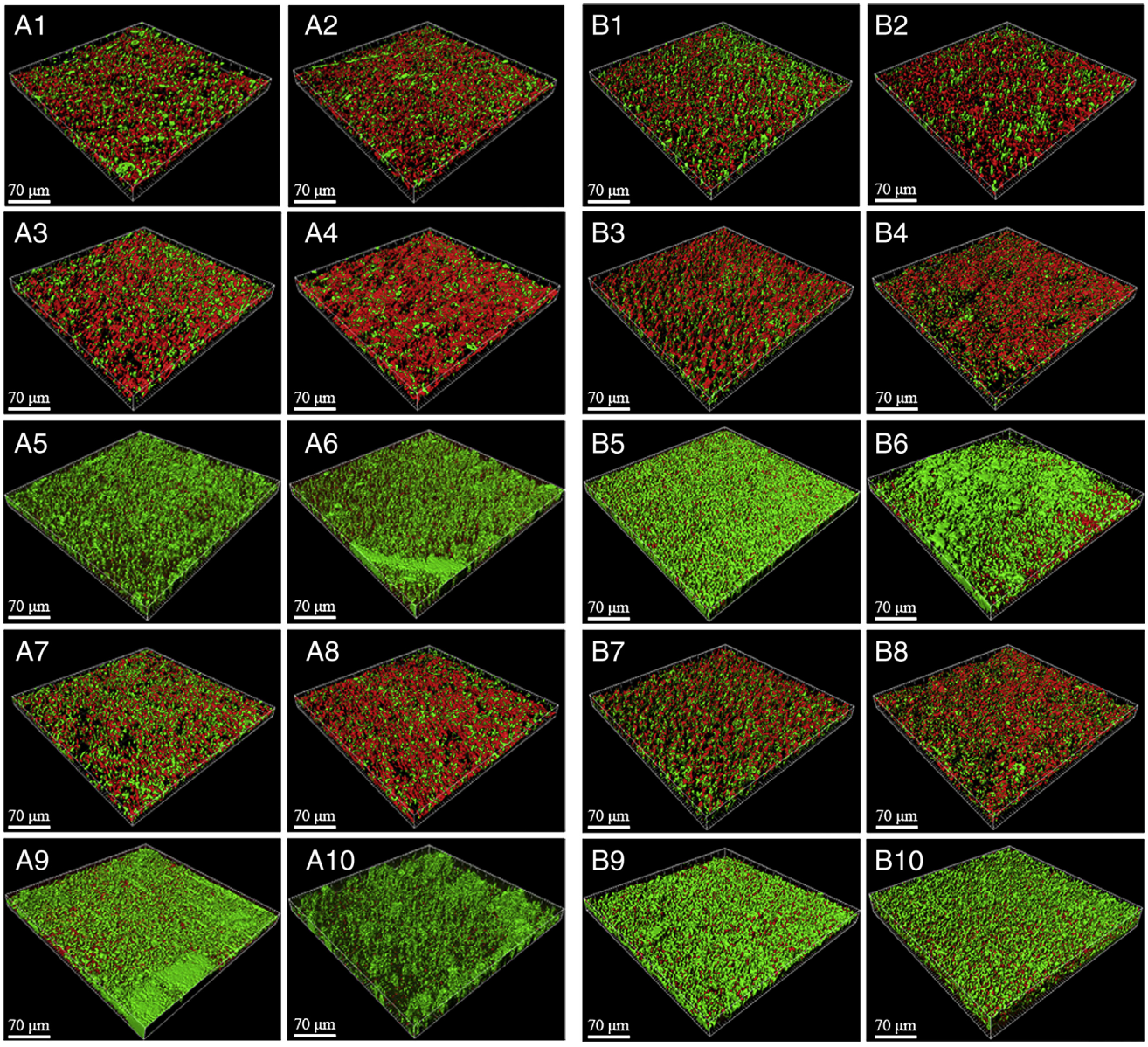

Figure 2. Confocal laser scanning microscopic images of 3-week-old (*A*) *E. faecalis* and (*B*) multispecies biofilms subjected to (*A1* and *B1*) 2 minutes of nonequilibrium plasma treatment, (*A2* and *B2*) 5 minutes of nonequilibrium plasma treatment, (*A3* and *B3*) 2 minutes of modified nonequilibrium plasma treatment, (*A4* and *B4*) 5 minutes of modified nonequilibrium plasma treatment, (*A5* and *B5*) 2 minutes of working gas with CHX, (*A6* and *B6*) 5 minutes of working gas with CHX, (*A7* and *B7*) 2 minutes of irrigation with CHX, (*A8* and *B8*) 5 minutes of irrigation with CHX, (*A9* and *B9*) 2 minutes of saline treatment, and (*A10* and *B10*) 5 minutes of saline treatment.

inactivation of bacteria (15–18). In addition, it has been reported that ultraviolet (UV) irradiation, intrinsic photodesorption, and etching (eventually enhanced by UV radiation) contribute to the inactivation processes (33). However, our previous studies showed that UV emission played a minor role in the inactivation of the bacteria (15, 21). The present study was the first attempt in which the modified nonequilibrium plasma combined CHX to a He/O_2 gas mixture to generate a plasma jet. It is hypothesized that Cl^- could be formed when chlorhexidine was ionized by a low-frequency radiofrequency supply at a high voltage. Although not known with certainty, it has been suggested that the combined use of sodium hypochlorite and CHX within the root canal resulted in an improved antibacterial effect because of the formation of "chlorhexidine chloride," which increased the ionizing capacity of the chlorhexidine molecule and may have played a role in more effective killing (19). The modified nonequilibrium plasma method was more effective in killing bacteria in both biofilms than the conventional nonequilibrium plasma and CHX in all experimental settings (Figs. 2 and 3). Although not examined in the present study, reactive oxygen species and ionized chlorhexidine chloride might also penetrate dentinal tubules more effectively than conventional disinfection.

Nonequilibrium plasma is active against microorganisms but is nontoxic to host tissues (34). The modified plasma in the present study killed between 65% and 80% of the biofilm bacteria within 5 minutes of exposure, which is among the highest killing rates reported in recent literature considering oral and endodontic biofilms (22, 30). A current study showed that no or only a few bacterial cells were detectable by scanning electron microscopic examination on the

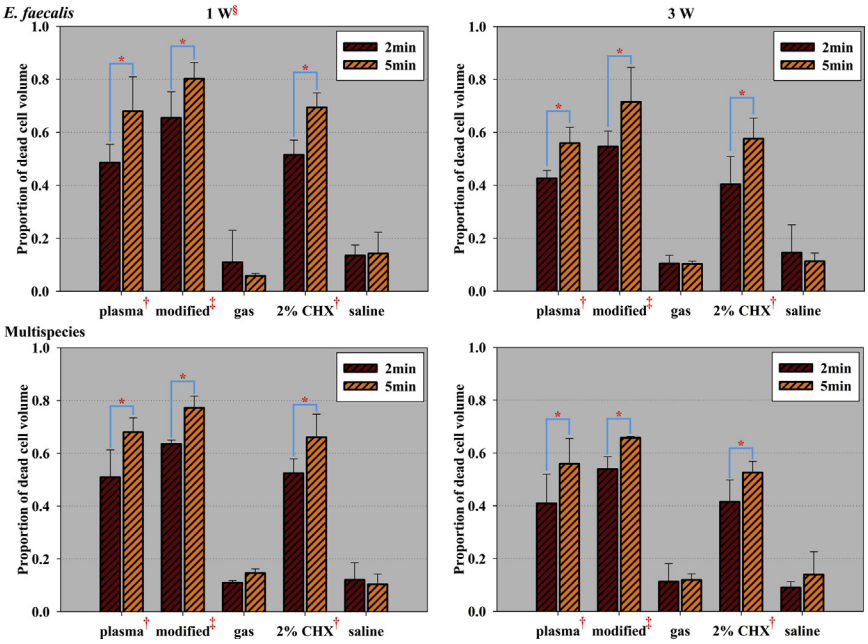

Figure 3. The proportion of the dead cell volume in the biofilms after the antibacterial treatments for 2 and 5 minutes. *A significant difference between exposure times ($P < .05$). †‡A significant difference between treatments ($P < .05$). §A significant difference between young and old biofilms ($P < .05$).

radicular dentin surface subjected to sonic or laser activation of 4% NaOCl (35). Thus, it seems that the combined use of irrigating solution with other disinfecting methods such as plasma, sonic, ultrasonic, or laser may improve the overall antimicrobial effect.

Further studies should be focused on optimizing the exposure time and other modifications of the substance cocktails used to generate the plasma stream to facilitate further the elimination of endodontic biofilm bacteria. The present study indicates that modified nonequilibrium plasma may be an effective method to attack root canal biofilms on dentin.

Acknowledgments

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

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