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Evaluation of Antibacterial Effects of Cold Atmospheric Plasma, Calcium Hydroxide, and Triple Antibiotic Paste on *Enterococcus faecalis* Biofilm in the Root Canal System: An In Vitro Study

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Abstract

Introduction: One of the essential factors in successful endodontic therapy is the effective cleaning and disinfection of the root canal. This study aimed to determine the effect of cold plasma on infected root canals with *Enterococcus faecalis* and compare its antibacterial effect with the conventional medicaments in vitro.

Methods: Sixty-tree single-root teeth were extracted. Canals were cleaned and shaped. Ten teeth were selected as the negative control randomly. The rest of the teeth were incubated at 37°C for 21 days to form *E. faecalis* biofilm. The specimens were divided into six groups; each group had 10 teeth. In group 1 (the positive control group of calcium hydroxide and triple antibiotic paste [TAP]), methylcellulose was placed in the root canal; in group 2, calcium hydroxide was placed in the root canal for 12 days; in group 3, 10 mg/mL of TAP was placed in the root canal for 12 days; in group 4, helium/oxygen plasma jet was used for 10 minutes. Group 5 was considered as a positive control of plasma, and group 6 was the negative control. After treatment, F4 Pro-Taper rotary file was used to collect root canal microbial biofilms. Bacterial suspensions were serially diluted, and the percentage of growth reduction for each group was obtained by dividing the logarithm of CFU/mL of each group by CFU/mL of the control of the same group.

Results: The CFU/mL of TAP and plasma-treated samples was significantly lower than that of the control groups; however, there were no significant differences between the control group and the samples treated by calcium hydroxide. The most percentage of CFU reduction was in the TAP-treated group compared with plasma and calcium hydroxide-treated groups.

Conclusion: The application of cold plasma effectively inhibited the growth of *E. faecalis* and reduced bacterial biofilm. Also, in the present study, 10 mg/mL of TAP caused the complete elimination of *E. faecalis*. Calcium hydroxide had the most negligible effect on *E. faecalis* biofilm elimination.

Keywords: Cold atmospheric plasma; *Enterococcus faecalis* biofilm; Root canal; Calcium hydroxide; Triple antibiotic paste

Introduction

The primary purpose of root canal treatment is to eliminate or reduce the pathogenic microorganism and bacterial biofilm of the root canal system. Obtaining a bacteria-free root canal is one of the challenges facing dentists.[1] Since the mechanical debridement alone cannot access all root canal surfaces, irrigation with chemicals and intracanal medications is necessary to eliminate bacterial biofilm.[2,3]*Enterococcus faecalis* is an arbitrary anaerobic gram-positive coccus[4] and the common bacterium isolated from the root canal in persistent infection, and it appears to be able to infect the canal alone in many cases.[5,6] The ability to form biofilm and penetrate through the dentinal tubules is one reason for its resistance to antimicrobial agents.[7,8] *E. faecalis* can penetrate dentinal tubules up to 1000 µm in-depth, while intracanal irrigations (NaOCl) can penetrate up to 300 µm into dentinal tubules.[9-11] For these reasons, various intracanal medications have

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improved canal disinfection.[12,13]

Calcium hydroxide (Ca(OH)2) is a broad-spectrum antibacterial agent that kills bacteria by producing alkaline environments. Traditionally, Ca(OH)2 is used widely as an interappointment intracanal medication.[14] However, a systematic review demonstrated that interappointment dressing with calcium hydroxide had no significant effect on reducing the root canal microorganisms.[15]

Triple antibiotic paste (TAP), a mixture of metronidazole, ciprofloxacin, and minocycline, has been used as an intracanal dressing, particularly in regeneration endodontic treatment. The regeneration procedure is a valuable treatment option due to induce root development in immature teeth with an open apex and thin root canal. The regeneration outcome depends on microbial elimination because apical repair will not happen in the presence of infected tissues. It was shown that the microbial profile of infected immature teeth is similar to that of primarily infected permanent teeth.[16]

Studies demonstrated the high efficacy of TAP in eradicating bacteria from the infected root canal, but its cytotoxic effect on stem cells was undesirable.[17,18] Because viable stem cells are necessary to achieve the successful outcome of regeneration treatment, investigations have been made to introduce methods with adequate bacterial elimination and minimal stem cell toxicity during regeneration endodontic treatment.[19]

Cold plasma is the partially ionized gas, recently used as a therapeutic approach in dentistry.[20-22] The plasma jet device generated reactive particles including ions, electrons, oxygen radicals utilizing a mixture of gases, typically helium, Argon, nitrogen, air or oxygen, and electric energy. These reactive particles interact with bacterial membranes thereby killing the bacteria.[23] Atmospheric pressure nonequilibrium plasmas jet generated plasma plums in the air under atmospheric pressure and near room temperature caused no damage to nearby vital tissue. These properties make plasma technology applicable in various biomedical fields, including sterilization, wound healing, growth factor release, tissue regeneration, and apoptosis of tumor cells.[24] Atmospheric pressure nonequilibrium plasmas can also be used as new, effective, safe root canal disinfection without damaging surrounding tissues.[25]

The objective of this study was to evaluate and compare the antibacterial effect of cold plasma, TAP, and calcium hydroxide on *E. faecalis* biofilm in the root canal systems.

Methods

Sample Preparation

sixty-three single-rooted extracted human teeth without root caries, internal or external resorption, fracture, or calcification, confirmed by radiography, were selected from the dental clinics and department of Shahid Beheshti University of Medical Sciences. Teeth extracted due to periodontal problems, orthodontic treatment, or coronal caries were collected in the department of oral and maxillofacial surgery of Shahid Beheshti Dental School. Then the teeth were cleaned and debrided, and the extra soft and hard tissues attached were removed. The samples were swamped in a 5.25% sodium hypochlorite solution (Taj, Iran) for surface disinfection for 24 hours. The teeth were then kept in sterile physiological serum 0.9% (Merck, Germany) at room temperature for two weeks until the beginning of the study. The teeth were decoronated at or below the cementoenamel junction (CEJ) using a low-speed diamond saw. Then, the specimen's length was measured, and they had approximately the same length (12-14 mm). The cementum covering the external root surface was removed using a diamond fissure bur in order to increase penetration of microorganisms into dentinal tubules. By passing k file # 15 (Dentsply Maillefer, Ballaigues, Switzerland), we ensured that the roots were single-canal and the patency of the apical foramen was established. To reduce the effect of anatomical and technical confounding variables, we prepared the root canals of all teeth with similar mechanical and chemical instrumentation methods as follows:

ProTaper rotary files (Dentsply Tulsa Dental; Tulsa, Oklahoma) up to F3 size with the Endo IT device (VDW, Munich, Germany) were used for root canal mechanical preparation according to the manufacturer's instructions.

The canals were irrigated with 1 mL of 5.25% NaOCl solution. After each instrumental procedure, the canals were irrigated with 17% EDTA (Aria Scratch, Asia Chemi Teb, Tehran, Iran) for 5 minutes, followed by 5.25% NaOCl (Taj, Iran) for 2 minutes. The last wash utilized 5 mL of physiological serum to remove all the chemicals. The teeth were dried with sterile paper points.

Then the apical root ends of the teeth were sealed with a composite resin (*Kerr*, Orange, CA, USA). Afterward, the specimens were divided into six groups (n = 10). Group 1: the positive control group of calcium hydroxide and TAP; group 2: calcium hydroxide was placed in the root canal; group 3: TAP was placed in the root canal; group 4: helium/oxygen plasma jet was used; group 5 was considered the positive control of plasma, and group 6 was considered the negative control.

Also, three samples were prepared for scanning electron microscopy (SEM) examination. One specimen was assigned to each plasma treatment, positive control and negative control. Using a diamond disk, we made two grooves in the buccal and lingual surfaces of these roots (Jota, Switzerland). Each sample was immersed in a test tube containing 1 ml of liquid culture medium Brain Heart Infusion Broth (BHI) (Merck, Germany). The tubes were then closed and inserted into the autoclave. An autoclave thermally sterilized the samples at a temperature of 121°C and 15 Pascal pressure for 15 minutes.

Biofilm Formation

Enterococcus faecalis (ATCC: 29212 from Persian Type Culture Collection) was cultured in BHI Agar at 37°C for 24 hours. Then the bacterial suspension was prepared according to 0.5 McFarland standard in BHI broth (1.5×10^8 CFU/mL). Optical absorption at 600 nm at a magnitude of 0.09 was determined to adjust the bacterial suspension turbidity to a magnitude of 0.5 McFarland with a spectrophotometer. The remaining samples were contaminated by adding 50 µL of prepared E. faecalis suspension to the sterilized test tube. All tubes were vortexed and incubated at 37 °C for 21 days to form bacterial biofilms and penetrate the dentin tubules. The culture medium (BHI) was changed every two days to confirm the bacterial growth, and all tubes were vortexed each time to deeper penetration of the culture medium and bacteria into the dentinal tubules. To confirm the growth of the microorganism and its purity, the negative control and some random samples passage on the BHI Agar medium (20 µL of the culture medium was cultured in streak method), and they were incubated for 24 hours. This subculture technique was repeated several times during 21 days of incubation.

Preparing Intracanal Medicines

Methylcellulose powder (MERCK neuro lab, Germany) was dissolved in distilled water to prepare a gel form antibiotic past. The concentration of methylcellulose gel in the pilot stage was 8% based on the Algarni and colleagues' study.[17] However, due to its high viscosity and difficult removal of the past from the root canal wall, a final concentration of 4% methylcellulose was used for the final test. One hundred and fifty milligrams of each ciprofloxacin (Temad Co, Tehran, Iran), metronidazole (Kimyagaran Emruz, Tehran, Iran), and minocycline (Hexal Co, Leverkusen, Germany) were mixed with 45 mL of sterile distilled water for preparation of 10 mg/mL TAP.[26] Then, the methylcellulose powder was added at a ratio of 4% and heated to 45°C for methylcellulose powder dissolution. A sterile magnet of a suitable size was inserted into the material container to prepare the homogenous mixture. The container was put on the Magnetic Stirrer machine (Tajhiz Gostar - Iran) for 2 hours. To prepare the neutral material for the positive control canal samples, we mixed distilled water and 4% methylcellulose . [27,28] To prepare calcium hydroxide, we added 2 g of calcium hydroxide (Golchadent, Iran) to 2 mL of sterile distilled water.

Intracanal Medicament Treatment of Endodontic Biofilm

After 21 days of incubation, the samples were transferred to the biological hood under entirely aseptic conditions to apply prepared medicaments. Each sample was removed and rinsed separately with 5 mL normal saline to remove any planktonic bacteria. After drying the samples with sterile gas, their outer surface was covered with two layers of nail varnish to simulate clinical conditions and create a cementum-like layer on the root surface.[29]

In the first group (10 samples as the positive control group), methylcellulose was inserted into the dental canals with Lentulo size 25 using the ENDO IT device. In the second group, calcium hydroxide was transferred to the root canals of 10 samples, and in the third group, TAP 10 mg/mL was inserted into the root canals of 10 samples, with the same procedure as the control group. Then, the adhesive wax for the coronal seal was used to reduce the probability of contamination of the samples. The samples were placed in 48-well plates, and they were incubated for 12 days at 37°C and 90% moisture.

After 12 days of incubation, the root canal specimens were gently washed for 1 minute with 5 mL of sterile saline to remove the medicaments in the medicament groups and the positive control group. Then the sterile ProTaper file F4 (150 rpm circumferential motion for 30 seconds) was used for sampling. The file was transferred to a sterile microtube containing 1 ML BHI medium, and it was incubated for 18 hours at a temperature of *37*°C. Then the bacteria grown in microtubes were serially diluted to 1: 10^{10} dilution. 100 µL of the diluted solution was inoculated onto BHI agar plates and incubated for 24 hours. Then, the counting colonial units (CFU) of *E. faecalis* colonies were counted using a colony counter machine (Teif Azma Teb, Iran).

Plasma Treatment of Endodontic Biofilm

A pilot test was performed to ensure the accuracy of the plasma device and determine the appropriate voltage and irradiation time. Thus, two plates infected with *E. faecalis* were irradiated with two different voltages of plasma, 8 and 10 kV. Each dish was subjected to plasma irradiation at a 14 kHz frequency for 1, 5, and 10 minutes.[30] Finally, according to the results, irradiation with 8 kV in 10 minutes were considered for the test. The plasma samples were fixed under the biological hood. Also, to reduce the handshake error and equalize the treatment conditions of the samples, the plasma jet device was fixed so that the plasma could be inserted directly into the canal without manual intervention. The distance between plasma head and the samples was 10 mm.[31,32] The samples were exposed to plasma irradiation at 8 kV and 14 kHz for 10 minutes. Plasma radiation consisted of 95% helium gas and 5% oxygen gas (Figure 1). Ten samples were considered as the plasma positive control group.

The biofilm was collected, and the colonies were counted in the same procedure as the medicament groups.

Preparation of the samples for Scanning Electron Microscopy

One specimen from each group of plasma treatment,

positive control and negative control was prepared for SEM analysis to investigate the structural changes of biofilms after plasma treatment and confirm biofilm formation in root canals.[33] The specimens were separated from the longitudinal groove (made by a diamond disc) of the root surface in two halves using a chisel. the samples were first placed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate (pH = 7.4) for 24 hours at 4°C to fix the bacteria, then they were dehydrated in ethanol. The samples were transferred to Razi Metallurgy Research Center for SEM examination.

Statistical Analysis

The data of the CFU count were transformed by log 10. All data were analyzed by SPSS software (ver. 20.0; SPSS Inc., Chicago, IL, USA), and the results were considered statistically significant when P value < 0.05. A one-way ANOVA test analyzed each group's bacterial CFU counts before and after the treatment. In addition, pairwise comparisons of treatment groups were performed by the post hoc test. To compare the rate of CFU reduction between the treatment groups, the percentage of reduction in the colony count was also calculated by the method:

$$RCC = \frac{CFUs(Before\ Treatment) - CFUs(After\ Treatment)}{CFUs(Befor\ Treatment)} \times 100$$

Results

The CFU/mL counting method was adopted to evaluate the antimicrobial effectiveness (Table 1). The normal distribution of data was confirmed. Statistical analysis showed a statistically significant difference in the CFU of *E. faecalis* between the TAP-treated samples and the control group. TAP had a significant antibacterial effect, so that all TAP-treated samples were sterilized against *E. faecalis* (P < 0.001) (Figure 2). No significant difference was observed between the calcium hydroxide group and the positive control group (P = 0.389). There was a statistically significant difference between the plasma and positive control groups (P < 0.001). The percentage of CFU reduction in each group is shown below:

$$\text{Calcium hydroxide:} \frac{8.88 - 9.03}{8.88} \times 100 = -1.69\%$$

$$TAP: \frac{8.87 - 0}{8.87} \times 100 = 100\%$$

$$Plasma = \frac{9.15 - 8.09}{9.15} \times 100 = 11.6\%$$

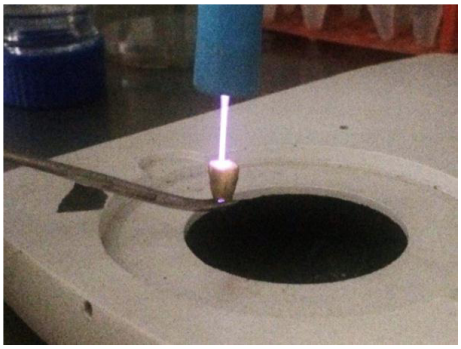

Figure 1. Plasma Radiation to the Canal of Dental Samples

Table 1. Mean of the Log CFU in experimental and control groups

Group	Mean of Log CFU	Standard Deviation	Standard Error
Calcium hydroxide	9.03	0.24	0.08
Control of calcium hydroxide	8.88	0.22	0.07
Triple antibiotic past	0	0	0
Control of triple antibiotic past	8.87	0.21	0.07
plasma	8.09	0.18	0.06
Control of plasma	9.15	0.26	0.08

Scanning Electron Microscopy

SEM images were taken at the apical one-third of the split root canals with different magnifications in the negative control group, positive control group, and plasma-treated group.

As shown in Figure 3 , the dentin tubules lacked bacterial and bacterial biofilm in the negative control group. In the positive control group, thick mature biofilm and their penetration into the dentinal tubules are visible (Figure 4). Although the biofilm remained in the root canal wall in the plasma-treated group, the regular structure of biofilm was destroyed and the open dentinal tubules were observed (Figure 5)

Discussion

The basis of successful root canal treatment with an

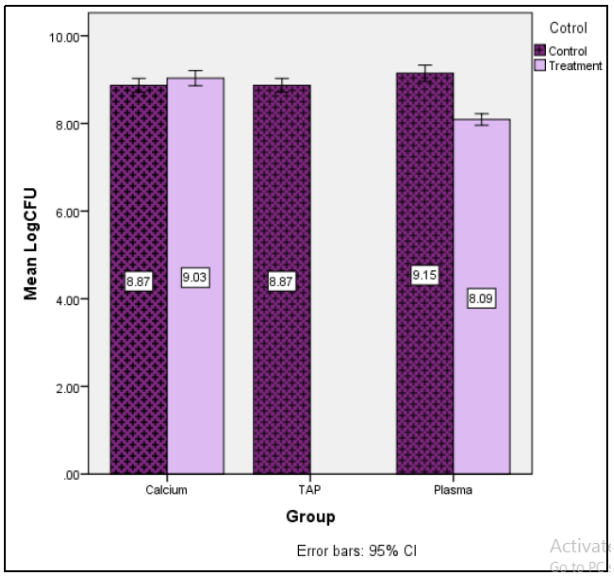

Figure 2. The Antibiofilm Effects of the Different Groups, Represented as the Mean ± Standard Deviation of the Log CFU

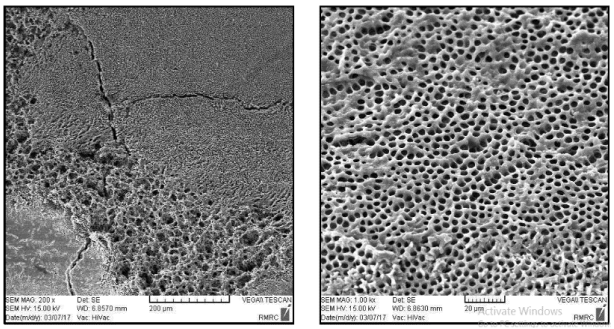

Figure 3. SEM Images From the Root Canal Wall in the Negative Control Group

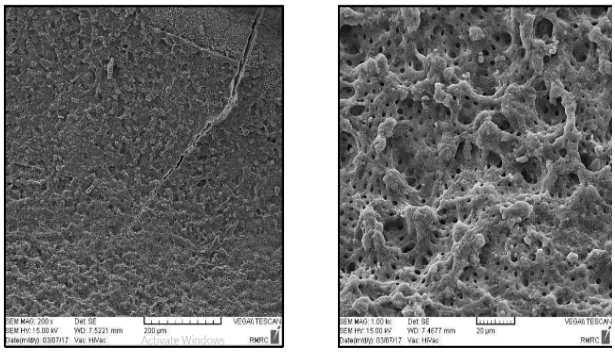

Figure 4. SEM Images in the Positive Control Group

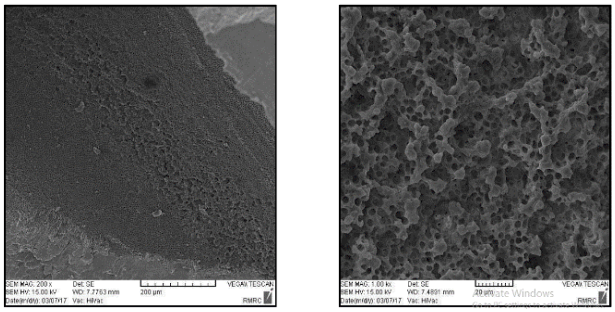

Figure 5. In-canal SEM Images of the Plasma-Treated Group

appropriate long-term prognosis is the elimination of bacteria and their products from the root canal system.[2] This study investigated the antimicrobial effects of plasma and TAP and calcium hydroxide on *E. faecalis* in the root canals. A 21-day-old biofilm of *E. faecalis* was used in this study due to the different antimicrobial effects of the medicaments on planktonic bacteria and bacteria in the biofilm. The bacteria that make up a biofilm are more resistant to antibiotics than similar microorganisms that grow in a planktonic state. The concentration of antibiotics required to kill biofilm bacteria is about 100 to 1000 times higher than the concentration required to kill the same species in the planktonic state.[34] A clinical TAP concentration of 10 mg/mL was an effective medicament in eliminating bacteria from the root canal system while allowing for the survival of stem cells.[18,19] In the regeneration treatment protocol, TAP is used as an intracanal medicament, but it has been shown that TAP, especially in high concentrations, has a toxic effect on the pulp stem cell.[35,36] Methylcellulose was used as a carrier for intracanal medications because it did not interfere with the antibiotic, did not have an antimicrobial effect itself, and did not interfere with the penetration of medicaments into the canal. It is also easily cleaned from the canal environment at the end of the treatment.[37] Based on the results of this study, 10 mg/mL TAP was significantly effective in killing *E. faecalis*, but calcium hydroxide did not decrease *E. faecalis* biofilm inside the root canal. Studies have demonstrated that calcium hydroxide has a limited effect on *E. faecalis* in the biofilms because *E. faecalis* is resistant to calcium hydroxide; in particular, it has an ability to resist high pH values due to a functioning proton pump that drives protons into the cell to acidify the cytoplasm.[34]

Latham et al[19] showed that TAP with a concentration of 10 mg/mL completely removed the *E. faecalis* biofilm from inside the canal, but calcium hydroxide could not remove the biofilm, and the result was the same as our study. Statistically significant differences were observed in the antimicrobial effect of cold plasma at 8 KV for 10 minutes. Plasma antibacterial effects were more than calcium hydroxide but had less antibacterial effects than TAP (For plasma, RCC = 11.53% and for TAP, RCC = 100%).

Similar to our study, Li et al[38] showed a greater antibacterial effect for plasma than calcium hydroxide, but in contrast to this study, the use of argon oxygen plasma for 12 minutes completely removed *E. faecalis* biofilm from the canal. The different result may be due to different bacterial sampling and plasma parameters. Du et al[39] examined the antimicrobial effects of atmospheric plasma in vitro for the treatment of root canals and concluded that the procedure significantly reduced *E. faecalis* biofilm in 10 minutes. Pan et al[30] declared that cold plasma was effective in killing *E. faecalis* biofilms in root canals.

In our study, the sampling was done by using a rotary file. This sampling method was different from other plasma-related articles.[38] In previous studies, the paper point or normal saline solution was used for sampling, which is suitable for the study of planktonic bacteria, not for an effect on bacterial biofilms. The rotary file sampling, which shaves the biofilm, shows more accurate results.

Despite the importance of laboratory studies in evaluating the performance of dental materials, the generalizability of the results in this study in the clinical and oral settings of patients is not without challenges because of many confounding variables in the oral environment that are very difficult to control. The in vitro nature of the research limits the generalizability of its findings in clinical and oral settings. Further studies

on other specific bacterial species and evaluation of different types of plasma devices are recommended for future studies.

Conclusion

Ten mg/mL of TAP has the strongest antibacterial effect, resulting in the complete removal of *E. faecalis* biofilm.

Also, the application of cold plasma to dental samples effectively inhibited the growth of *E. faecalis* and decreased the biofilm level of bacteria. Plasma could be as canal disinfectants, especially in regeneration treatments, because plasma has no adverse effect on stem cells[23]; however, using plasma alone is not efficient enough to completely remove the bacterial biofilm.

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Conflict of Interests

The authors declare no conflict of interest.

Ethical Considerations

Ethical approval was obtained from the research ethics committee of Shahid Beheshti University (IR.SBMU.RETECH.REC.1395.549).

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