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Efficacy of Atmospheric Pressure Plasma as an Antibacterial Agent Against Enterococcus Faecalis in Vitro*

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Abstract Enterococcus faecalis (E. faecalis) is a microorganism that can survive extreme challenges in obturated root canals. The aim of this study was to evaluate the efficacy of a non-thermal atmospheric pressure plasma plume against E. faecalis in vitro. A non-thermal atmospheric pressure plasma jet device which could generate a cold plasma plume carrying a peak current of 300 mA was used. The antibacterial efficacy of this device against E. faecalis and its biofilm under different conditions was detected. The antibacterial efficacy of the plasma against E. faecalis and Staphylococcus aureus (S. aureus) was also evaluated. After plasma treatment, the average diameter of inhibition zone on S. aureus and E. faecalis was 2.62±0.26 cm and 1.06±0.30 cm, respectively ($P < 0.05$). The diameter was increased with prolongation of the treatment duration. The diameters of inhibition zone of the sealed Petri dishes were larger than those of the uncovered Petri dishes. There was significant difference in colony-forming units between plasma group and control group on E. faecalis biofilm ($P < 0.01$). The transmission electron microscopy revealed that the ultrastructural changes cytoderm of E. faecalis were observed after treatment for 2 min. It is concluded that the non-thermal atmospheric pressure plasma could serve as an effective adjunct to standard endodontic microbial treatment.

Keywords: non-thermal atmospheric pressure plasma jet, Enterococcus faecalis, Staphylococcus aureus

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1 Introduction

Enterococcus faecalis (E. faecalis), a facultative anaerobic gram-positive coccus, has been frequently found in obturated root canals exhibiting chronic apical periodontitis, and been isolated in 23% to 70% of the positive cultures [1]. It is a group of bacteria cultured from periapical lesions refractory to endodontic treatment and even isolated from endodontic cases requiring retreatment [2].

E. faecalis, moreover, can enter the viable but non-cultivable (VBNC) state, to tolerate or adapt to harsh environmental conditions and resuscitate upon returning to favorable conditions [3]. Following pre-exposure to sublethal stress condition, E. faecalis becomes less sensitive to normally lethal levels of heat, ethanol, hydrogen peroxide, acidity, and calcium hydroxide; furthermore, "cross-protection" is pronounced against diverse challenges [4,5]. Therefore, it is so important to seek an alternative disinfection technique to inactivate E. faecalis during root canal treatment.

A new-type plasma jet device, which can generate a cold plasma plume with high discharge current, has been successfully used to inactivate the Staphylococcus aureus (S. aureus) [6]. In this study, a series of experiments were designed to evaluate the efficacy of the atmospheric pressure plasma plume against E. faecalis.

2 Experimental design

2.1 Microorganism

E. faecalis strains (ATCC29212) and S. aureus strains (ATCC25923) (China Committee of Culture Collection for Microorganisms, CCCCM) were taken from −80°C stocks and plated onto Todd Hewitt Broth (THB, Becton, Dickinson and Co., USA) supplemented with agar of 1.5%. They were incubated in aerobic atmosphere at 37°C for 24 h respectively. An overnight pure culture of E. faecalis grown in brain heart infusion (BHI) medium was adjusted to 0.5 McFarland scale (1.5×10^8 CFU/mL) in 2.0 mL sterile physiological saline. A suitable turbidity of bacterial suspension

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and Petri dishes with BHI agar plate were prepared for later experiments.

2.2 Atmospheric pressure plasma jet device

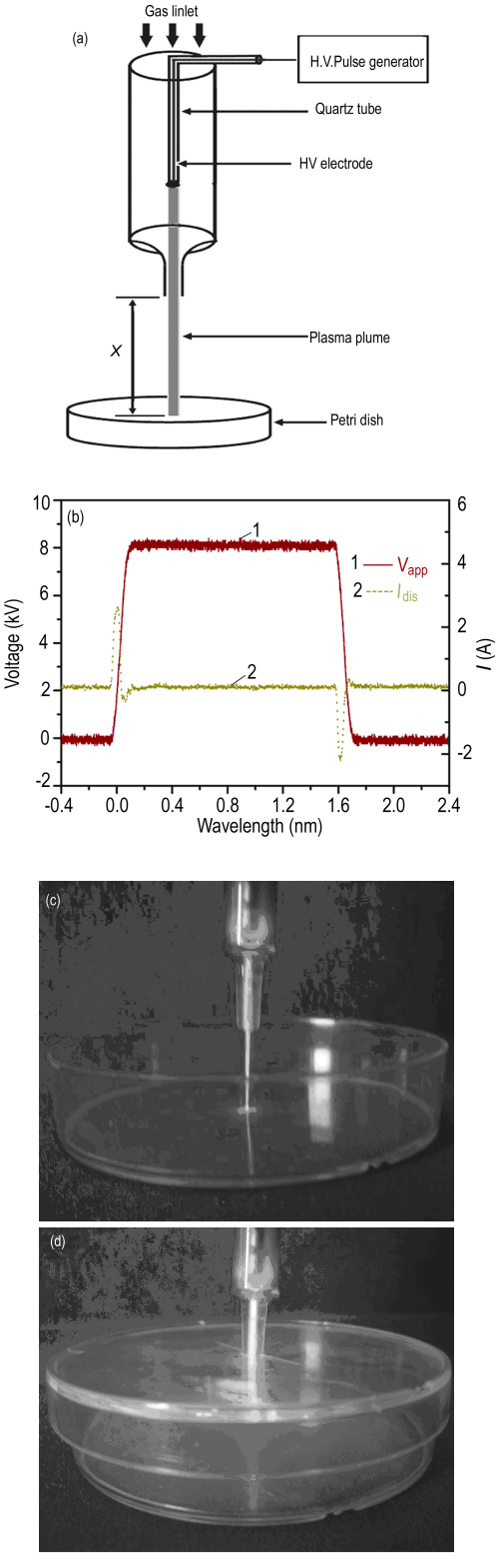
The atmospheric pressure plasma jet device was built up and described previously [7,8]. A helium-oxygen mixture (He/O_2) were injected into the hollow barrel and a HV pulsed dc voltage, with an amplitudes of up to 10 kV, repetition rate of up to 10 kHz, and pulse duration varied from 200 ns to dc, was applied to the HV electrodes. The homogeneous plasma was generated in front of the quartz tube's end, as shown in Fig. 1(a) and (b). In all inactivation experiments, the pulse frequency of 10 kHz, pulse width t_{pw} of 1.6 μs, and applied voltage V of 8 kV were fixed. The tip of the device was designed with a syringe needle. The diameters of the syringe and syringe nozzle were about 6 mm and 0.7 mm respectively. The inner diameter and the length of the needle was 200 μm, and 3 cm respectively. The distance between the jet nozzle and the bacterial samples was 10 mm.

2.3 Antibacterial efficacy of the atmospheric pressure plasma jet

Ten Petri dishes with BHI agar plate were prepared. A total of 100 μL suspension of 1×10^6 CFU/mL of the diluted E. faecalis and S. aureus were cultured on five agar plates respectively. These (ten) Petri dishes were treated by the plasma plume with helium/oxygen (2%) at a flow rate of 2 L/min for 5 min without lids respectively, as shown in Fig. 1(c). Immediately after the plasma treatment, E. faecalis groups were incubated in an anaerobiotic condition, and S. aureus groups in an aerobic condition at 37°C for 24 h. The diameters of inhibition zones were measured by slide gage in four directions.

Twenty-five Petri dishes with BHI agar plate were prepared and divided into five groups ($n = 5$ for each). The diluted E. faecalis of 2.5×10^5 CFU were evenly spread over each agar plate. The Petri dishes in groups A, B and C were treated by the plasma plume with helium/oxygen (2%) at a flow rate of 2 L/min for 5 min, 10 min and 15 min without lids, respectively. In the control group D, the samples were treated for 15 min by the working gas flowing at the same flow rate with the plasma turned off. In the negative control group E, no treatment was given, as shown in Fig. 1(c). They were incubated instantly in anaerobiont at 37°C for 24 h. The diameters of inhibition zones were measured.

Ten Petri dishes with BHI agar plate were prepared and divided into two groups ($n = 5$ for each). The diluted E. faecalis of 1.5×10^5 CFU were evenly spread over each agar plate, and each of them was treated with helium/oxygen (2%) at a flow rate of 1 L/min for 5 min. In group 1, the Petri dishes were sealed (Fig. 1(c)), and in group 2 the Petri dishes were uncovered (Fig. 1(d)). All Petri dishes were instantly incubated in anaerobiotic condition at 37°C for 24 h and the inhibition zones were measured.



(a) Experimental design. "X" represents the distance between the jet nozzle and the bacterial samples, (b) Voltage and current waveforms in the experiments, Photographs of the plasma plume treatment without lids (c) in Petri dishes and (d) in sealed Petri dishes

Fig.1 Sketch of experimental principle of the atmospheric pressure plasma jet

2.4 Observation under the transmission electron microscopy (TEM)

Three groups were set up ($n=3$) and treated with helium/oxygen (2%) at a flow rate of 1 L/min for 2 min, 4 min and 8 min respectively. Each Petri dishe was rinsed instantly and thoroughly by 0.1 M phosphate buffered saline (pH 7.0) and centrifuged at 12000 r/min for 8 min. The precipitates were collected, fixed and dehydrated routinely. Finally, the samples were embedded in Quetol 651 resin and visualized under TEM (CM100, FEI/Philips, Netherlands).

2.5 Antibacterial efficacy of atmospheric pressure plasma jet against E. faecalis biofilms

A total of 20 μL of the suspension of diluted E. faecalis with a concentration of 1×10^6 CFU/mL were seeded on the sterile cellulose nitrate membrane filters, which were placed on the surface of BHI agar plates. Plates covered with membranes were incubated at 37°C for 48 h in an aerobic atmosphere for later experiments. The membranes in experimental group ($n=10$) were treated with helium/oxygen (2%) at a flow rate of 1 L/min for 10 min, while those in control group ($n=10$) were treated for 10 min with the working gas flowing at the same flow rate with that for the plasma turned off.

The membranes in these two groups were rinsed with sterile physiological saline for 1 min. Ten-fold sterile dilutions were generated in reduced transport fluid, and then incubated in BHI plate at 37°C for 24 h. CFU per membrane were calculated.

The membranes of these two groups were fixed with 0.4% formaldehyde, rinsed three times with distilled water and dehydrated in a series of 30%, 50%, 70%, 90%, 96% and 100% ethanol. They were coated with gold and observed under the scanning electron microscopy (SEM, s-570, Japan).

2.6 Statistical analysis

All in vitro assays were carried out in triplicate. Experimental data were expressed as means ±SD. All data were analyzed via analysis of variance tests (ANOVA), with pairwise comparisons made by least significant difference procedure (LSD) or parametric student's t-test. The results were considered statistically significant when $P<0.05$. Correlation analysis was performed using SPSS statistical software (version 13.0).

3 Experimental results

3.1 Antibacterial efficacy under different conditions

In order to compare the antibacterial efficacy of the atmospheric pressure plasma jet on different bacteria, a normal germ, S. aureus was used as the control. The inhibition zones were observed clearly after atmospheric-pressure plasma treatment, as shown in Fig. 2(a). The statistical analysis showed that the average diameter in S. aureus group and E. faecalis group was 2.62±0.26 cm and 1.06±0.30 cm respectively shown in Fig. 2(d), with the difference being statistically significant between two groups ($P=0.000$).

Under the stable parameter conditions, the average diameter of inhibition zone at 5 min, 10 min and 15 min was 1.48±0.24 cm, 3.64±0.27 cm and 8.16±0.51 cm in groups A, B, and C, respectively. In the two control groups, there was no inhibition zone. The ANOVA analysis revealed that there was no statistically significant difference among groups A, B, and C, as shown in Fig. 2(b) and (e). Furthermore, the results implied a double increase in diameter while the working time was prolonged by 5 min.

The antibacterial efficacy in the sealed Petri dishes was better than that of patent Petri dishes. The diameters of inhibition zone in the sealed Petri dishes were larger than those of the patent Petri dishes, shown in Fig. 2(c).

3.2 Observation under TEM

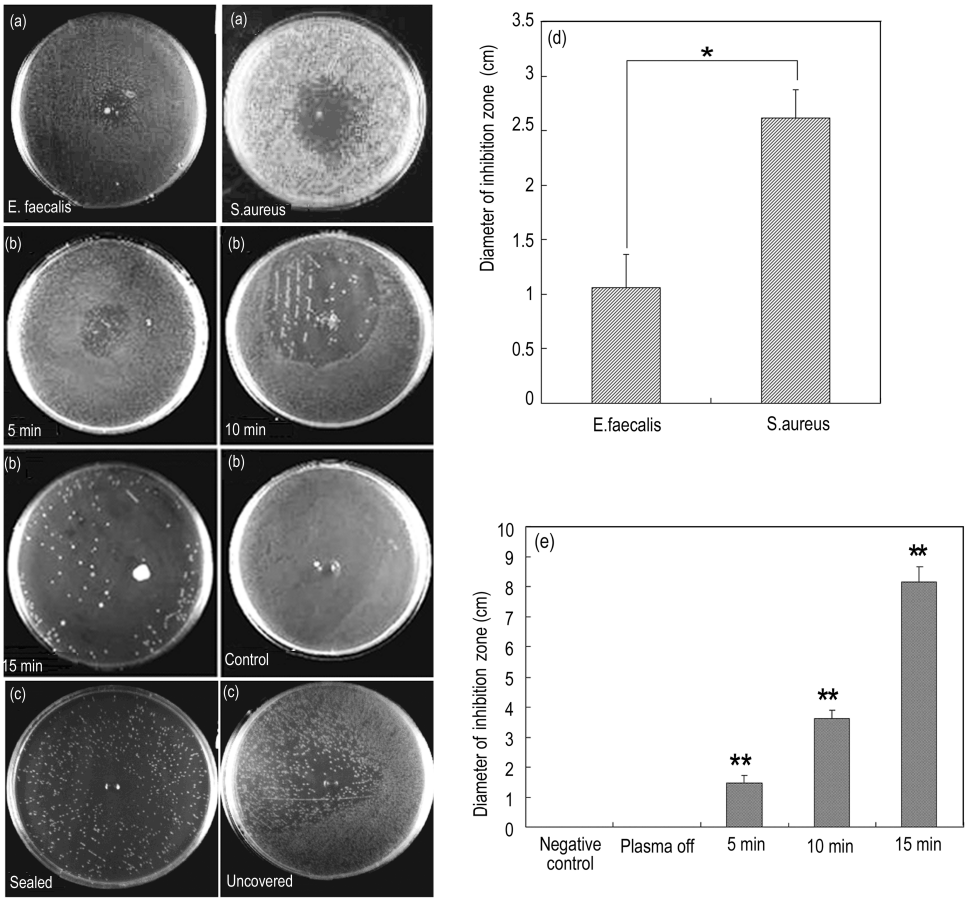
The ultrastructural changes in E. faecalis could be observed under TEM after treatment with a plasma plume, as shown in Fig. 3. After treatment for 2 min, 85% of the cytoderm of E. faecalis started to change. 90% of the cytoderm appeared a lysis and the kytoplasm generated vacuolization until treated for 8 min.

3.3 Antibacterial efficacy of plasma jet against the E. faecalis biofilms

E. faecalis was seeded on cellulose nitrate membrane filter after incubation for 48 h. The biofilm of E. faecalis began to take shape. Nearly all the E. faecalis colonies and biofilms were eliminated after the plasma plume treatment. The residual bacterial colonies could be observed under SEM after treatment but the structure was damaged, and matrix-like material became sparse, as shown in Fig. 4(a)∼(c). All the tests showed a complete removal of living bacteria from the cellulose nitrate membrane after the treatment. CFU of E. faecalis in experiment group had a 100-fold decrease as compared with that for control group, as shown in Fig. 4(d).

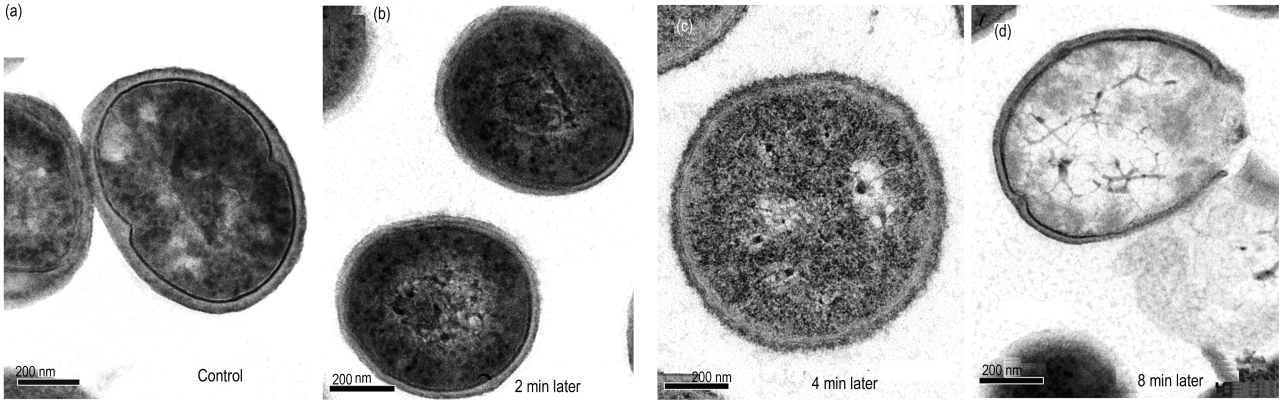
4 Discussion

In the past few years, E. faecalis has been the focus both in medicine and dentistry. As a recognized pathogen in post-treatment endodontic infections, E. faecalis is frequently isolated both in mixed flora and monocultures. Eradication of E. faecalis from the root canal is difficult with chemomechanical preparation, disinfectant irrigation or antibacterial dress-



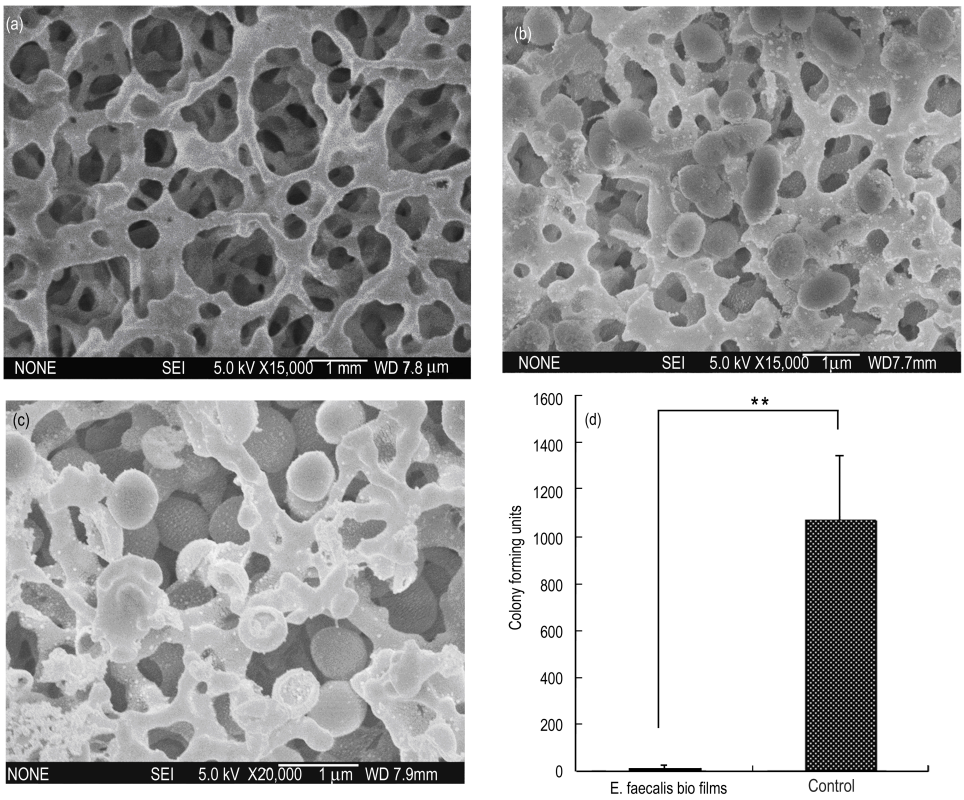
(a) Diameter of inhibition zones in E. faecalis and S. aureus, and (d) the related statistical analysis, (b) E. faecalis samples treated with different durations, and (e) the related statistical analysis, (c) E. faecalis samples treated for 5 min with helium/oxygen (2%) at a flow rate of 1 L/min in the uncovered dish and sealed dish. In (d) and (e), $^{*}P < 0.05$, $^{**}P < 0.01$, one-way ANOVA and LSD comparison between groups, and parametric student's t-test comparison between two groups

Fig.2 Antibacterial efficacy of the atmospheric pressure plasma jet



(a) E. faecalis was oval-shaped cell with clear cell structure under TEZM. The diameter of the cell was 0.5 μm to 1.0 μm with smooth cell walls close to the cell membrane, (b) After treated for 2 min, the cell surface became rough and wrinkled, and the cell wall became fuzzy under TEM, (c) After treated for 4 min, the cell shell became fuzzy, swelling, and its edge became rough. The cellular core became osteoporosis and its dyeing became shallow under TEM, (d) After treated for 8 min the cells became swelling, vacuolar degeneration occurred in cytoplasm, cell wall was ruptured, and the cytoplasmic contents outflowed. Some of the debris was completely dissolved

Fig.3 Ultrastructural changes in bacteria after treatment with plasma plume



(a) Nitrate membrane filter (×15 000), (b) Biofilm of E. faecalis began to take shape after incubation for 48 h (×15 000), (c) Nearly all the E. faecalis colonies on the biofilm were affected after the plasma plume treatment. The structure of residual E. faecalis was damaged; the shape of bacteria was shrunk, and the surface was rough and dry wrinkle (×15 000), (d) Statistical analysis on CFU between E. faecalis biofilms and controls ($^{**}P < 0.01$, parametric Student's t-test comparison between two groups)

Fig.4 E. faecalis biofilm on cellulose nitrate membrane filter under SEM

ings [9]. Therefore, advanced method to eradicate the E. faecalis and its biofilm should be further investigated.

E. faecalis is not sensitive enough to some traditional sterilized methods, such as heat, acid, calcium hydroxide, and UV irradiation [7]. The plasma jet device used herein could generate a cold plasma plume with high discharge current and without any toxic residue after treatment. The plasma generated by this device is an atmospheric pressure non-equilibrium plasma (APNPs) and has been confirmed sterilizing to S. aureus [6]. In order to eradicate the E. faecalis in root canal system, the tip of the device had to be replaced with syringe needle. Fortunately, according to the result of this study, it could bring about an obviously antibacterial activity against E. faecalis and its biofilm in spite of the plasma plume was as thin as a needle.

The results of this study indicated that longer treating duration could lead to a better antibacterial efficacy. There was an obvious efficacy at the center of plasma plume-treated samples after treated for 10 min. E. faecalis could be killed completely after treated for 15 min, which was consistent with that reported in the literature [10,11]. However, in this study the same plasma plume showed different antibacterial efficacy for E. faecalis and S. aureus. The antibacterial efficacy of the plasma plume against E. faecalis seemed to be lower than that for S. aureus. This stubborn ability of E. faecalis to endure harsh environment had also been elucidated in other studies [1,2]. There were always a few residual E. faecalis colonies scattering in the inhibition zone after the plasma treatment, which might be attributed to the fact that when bacteria were spread over the agar plate in dishes, some E. faecalis left and hided in agar, but the plasma plume could not penetrate deeper to kill these coated bacteria.

It was found that the antibacterial efficacy of the plasma plume in sealed Petri dishes was clearly higher than that in patent Petri dishes. LU [6] reported that the electrons, ions and oxygen free radicals played important roles in antibacterial efficacy in the discharge area. Only oxygen free radicals acted to antagonize the bacteria both in both after glow area and farther area [12]. For oxygen-free radicals in sealed Petri dishes can be reserved longer, the concentration of the charged particles O_2^-, including the ozone (O_3), metastable state O_2, and atom oxygen will be raised, which plays the main role in the inactivation process [8]. That's why the antibacterial effect is more notable in sealed Petri dishes than in uncovered dishes. Especially, the ozone (O_3) has been proved effective for the sterilization of cavities, root canals, periodontal pockets, and herpetic

lesions [13]. It has also been utilized in dental clinical treatment for many years.

Under TEM, it was observed that the surface of some E. faecalis cells became roughness and wrinkles after treated with the plasma plume for 2 min, shown in Fig. 3. After the treatment with plasma plume for 4 min the cell wall became fuzzy, swelling, and its edge became rough. The cellular core became osteoporosis and its dyeing became shallow. Some cell's wall became cloudiness and was ruptured for a prolonged treating duration.

E. faecalis has the ability to form biofilms on the walls of root canals [14~16]. A kind of biofilm model, membrane filter disc model, was used to explore the efficacy of plasma plume treatment in this study. This membrane filter disc model has been used by many researchers to co-evaluate antibacterial efficacy of the agents against the bacterial biofilm [17~19]. It has the advantage of forming biofilm on standardized surfaces, so the antibacterial efficacy of the plasma plume can be more accurately assessed.

Under SEM, it was clearly observed that the biofilms of E. faecalis began to take shape initially after culture for 48 h, and the construction of biofilms was damaged after treatment with the plasma plume.

5 Conclusion

Based on the results of this in vitro study, it is concluded that the plasma plume has a significantly antibacterial efficacy against E. faecalis and their biofilms. Our results demonstrate that the antibacterial efficacy of the APNPs is inspiring, and further study is needed before APNP could be adopted to disinfect root canal in oral clinic.

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