Plasma Chemistry and Plasma Processing
https://doi.org/10.1007/s11090-022-10244-4

ORIGINAL PAPER

Effect of Plasma Activated Water in Caries Prevention: The Caries Related Biofilm Inhibition Effects and Mechanisms

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Received: 22 February 2021 / Accepted: 5 March 2022

Abstract

Streptococcus mutans (*S. mutans*) has been implicated as the primary cariogenic species of dental caries in humans. The purpose of this study was to explore the inhibition effects of plasma activated water (PAW) on *S. mutans* biofilm and the mechanisms. *S. mutans* biofilm model was established on bovine enamel discs in vitro. Both 12 h old and 48 h old biofilm were constructed. The samples were placed into sterile distilled water and treated with cold plasma or air for 10 min. 0.12% chlorhexidine (CHX) and phosphate buffer saline (PBS) were used as positive and negative control respectively. PAW was confirmed to have strong inactivation effect on *S. mutans* biofilms. The number of CFU was significantly reduced below the detection limit in both 12 h and 48 h PAW groups, and CLSM images were consistent with CFU results. SEM images showed cells in disorder and burst open with the appearance of melted clusters after PAW treatment. The ORP value of PAW increased obviously with time, while the pH value decreased obviously. The OES analysis indicated that the atomic oxygen (O) emissions at 777 nm and 844 nm and OH emissions at 306–309 nm were detected. Amounts of H_2O_2, NO_2^- and NO_3^- were detected, which might be major excited reactive species in PAW.

Keywords Plasma activated water (PAW) · *Streptococcus mutans* (*S. mutans*) · Biofilm · Dental caries

Introduction

Dental caries is considered to be the third serious disease that is hazardous to human health. Dental caries is one of the most prevalent biofilm-induced oral diseases, which affects 2.4 billion adults and 621 million children worldwide [1]. *Streptococcus mutans* (*S. mutans*) is considered to be the key etiological agent of dental caries among the various

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bacterial species which is the primary exopolysaccharides producer in oral cavity and can cause the subsequent coaggregation of more fastidious organisms to colonize the cariogenic biofilm [2, 3].

How to eliminate caries-causing bacteria effectively has long been a challenge for both dentist and caries susceptibility population. However, the current procedures for treating dental caries are not always guaranteed to successfully remove all residual dentin tissue microorganisms [4, 5]. Therefore, the expectation of disinfection strategy to prevent dental caries and the secondary caries after restoration has long been a goal of dentistry. As to caries susceptibility population, tooth brushing had been recognized to be the most common and important approach. However, the adjacent surface and the fossa, groove and spot space of the tooth cannot be efficiently cleaned. It is really a disaster for the caries susceptibility population.

Therefore, chemotherapeutic strategies have been also considered, and among them, CHX has been recommended for dental biofilm treatment in clinic [6, 7]. However, CHX may cause changes in taste, mouth discoloration, mucosal irritation, and desquamation of the gums [6, 8]. Due to the undesirable side effects and insufficient biofilm suppression associated with CHX, the search for new anti-biofilm mouthwashes remains a continuing research focus.

Plasma activated water (PAW) is considered a green and prospective solution for biotechnology applications due to the transient nature of its biochemical activity [9]. It was first reported about the antibacterial effect of PAW in 2007 [10]. The preliminary results of PAW against *S. mutans* in planktonic culture have been verified [11, 12]. However, the main form of bacteria lived in the oral environment is biofilm, which is closely attached to the dentin surface and highly resistant than planktonic [13]. Meanwhile, the presence of extracellular polymeric substance (EPS) with its local barriers and altered microenvironment could reduce drug access, trigger bacterial tolerance to antimicrobials and enhance the mechanical stability [14]. More important, the bovine teeth and non-stimulated saliva from donors were applied to simulate the real clinical situation at present study, instead of using hydroxyapatite discs or artificial saliva or directly making biofilm to form on material surface without acquired enamel pellicle that widely used in previous studies [15]. The acquired enamel pellicle is a bacteria-free, organic film formed in vivo on the tooth surface due to selective adsorption of salivary proteins and glycoproteins [16]. The present study with bovine teeth infected with *S. mutans* biofilm is closer to the real clinical level and more acceptable by the clinicians. During biofilm formation, bacterial colonization starts with the adhesion of pioneer bacteria, mostly streptococci and *Actinomyces* spp., to the salivary pellicle of the teeth [17].We have either searched similar methods that use natural saliva to form acquired enamel pellicle in previous papers on bactericidal effect of PAW.

In our previous study, the cold plasma jet cannot be activated under water and the working gas is Ar/O_2, so the reactive oxygen species (ROS) cannot fully react within water [11]. Thus, dielectric barrier structure with hollow electrodes (HEDBS) was applied in this study.

The aim of this study was to evaluate the antimicrobial effect of PAW on *S. mutans* biofilm against conventional methods at bovine dentin and explore the main antimicrobial mechanisms.

Materials and Methods

Cold Plasma Device

The equipment used in this study was an air plasma generator with hollow electrodes structure. A detailed description of the working principle of HEDBS has been introduced in our previous study [18]. Compressed air with 260 L/h gas flow rate was injected into the quartz tube, and the high voltage electrode is connected to a power source with 20 kHz. A homogeneous plasma is generated in the discharge gap of 0.5 mm and a plasma jet reaching lengths of up to 7 mm long is ejected through the end outlet of 0.5 mm.

Establishment of *Streptococcus mutans* Biofilm

Bovine Enamel Disc Preparation

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 0.1% thymol solution at 4℃ until being used. Each bovine root was horizontally cut at the cemento-enamel junction (CEJ) with diamond burs under water cooling. To allow each biofilm to grow in the same substrate, the labial enamel of each specimen was polished by 800 grit, 1000 grit, 1500 grit, 3000 grit and 5000 grit silicon carbide paper in sequence. The enamel discs were then shaped with a fine annular bur with inner diameter of 4 mm and a carbide bur under water cooling to create a round enamel disc with a diameter of 4 mm and thickness of 2 mm. The prepared enamel discs were immersed in PBS solution and ultrasonic washed for 15 min to remove smear layer and then stored in a glass vial containing 0.9% sodium chloride solution for sterilization in an autoclave at 121℃ for 20 min.

Saliva Collection and Acquired Enamel Pellicle Formation

Non-stimulated saliva from 30 healthy donors was collected, pooled and then centrifuged (10,000 × g) for 15 min at 4 ℃. The supernatant was mixed with PBS solution by 1:1 and the mixture was stored at − 20 ℃. Immediately before starting the experimental session, saliva was filtered with a 0.45 μm and 0.22 μm filtrum successively.

The prepared enamel discs were placed in 24 well plate. Then, 1 mL of saliva solution was added to each well, and the plates were placed in 37 ℃ in a 5% supplemented CO_2 environment for 12 h. The acquired enamel pellicle would form on the surfaces of the discs.

Biofilm Incubation Protocol

The strain of *S. mutans* UA159 was used in this study. It was cultured in Brain Heart Infusion (BHI) (Oxoid, Basingstoke, UK) plate for 48 h at 37 ℃ in a 5% supplemented CO_2 environment, and then kept at 4℃ subsequently. A single colony was obtained from the plate and transferred to 1 mL BHI added with 1% sucrose and incubated for 12 h at 37 ℃ in a 5% supplemented CO_2 environment, until the bacterial population reached 1.0×0^7 CFU/mL to 1.0×10^8 CFU/mL.

Bacterial suspension of 100 μL was added to 3 mL BHI with 1% sucrose within a 12-well plate. An enamel disc on which acquired enamel pellicle has formed was

Table 1 The experimental groups of different treating methods (n = 10/group)

Group (12 h biofilm)	Treatment	Group (48 h biofilm)	Treatment
Group 1	PAW	Group 5	PAW
Group 2	0.12% CHX	Group 6	0.12% CHX
Group 3	Blank control (Air)	Group 7	Blank control (Air)
Group 4	Negative control (PBS)	Group 8	Negative control (PBS)

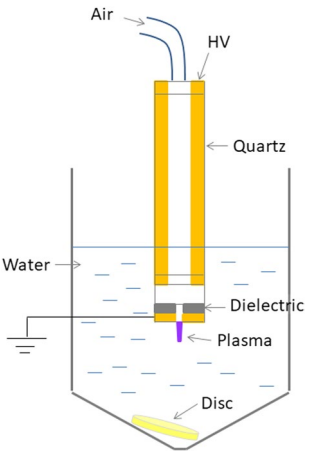

Fig. 1 Schematic of the cold plasma device and the treatment process

transferred to a new well with 1 mL PBS to wash down unattached saliva, and then transferred to the well with bacterial suspension and incubated at 37 ℃ in a 5% supplemented CO_2 environment for 12 h (immature biofilm) or 48 h (mature biofilm).

The Effect of the *Streptococcus mutans* Biofilm Treated by PAW

As Table 1 shows, groups 1–4 were incubated for 12 h, and groups 5–8 were incubated for 48 h, representing for young and mature biofilm respectively. The discs were transferred to a new well with 1 mL PBS to erase unattached bacteria. In groups 1 and 5, the discs were respectively transferred to centrifuge tubes with 20 mL sterile deionized water, and PAW was generated by inserting the tube of the cold plasma device into sterile deionized water and locating the tip of the tube approximately 10 mm below the liquid surface (Fig. 1). In groups 2 and 6, the same device was used as above, with air into the water, without cold plasma generated. In groups 3 and 7, the discs were transferred to 20 mL 0.12% chlorhexidine (CHX). In groups 4 and 8, the discs were in PBS, with no further treatment. All of the groups were treated for 10 min.

Biofilm Viability

Colony Forming Unit (CFU) Assay After the above treatments, each of the specimens was transferred to a new well with 1 mL sterile PBS, and then the total biofilm was collected. The biofilm was transferred into 1 mL PBS. The centrifuge tubes were shaken vigorously for 1 min in order to make the biofilm evenly dispersed in PBS. The number of *S. mutans* was counted by an EasySpiral instrument (Interscience, France).

Confocal Laser Scanning Microscopy (CLSM) analysis After the specified treatments, specimens were gently rinsed with 1 mL sterile PBS. LIVE/DEAD Back Light Bacterial Viability Kit (Invitrogen, Carlsbad, CA) was used following the manufacturer's instructions. The biofilm was stained and kept in dark at 4℃ for 15 min. The fluorescence from stained bacterial cells adherent to the enamel discs was observed and imaged by CLSM (A1R-si; Nikon, Tokyo, Japan) with the excitation/emission wavelengths of argon ion laser 488/543 nm. Three randomly selected areas were examined and three dimensional (3D) reconstructions analysis was obtained for each biofilm specimen.

Biofilm Morphology Analysis

Scanning electron microscopy (SEM, S-4800; Hitachi, Tokyo, Japan) was introduced to study structural changes of the biofilm in groups 1–8. The specimens were immersed in 2.5% glutaraldehyde for 24 h at 4℃ and a gradient of dehydration in 30%, 50%, 70%, 90% and 100% ethanol. Subsequently, they were dried naturally, fixed with electrically conductive silicone, and sputter-coated with gold–palladium for examining.

Mechanisms for Bactericidal Effect of PAW

Oxidation–Reduction Potential (ORP) and pH Measurement

To evaluate the oxidizability of PAW and the concentration of oxidizers, ORP of the PAW was measured using a redox-sensitive electrode (LE501, Mettler Toledo, Switzerland) at room temperature (25℃). Both of pure water and water with 12 h old or 48 h old biofilm in it were treated with plasma and measured. The ORP value of water was considered to be the "zero" point, where the addition of oxidizing chemicals will result in higher values. The pH value of the PAW was measured using a microprocessor pH-meter (pH 213, Hanna Instruments, Woonsocket, RI, USA).

Major Reactive Species Measurement

To measure the major short-lived reactive species (O, $^{\cdot}OH$) generated in the water by the cold plasma equipment, conventional optical emission spectroscopy (OES) was applied with a fiber optic spectrometer (Avantes, Eerbeek, Netherlands). A quartz tube was placed at a distance of 5 mm away from the nozzle. The dispersed emission spectra were recorded in the 200–1000 nm range by a 2048-pixel charge-coupled device (CCD) detector array, and transferred to a computer for further analysis. Detailed OES detection method of the cold plasma can be found in our previous study [19, 20].

The concentrations of three representative long-lived reactive species (H_2O_2, NO_2^-, NO_3^-) in PAW induced by cold plasma were measured. H_2O_2 and NO_2^- were measured

respectively using a Hydrogen Peroxide Assay Kit (Beyotime, Jiangsu, China) and a Nitrite Assay Kit (Jiancheng, Nanjing, China). 100 µL of 1 mol/L hydrochloric acid was added into the Eppendorf centrifuge tube with 5 mL of PAW and subsequently, 10 µL of 0.8% sulfamic acid was added. The concentration of NO_3^- was determined by measuring the absorbance at 220 nm with an UV–Vis Spectrophotometer (Cary 60; Agilent, America) at room temperature.

Statistical Analysis

The statistical analysis was determined by using one-way analysis of variance, followed by post hoc multiple comparisons using the SPSS statistics software (version 20.0; SPSS, Inc., USA). The results were considered statistically significant when $p < 0.05$.

Results

The Determination of Bacterial Viability

The *S. mutans* biofilm model has been established successfully on the bovine enamel disc (Fig. 2A). After coated with acquired enamel pellicle (Fig. 2B), *S. mutans* biofilm was formed on the discs. Cell blocks can be seen on 12 h-old biofilm (Fig. 2C, D), and the structure of biofilm became more compact and complex after 48 h (Fig. 2E, F). Thus, the 48 h old biofilm were more resistant than the 12 h old biofilm.

Figure 3 shows the survival of 12 h-old and 48 h-old *S. mutans* biofilm with different treatments. It can be seen that the survival of microbes being reduced for about 7–8 log after PAW treatment. It was also found that in 12 h groups, there was no significant difference in the disinfection effect between PAW and CHX treatments ($p > 0.05$), in both of which all *S. mutans* bacteria was killed. However, in 48 h groups, PAW treatment exhibited

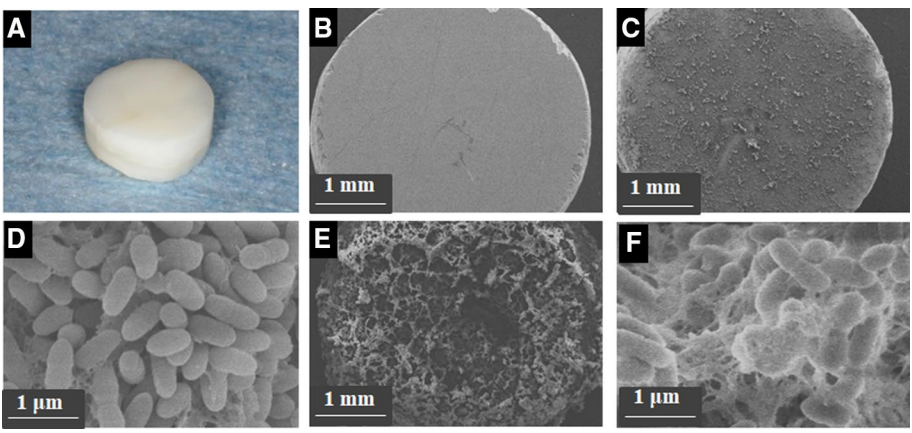

Fig. 2 *S. mutans* biofilms model. **A** Picture of bovine enamel disc. **B** Bovine enamel coated with acquire enamel pellicle. **C**, **D** 12 h old *S. mutans* biofilm. **E**, **F** 48 h old *S. mutans* biofilm

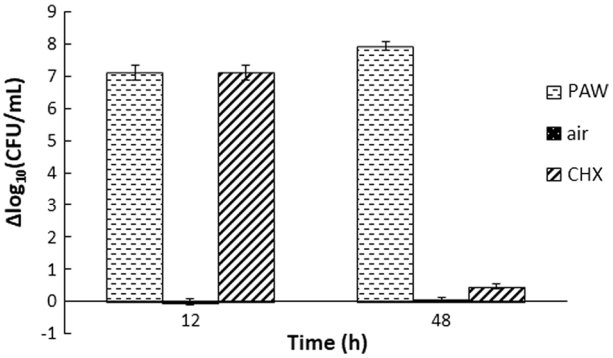

Fig. 3 Inactivation effect of PAW for 12 h old and 48 h old *S. mutans* biofilms ($\Delta\log_{10}$(CFU/mL) = $\log_{10}$control − $\log_{10}$PAW/air/CHI)

significantly better disinfection effect than CHX ($p < 0.05$). Air groups both for 12 h and 48 h showed no significant difference in comparison to the blank control ($p > 0.05$).

CLSM

In 12 h groups, biofilm treated with PAW and CHX showed red fluorescence, while control group and air group showed green fluorescence, meaning that the proportion of dead cells in PAW group and CHX group was higher than the other two groups. In addition, it was noticed that the thickness of the 12 h old biofilm after PAW treatment (Fig. 4A) was 70 μm, while the other groups (Fig. 4B, C, D) were 100 μm in thickness, which demonstrated that PAW may contribute to destruction of 12 h old biofilm structure. However, in 48 h groups, red fluorescence was detected only in biofilm treated with PAW, while all the other three groups all showed green fluorescence. The thickness of the biofilm of all groups was 160 μm, possibly because the firm structure of mature biofilms could not be easily disintegrated.

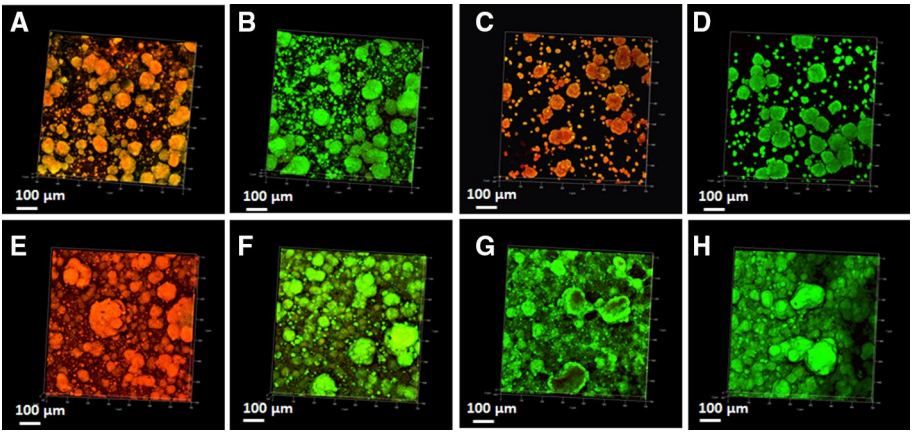

Fig. 4 Confocal laser scanning microscopic images for 12 h old (**A**–**D**) and 48 h old (**E**–**H**) *S. mutans* biofilms. **A** and **E** were treated by PAW. **B** and **F** were treated by air. **C** and **G** were treated by CHX. **D** and **H** were control groups

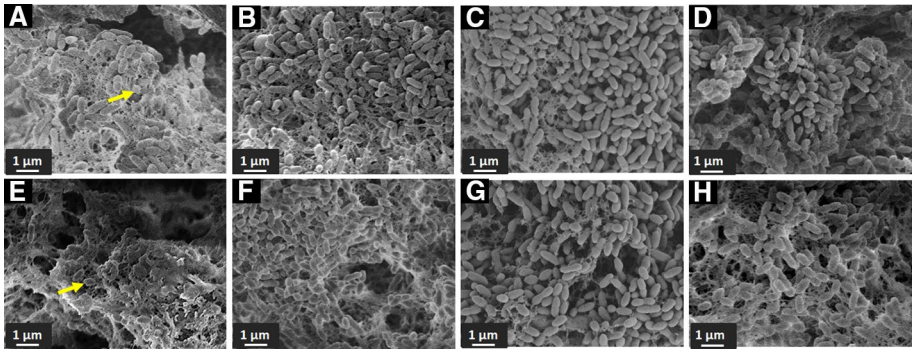

Fig. 5 SEM images for 12 h old (**A**–**D**) and 48 h old (**E**–**H**) *S. mutans* biofilms. **A** and **E** were treated by PAW. **B** and **F** were treated by air. **C** and **G** were treated by CHX. **D** and **H** were control groups

Biofilm Morphology Analysis

SEM images confirmed that PAW killed bacteria by inducing cell membrane damage [21]. It can be seen that in 12 h groups (Fig. 5A–D), the biofilm treated with PAW seemed to be less dense and thinner than the other groups, which was consistent with the results shown in CLSM. The physical size of *S. mutans* cells was around 1 μm in length. The arrangement of cells in the biofilm treated with PAW became in disorder, and surface roughening of the cell walls was observed. In 48 h groups (Fig. 5E–H), PAW treatment resulted in a significant alteration in morphology changes when compared with the other groups. The bacteria cells burst open after PAW treatment, and the bacterial cells were nearly indiscernible, with the appearance of melted clusters. Because of the cell structural damage observed in the SEM images (Fig. 5E), the CFU shown in Fig. 3 indicate that the bacterial cell structures were completely destructed and as a result the bacteria were almost completely killed.

ORP and pH Measurement

In Fig. 6, "0" minute represents the untreated control. After treated with plasma for 0.5 min, the ORP value of PAW has rised obviously, and reached 545.8 ± 9.7 mV after

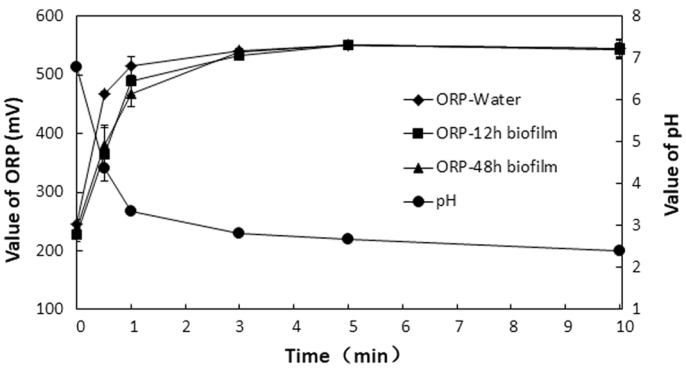

Fig. 6 Values of ORP and pH of PAW as time of water treated by plasma from 0 to 10 min

10 min. The ORP value of PAW with biofilm in it was lower than that of pure water when treated with plasma within 3 min, which got nearly the same after 3 min. Meanwhile, the pH value decreased obviously in the first 1 min, and dropped to 2.40 ± 0.02 after 10 min.

Major Reactive Species Measurement

The OES for PAW is shown in Fig. 7. The atomic oxygen (O) emissions at 777 nm and 844 nm was presented in the emission spectra. Meanwhile, $^{\cdot}OH$ (306–309 nm) emissions were also detected.

In present study, it was indicated that O and $^{\cdot}OH$ were detected.

The concentrations of H_2O_2 and NO_2^- in PAW as time are shown in Fig. 8. The H_2O_2 concentration increased gradually to 36.21 ± 3.36 μmol/L after 10 min treatment. It was discovered that the NO_2^- concentration obviously increased to 790.34 ± 120.58 μmol/L after 3 min, and then reduced slightly to 671.49 ± 112.89 μmol/L. The concentration of NO_3^- increased to 105.82 ± 1.30 μmol/L after 10 min treatment.

Discussion

When this *S. mutans* biofilm model was exposed to PAW for 10 min, better germicidal efficiency was attained. The results indicated that PAW was an effective disinfection agent for both immature and mature *S. mutans* biofilm. Interestingly, as a positive control, CHX showed similar disinfection effect with PAW on 12 h old biofilm, but had nearly no disinfection effect for 48 h biofilm, which indicated that the disinfection effect with CHX on *S. mutans* biofilm was limited. Therefore, PAW treatment shows significant disinfection effect on mature *S. mutans* biofilm. These results are consistent with Koban I [22]. Meanwhile, recent studies have shown that the antimicrobial capacity of PAW may decline with storage time and temperature [21, 23–25]. As to the treatment time, it has been shown that extending the exposure time could improve the antibacterial activity [21]. In this study, the bovine enamel discs with *S. mutans* biofilm were put into the centrifuge tubes and plasma was blew just into the water in these centrifuge tubes, which indicated that *S. mutans* biofilm was treated with PAW in real time, in order to get the best bactericidal activity of PAW. If PAW could be used in clinical to prevent caries in future, we would make it a portable plasma generated device that could be used in oral cavity and PAW would be used in real time, just as a new-type oral irrigator.

When the tube was inserted to water, plasma was generated and blowing in water. To distinguish PAW treatment effects from the air blowing, *S. mutans* biofilm were exposed to the flowing air without plasma generated and it can be seen that air blowing did not cause obvious survival rate decrease in the number of bacteria in this study. Similar results were found that the gas blowing did not affect the cell surviving [26]. Therefore, we totally excluded the mechanical influence on the bactericidal activity of PAW.

Meanwhile, the cooper electrode was applied in present study. The copper ions and copper complexes have certain bactericidal ability, which would release into the solution during the treatment. However, a limited concentration of copper ions were detected and our previous study had verified that PAW was basically non-corrosive to copper [19, 27]. Thus, we excluded copper ions and copper complexes as major inactivation agents.

The disinfection effect of PAW was further substantiated with CLSM images, which were consistent with the CFU results. In previous studies [28], plasma was found to

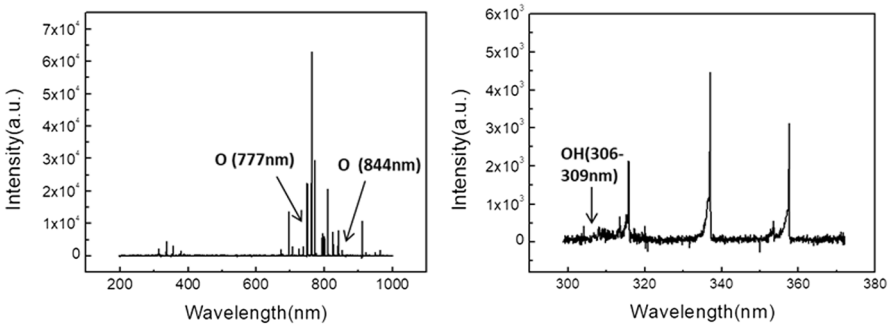

Fig. 7 Optical emission spectroscopy (OES) of PAW produced by the plasma device

penetrate into biofilm in 15 μm thick biofilm. However, from Fig. 4A, E, the bacteria in biofilm was completely inactivated, confirming that PAW may demonstrate better biofilm permeability than plasma, with the thickness of at least 160 μm. It was found that some small plasma-generated molecules could successfully penetrate the extracellular matrix and reach the interior of the biofilm, leading to cell death [29]. Penetration of plasma species into samples depends on several factors, including the type of plasma, delivery mode and the gas composition to make the plasma and resulting PAW [30].

Although many studies have been carried out on the mechanism of disinfection effect of PAW, the exact mechanism is not yet clearly understood. SEM images provided a morphological visualization of the specimen surfaces. The results of the morphological analysis confirmed the findings of Chen, that PAW killed bacteria through the mechanism by inducing cell membrane damage [21]. It seems that the damage of bacteria in PAW treated 48 h old biofilm was more severe than that of 12 h old biofilm. Combining with the results of CLSM, it was not difficult to understand. The reactive species generated in PAW could damage biofilm extracellular matrix and the bacteria itself, which lead to the biofilm structure destructed and the destroyed bacteria separated from attached surfaces [21]. The thickness of 12 h old biofilm became thinner after PAW treatment (Fig. 4A), signifying that the biofilm structure has been destroyed severely and a mass of bacteria has broken away. On the contrary, the structure of 48 h old biofilm was more stable, so that the bacteria was still attached to the biofilm even if it had been killed, and the thickness of biofilm was the same as control. Therefore, the more severe damage of 48 h old biofilm was actually an illusion, just because the broken bacteria was still on the surface in the 48 h old biofilm while it broke away in the 12 h old biofilm.

The ORP value is an important indication represent total reductants. High ORP value can break down the balance of the external and inner membranes of bacteria [31, 32]. As shown in Fig. 6, the ORP value of water rised from initial 245.9 to 545.8 mV, which indicated a large number of oxidants were generated. We also noticed that the ORP value has risen obviously within 1 min, from 245.9 to 515.7 mV, indicating that the oxidants has been generated in very short time and reached saturation point in water quickly. However, the ORP value of water with biofilm in it was lower than that of pure water within the first three minutes, demonstrating that the biofilm could undertake a part of the oxidative stress in water through interacting with the ROS generated. As some of reactive species of PAW was short lived [15, 33–35], we made plasma continuing to be generated for 10 min in

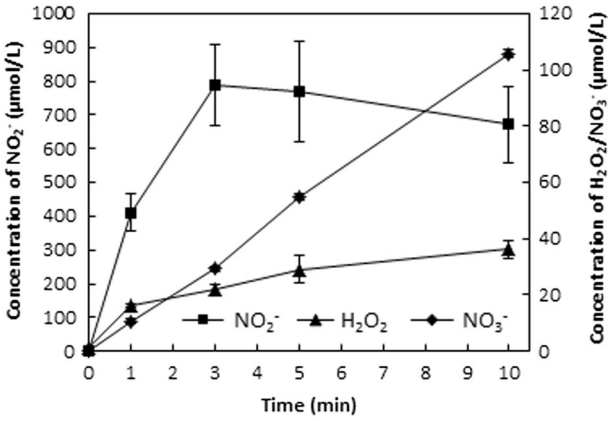

Fig. 8 Concentrations of H_2O_2, NO_3^-, and NO_2^- in PAW

order to maintain a certain level of some reactive species, so as to make *S. mutans* biofilm deal with liquid with high ORP value for nearly 10 min.

To further explore the major reactive species generated, optical emission spectra was applied. A series of physical and chemical reactions are consequently triggered so as to producing PAW, which contains amount of ROS and reactive nitrogen species (RNS), mainly including short-lived reactive species such as oxygen atoms (O), hydroxyl radical (OH), superoxide (O_2^-), peroxynitrite ($ONOO^-$), and long-lived reactive species including hydrogen peroxide (H_2O_2), nitrite (NO_2^-), nitrate (NO_3^-), ozone (O_3), and so on [36–39].

ROS could induce obvious oxidative stress response, mainly including superoxide anion radical ($O_2^{-\cdot}$), hydroxyl radical ($^{\cdot}OH$), atomic oxygen (O), ozone (O_3), hydroperoxy radical ($HOO^{\cdot}$), and hydrogen peroxide (H_2O_2) [36–38]. In our study, it was indicated that O and $^{\cdot}OH$ were present. It was stated that O and $^{\cdot}OH$ were among the most reactive and toxic species, which could damage cell-membrane integrity and cause protein and DNA leakage that consequently lead to bacterial cell death [11, 40]. Zhang stated that O was a chemically reactive species that is detrimental to biological molecules and was the most effective inactivation agent, while other reactive species were less important [27]. The pathways and destruction are as follows [30]: $O+e^- \rightarrow 2O+e^-$; $O_2+M^* \rightarrow 2O+M$; $O_2+H \rightarrow {}^{\cdot}OH+O$; O_2+N_2 (A, B, C) $\rightarrow 2O+N_2(X)$; $O+O_2+M \rightarrow O_3+M$; $O+O+M \rightarrow O_2+M$. It was showed that $^{\cdot}OH$ had strong oxidizing ability in aqueous environment and performed well for bacterial inactivation even though its concentration is significantly lower than that of other species, indicating that $^{\cdot}OH$ was important for the bioactivity of PAW [40–42]. The pathways and destruction are as follows [11, 30]: $H_2O+e^- \rightarrow {}^{\cdot}OH+{}^{\cdot}H+e^-$; $O_2+H \rightarrow {}^{\cdot}OH+O$; $OH^- \rightarrow {}^{\cdot}OH+e^-$; ${}^{\cdot}OH+{}^{\cdot}OH \rightarrow H_2O_2$; ${}^{\cdot}OH+H_2O_2 \rightarrow HO_2+H_2O$; $N+{}^{\cdot}OH \rightarrow NO+H$.

As the short half-life and high reactivity, it is difficult to directly measure the concentrations of short-lived reactive species in PAW. However, concentrations of the long-lived reactive species could be measured so as to reflect the evolution trend of the short-lived ones to a certain degree. As an oxidant with strong antibacterial activity, H_2O_2 can react with bacterial components and cells could not survive H_2O_2 even at a very low concentration [40, 43]. It was discovered that the NO_2^- concentration obviously increased to 790.34 ± 120.58 μmol/L after 3 min, and then reduced slightly to 671.49 ± 112.89 μmol/L, because a series of reactions would occur and eventually produce NO_3^- [11, 19]. The aqueous solution could be acidified by NO_2^- and NO_3^-, synergistic

bactericidal effects may be thereby produced [44]. The pathways and destruction are as follows [19, 39]: $H_2O + H_2O + e^- \rightarrow H_2O_2 + H_2 + e^-$; $^{\cdot}OH + {}^{\cdot}OH \rightarrow H_2O_2$; $N_2 + O \rightarrow NO + N$; $2NO + O_2 \rightarrow 2NO_2^-$; $NO_2^- + H_2O_2 + H^+ \rightarrow NO_3^- + H_2O + H^+$.

Conclusion

In conclusion, PAW was shown to be an effective disinfection agent for mature and immature *S. mutans* biofilm in vitro. ROS and RNS may play an important role in disinfection process of PAW, resulted in the damage of cell-membrane integrity. The PAW has the potential for cavity disinfection after caries excavation to prevent secondary caries and as an alternative to help caries susceptible population. However, there are still some limitations, such as the caries generally consists of poly-microbial biofilms and the in vivo effects have not been verified.

Acknowledgements This study was supported by Foundation of Peking University School and Hospital of Stomatology (Grant No. PKUSS20170111) and National Natural Science Foundation of China (Grant No. 81901064), Natural Science Foundation of Beijing Municipality (Grant No. 7204265) and Beijing Nova Programme Interdisciplinary Cooperation Project (Grant No. Z191100001119009). The authors are highly thankful to Center Laboratory and Material Laboratory of Peking University School and Hospital of Stomatology and Chemistry Department of Peking University for providing technological assistance of this research project.

Declarations

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

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