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In vitro studies of the antimicrobial effect of non-thermal plasma-activated water as a novel mouthwash

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The aim of this study was to evaluate the antimicrobial effects of non-thermal plasma-activated water (PAW) as a novel mouthwash in vitro. Three representative oral pathogens – *Streptococcus mutans, Actinomyces viscosus* and *Porphyromonas gingivalis* – were treated with PAW. The inactivation effect was evaluated using the colony-forming unit (CFU) method, and the morphological and structural changes of a cell were observed by scanning electron microscopy and transmission electron microscopy (TEM). The physicochemical properties of PAW were analysed, and its influence on the leakage of intracellular proteins and DNA was evaluated. The results showed significant reduction of *Streptococcus mutans* within 60 s, of *Actinomyces viscosus* within 40 s, and of *Porphyromonas gingivalis* in less than 40 s. Scanning electron microscopy and TEM images showed that the normal cell morphology changed by varying degrees after treatment with PAW. Intracellular proteins (280 nm) and DNA (260 nm) leaked from all three species of bacteria after treatment with PAW. Reactive oxygen species (ROS), especially atomic oxygen (O), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2), were generated and led to strong oxidative stress and cell damage. These results suggest that PAW has potential use as a novel antimicrobial mouthwash.

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According to the World Health Organization (WHO), dental caries and periodontal diseases are considered to be the most important global oral health issues and major causes of tooth loss (1). The oral cavity is colonized by diverse microflora, and several species of bacteria have been implicated as causative agents of dental caries and periodontal diseases (2). *Streptococcus mutans* is a gram-positive, facultative anaerobic bacterium (3, 4) and the most important cariogenic agent for the development process of dental caries (5, 6). *Actinomyces viscosus* is a common gram-positive bacterium in the oral cavity and the primary colonizer of human tooth surfaces, which plays an important role in plaque formation and growth (7). *Porphyromonas gingivalis* is associated with aggressive periodontitis (8, 9) and is implicated as a significant pathogen in human periodontal diseases (10–12). Therefore, it is necessary to inhibit and remove these pathogenic bacteria.

Mouthwashes are widely used in oral health-care as affiliate products (13). The use of antimicrobial mouthwash has been proposed as an effective means to control the levels of oral pathogenic bacteria. Among the current mouthwashes, chlorhexidine (CHX) has been widely used in clinics (14, 15). However, CHX has a number of side effects, such as mucosal erosion, tooth staining, taste disturbance, and swelling of the parotid gland. Thus, formulations or compound mouthwashes without these side effects have long been desired, especially for those who have undergone oral surgery (16–18).

Many previous studies have realized that non-thermal plasma is a promising technology because of the existence of ultraviolet (UV), reactive oxygen species (ROS), and reactive nitrogen species (RNS) (19, 20). Plasma-activated water (PAW) is water treated by cold plasma with multibiological functions. Kamgang-Youbi *et al.* have shown that PAW can be obtained by exposing distilled water to gliding arc discharges (21). This is because the radical species of plasma (principally ·OH and ·NO) are precursors of other longer-lifespan species [hydrogen peroxide (H_2O_2), nitrous acid (HNO_2), and nitric acid (HNO_3)] that are responsible for lethal effects (22, 23). Compared with the direct use of a plasma jet, the dangerous factors of electrical current, thermal damage of tissue, and UV irradiation can be avoided (24).

The aim of this study was to investigate the inhibitory effect of PAW on *S. mutans*, *A. viscosus*, and *P. gingivalis*, and its main mechanisms of action.

Material and methods

Preparation of PAW

The cold plasma-jet device consists of a quartz tube and an outer copper foil that surrounds a quartz tube. The outer copper foil is connected to a 10-kHz sinusoidal high-voltage source with an 18-kV peak-to-peak voltage. The quartz tube has outer and inner diameters of 0.9 and 0.8 cm, respectively. Figure 1A shows a schematic diagram of the plasma device, and Fig. 1B shows a photograph of the PAW generation process. Premixed argon (Ar) and oxygen (O_2) [Ar/O_2 (98%:2%; v/v); referred to as Ar/O_2 (2%) from here on] was used as the working gas with a flow rate of 5 l min^{-1}. More details can be found in our previous study (25). Ten millilitres of sterile distilled water was treated with non-thermal plasma for 20 min to obtain PAW.

Bacterial strains and culture conditions

Streptococcus mutans UA159 was grown on a Brain–Heart Infusion (BHI) (Oxoid, Basingstoke, UK) plate and incubated aerobically in an atmosphere of 5% CO_2 at 37°C for 48 h. Then, a single colony was picked and placed into a 1.5 ml EP tube (Eppendorf, Hamburg, Germany) containing 1 ml of BHI broth and incubated at 5% CO_2 and 37°C for 12 h until the density of bacteria was between 1×10^7 colony-forming units (CFU) ml^{-1} and 5×10^7 CFU ml^{-1}. *Actinomyces viscosus* ATCC 19246 and *P. gingivalis* ATCC 33277 were *grown anaerobically at* 37°C on BHI sheep blood agar plates supplemented with 1 μg ml^{-1} of haemin, 1 μg ml^{-1} of L-cysteine, 5 μg ml^{-1} of yeast extract, and 5 μg ml^{-1} of vitamin K. After 2 d (*A. viscosus* ATCC 19246) and 7 d (*P. gingivalis* ATCC 33277), a single colony was inoculated into 10 ml of BHI broth containing 5 μg ml^{-1} of yeast extract and 5 μg ml^{-1} of vitamin K. Batch cultures were grown anaerobically in an MGC AnaeroPack Series (Mitsubishi Gas Chemical Company, Tokyo, Japan) at 37°C for 2 d (*A. viscosus* ATCC 19246) or 3 d (*P. gingivalis* ATCC 33277) until the density of bacteria was between 1×10^7 and 5×10^7 CFU ml^{-1}.

Disinfection procedure

For each bacterial species, 100 μl of cultured bacterial suspension was added into 9.9 ml of fresh PAW and treated for 10, 20, 40, 60, or 120 s. Then, 10-fold serial dilutions of 100 μl of the PAW-treated bacterial suspensions were immediately plated on an agar plate. The bacterial suspension treated with sterile distilled water was used as the negative control. To evaluate the response of *S. mutans*, *A. viscosus*, and *P. gingivalis* to liquid acidity, nitric acid was used to preset the pH of the water to pH 3. The three strains were treated with nitric acid (pH 3) for 60 and 120 s. The disinfection of bacteria was evaluated by counting the number of CFUs on a petri dish. Each experiment was performed at least three times for each condition.

Scanning electron microscopy

Bacterial suspensions (10 ml) were centrifuged at 2400 ***g*** for 10 min. The supernatant was discarded and the precipitate was treated with 10 ml of PAW (experimental group) or distilled water (control group) for 40 min. Both groups were fixed overnight with 2.5% glutaraldehyde, dehydrated sequentially in ethanol (30%, 50%, 70%, 80%, 90%, and 100%) and dried at 37°C for 12 h. The bacterial suspensions placed on glass slides before being prepared for SEM. All samples were coated with gold-palladium and evaluated with a scanning electron microscope (S-4800; Hitachi, Tokyo, Japan).

Transmission electron microscopy

Bacterial suspensions (10 ml) were centrifuged at 2400 ***g*** for 10 min. The supernatant was discarded, and the precipitate was treated with PAW (experimental group) or distilled water (control group) for 40 min. All prepared bacterial suspensions were fixed for less than 2 h with glutaraldehyde, and then washed five times with 0.1 M PBS (pH 7.2). The bacterial suspensions placed on glass slides before being prepared for TEM. Then, they were post-fixed for 2 h with 1% osmium tetroxide and washed five times with PBS. After that, bacterial suspensions were dehydrated in graded ethanol (50%, 70%, 85%, 95%, and 100%), embedded for 2 h in a fixative (50% absolute ethanol and 50% embedding medium), and then embedded for 12 h with a pure embedding medium. Finally, all samples were dried at 60°C for 24 h. Thin sections of the specimens were viewed with a transmission electron microscope (JEM-1400; JEOL, Tokyo, Japan).

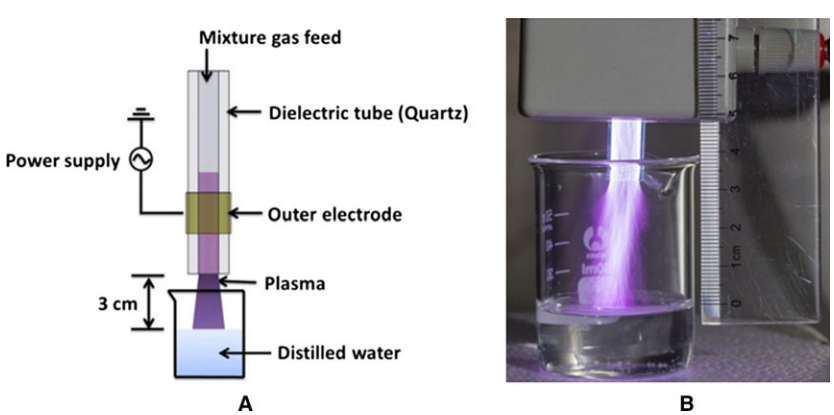

Fig. 1. (A) Schematic diagram of the experimental arrangement. (B) Photograph of the plasma-activated water (PAW) generation process.

DNA and protein leakage

To investigate the leakage of intracellular proteins and DNA after treatment with PAW, a UV-light spectrophotometer (DU 730 Nucleic Acid/Protein Analyzer; Beckman Coulter, Brea, CA, USA) was used. The group with sterile water treatment was used as the negative control. After treatment for 10, 20, 40, 60, and 120 s, the samples were filtered with 0.22-μm syringe filters to remove the bacteria. The wavelengths detected were 260 nm (DNA absorbance) and 280 nm (protein absorbance) (3).

Evaluation of physicochemical properties

The temperature of the PAW was evaluated using a portable intelligent data logger (TH-210; High-chance High-tech Science, Beijing, China), and the pH value of the PAW was monitored using a microprocessor pH-meter (pH 213; Hanna Instruments, Woonsocket, RI, USA). Hydrogen peroxide (H_2O_2) is thought to be related to the antimicrobial process of PAW. Thus, the H_2O_2 concentrations within 20 min were measured using a Hydrogen Peroxide Assay Kit (Beyotime, Jiangsu, China).

Optical emission spectroscopy

The contents of the plasma were analysed by optical emission spectroscopy (OES) using a Multi-Channel Fibre Optic Spectrometer (AvaSpec-2048-8-USB2; Avantes, Eerbeek, the Netherlands). The end of the fibre-optic cable was applied to record the light signals at the bottom of the quartz tube at approximately 5 mm from the plasma jet. The operational details can be found in our previous work (26, 27).

Results

Inactivation of PAW

Figure 2 and Table 1 show the cell-survival curves of *S. mutans*, *A. viscosus*, and *P. gingivalis* treated with PAW and distilled water. After 20 s of treatment with PAW, *P. gingivalis* was the inactivated to a degree corresponding to a 5-log reduction, while *S. mutans* and *A. viscosus* needed 40 and 60 s, respectively, of treatment with PAW to achieve a similar level of reduction (Fig. 2). The numbers of *S. mutans* and *A. viscosus* showed a slight increase, while the number of *P. gingivalis* decreased by about 50% (Table 1), perhaps because of the low concentration of oxygen in the water and the fact that oxygen could pass into the water through contact with water surface (28). Acid water inactivated *P. gingivalis* gradually over 120 s (Table 2). These results demonstrate that PAW can inactivate *S. mutans*, *A. viscosus*, and *P. gingivalis* effectively.

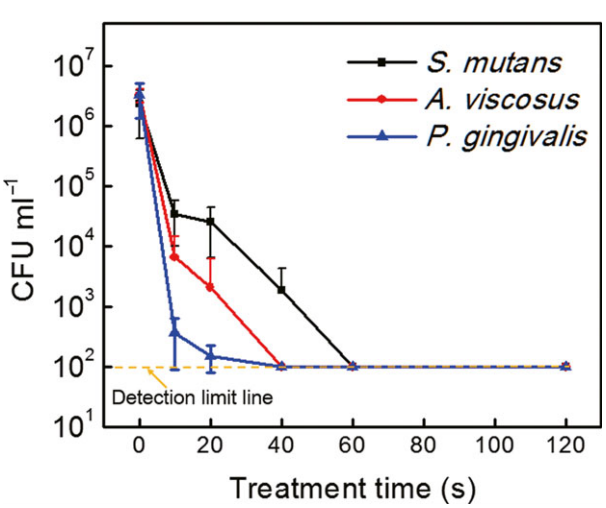

Fig. 2. Disinfection efficacy evaluation of the plasma-activated water (PAW) treatment group. *A. viscosus*, *Actinomyces viscosus*; CFU, colony-forming units; *P. gingivalis*, *Porphyromonas gingivalis*; *S. mutans*, *Streptococcus mutans*.

Table 1

Disinfection efficacy evaluation of the group treated with distilled water: a limited disinfection effect was observed

Time (s)	*S. mutans*	*A. viscosus*	*P. gingivalis*
0	2.29 ± 1.67	3.13 ± 0.50	1.90 ± 0.53
10	3.87 ± 1.27	4.25 ± 1.75	1.67 ± 0.34
20	4.10 ± 0.87	3.50 ± 2.00	1.23 ± 0.33
40	4.03 ± 0.48	4.00 ± 1.50	1.17 ± 0.47
60	2.35 ± 0.15	3.75 ± 0.75	1.30 ± 0.25
80	2.35 ± 0.15	3.75 ± 0.74	1.33 ± 0.25
120	3.60 ± 0.28	4.00 ± 0.50	1.03 ± 0.45

Values are given as (means ± SD) × 10^6 .
A. viscosus, *Actinomyces viscosus*; *P. gingivalis*, *Porphyromonas gingivalis*; *S. mutans*, *Streptococcus mutans*.

Table 2

Inactivation of Streptococcus mutans, Actinomyces viscosus, and Porphyromonas gingivalis *with acid water (pH 3), which has limited inactivation effect on* S. mutans *and* A. viscosus

Time (s)	*S. mutans*	*A. viscosus*	*P. gingivalis*
0	3.28 ± 1.48	2.93 ± 0.48	2.32 ± 0.92
60	2.77 ± 0.88	2.87 ± 0.47	1.98 ± 0.71
120	2.39 ± 1.06	2.60 ± 0.35	0.46 ± 0.12

Values are given as (means ± SD) × 10^6.

Scanning electron microscopy and TEM investigations

From the scanning electron microscopy images (Fig. 3A,B), the treatment of *S. mutans* with PAW resulted in no obvious surface morphology change. In the case of *A. viscosus*, the surface morphology changed from flat to shrunken. The surface morphology of *P. gingivalis* changed significantly after treatment with PAW, and showed distortion and shrinkage. However, from the TEM images (Fig. 3C,D), the normal cell structure of *S. mutans* was fuzzy, and the complete form was not visible after treatment with PAW. Under the same conditions, the *A. viscosus* cell edge was indistinct, and *P. gingivalis* showed morphological diversity and cytoplasm shrinkage.

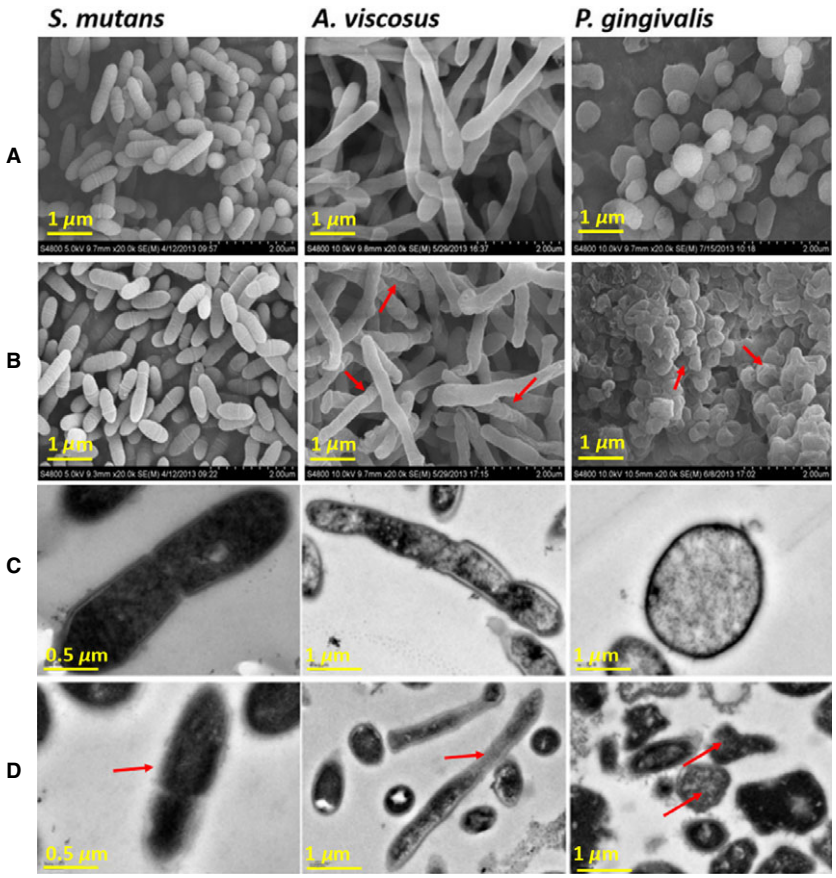

Fig. 3. Scanning electron microscopy (A, B) and transmission electron microscopy (C, D) images of *Streptococcus mutans*, *Actinomyces viscosus*, and *Porphyromonas gingivalis* before (A, C) and after (B, D) treatment with plasma-activated water (PAW). The red arrows indicate the obvious surface morphology change after PAW treatment compared with the control group.

DNA and protein leakage

The permeability change of the cell membrane might lead to the leakage of intracellular DNA and proteins. Figure 4 shows the leakage of both intracellular protein and DNA from *S. mutans*, *A. viscosus*, and *P. gingivalis*. For all three strains of bacteria, treatment with PAW led to marked leakage of DNA and protein in the first 10 s, with the absorbance peak intensity rapidly increasing for both protein (260 nm) and DNA (280 nm). When the PAW treatment time extended beyond 10 s, the absorbance peak intensity levelled off or even declined. Treatment with distilled water did not lead to a significant change in the leakage of DNA and protein from *S. mutans* and *A. viscosus*; however, slight leakage of DNA and protein were observed from *P. gingivalis*. The leakage of DNA and protein further confirmed the results of scanning electron microscopy and TEM that

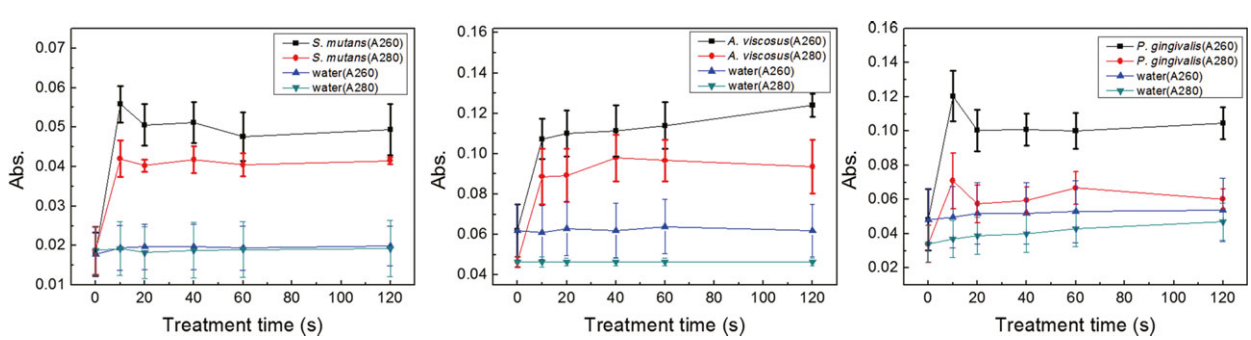

Fig. 4. Leakage of intracellular protein and DNA from *Streptococcus mutans, Actinomyces viscosus*, and *Porphyromonas gingivalis* after treatment with plasma-activated water (PAW). Abs., absorbance.

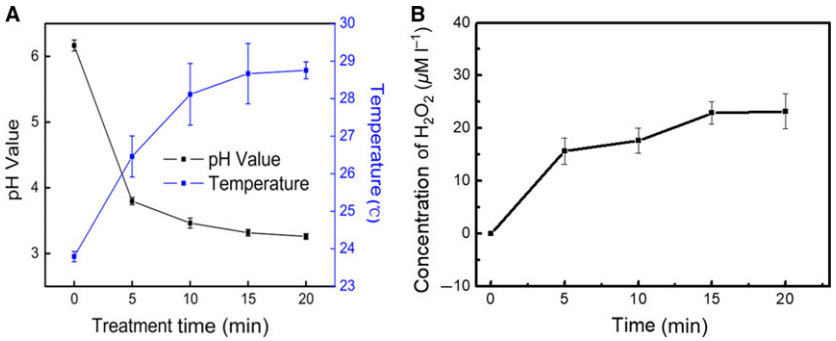

Fig. 5. Physicochemical property evolution of the water treated with Ar/O_2 (98%:2%; v/v) plasma. (A) The water temperature was approximately 29°C and the pH of the plasma-activated water (PAW) decreased from pH 6 to approximately pH 3 after 20 min of treatment. (B) The concentration of hydrogen peroxide (H_2O_2) increased to 23.18 μM l^{-1} after 20 min.

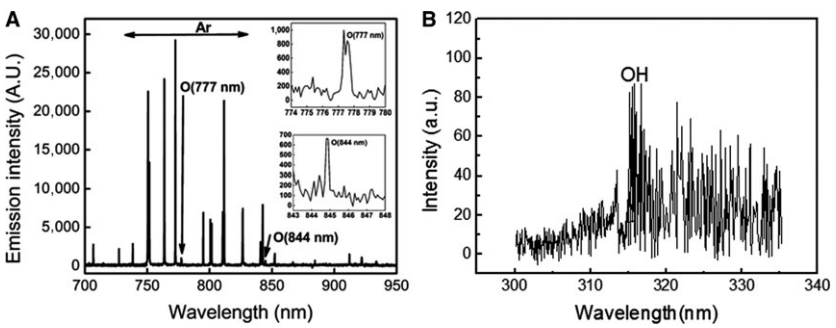

Fig. 6. End-on optical emission spectra of the plasma microjet ranging from 300 to 950 nm. The reactive species atomic oxygen (O) (at 777 and 844 nm) (A) and hydroxyl radical (·OH) (315–316 nm) (B) were detected. A.U., absorbance units. Ar, argon.

treatment with PAW could reduce the integrity of the bacterial membrane.

Physicochemical properties of PAW

The temperature and pH of PAW were measured. No drastic increase in the temperature of PAW was observed during the 20 min of plasma treatment. The water temperature was approximately 29°C after 20 min (Fig. 5A). However, the pH of plasma-treated water decreased rapidly during the first 10 min. After 20 min of plasma treatment, the pH value of PAW had decreased from pH 6 to approximately pH 3 (Fig. 5A). As shown in Fig. 5B, the concentration of H_2O_2 increased sharply to 15.64 μM l^{-1} within 5 min and then showed a more gradual increase thereafter, to reach 23.18 μM l^{-1} after 20 min.

Optical emission spectroscopy

Figure 6 shows the emission spectrum generated by Ar/O_2 (2%) plasma. The whole emission spectrum was dominated by Ar lines. Atomic oxygen (O) lines at 777 and 844 nm (Fig. 6A insets), as well as hydroxyl radical (·OH) lines at 316 nm (Fig. 6B), were detected. The chemical formula is as follows:

$$O_2 + e^- \rightarrow 2O + e^-,$$
$$H_2O + e^- \rightarrow \cdot OH + \cdot H + e^-.$$

Discussion

Dental caries and periodontal diseases are two of the most troublesome ailments affecting many people, and both are infectious bacterial diseases. Thus, preventive measures can be taken by controlling bacterial infections (29). Non-thermal plasma has been extensively used to inactivate biological species (26), and PAW has been proven to have a pronounced disinfection effect (27, 30). Thus, three species of oral bacteria associated with caries (*S. mutans* and *A. viscosus)* and periodontal diseases (*P. gingivalis)* were killed by PAW in this study.

As shown in Fig. 2, the total CFU count of all three species of bacteria decreased from 5×10^6 CFU to 5×10^2 CFU within 60 s, with a disinfection rate of up to 99.99%. The results showed that the disinfection effect of PAW is unique for each species of bacterium, from strong to weak in the order: *P. gingivalis* > *A. viscosus* > *S. mutans*. This difference in efficacy might be attributed to the variable structure and characteristics of the bacteria. As shown by the scanning electron microscopy and TEM results, the morphology of PAW-treated *P. gingivalis* indicated severe cell rupture and both *A. viscosus* and *S. mutans* showed cell shrinkage. Aerobic bacteria (*S. mutans*) are less sensitive than anaerobic bacteria (*A. viscosus* and *P. gingivalis)* when exposed to the same oxidative stress. Moreover, *S. mutans* and *A. viscosus* belong to the category of gram-

positive bacteria, and the thickness of the cell wall is approximately 20–80 nm (31). *Porphyromonas gingivalis* belongs to the category of gram-negative bacteria, and the thickness of the cell wall is approximately 10 nm (32). The peptidoglycan mesh-like layer outside the plasma membrane forming the cell wall of gram-positive bacteria can be up to 50 layers in thickness, whereas in gram-negative bacteria its thickness is only one to three layers. The tough cell wall of gram-positive bacteria has a higher resistance to virulence factors and maintains the cell morphology. The leakage of intracellular DNA and proteins was lowest from *S. mutans* of all three species (Fig. 4), which indirectly proves the importance of the cell wall in the disinfection effects of the three bacteria.

The bacterial membrane serves as a key structural component for resisting biocidal challenges. The leakage of intracellular DNA and proteins is a good indicator of membrane damage (33). As shown in Fig. 4, PAW treatment could lead to a dramatic release of intracellular DNA and proteins. The optical emission spectra results indicate that O (Fig. 6A) and ·OH (Fig. 6B) are present. Because O and ·OH are among the most reactive and toxic species, their presence could lead to lipid peroxidation of the lipid bilayer in the bacterial membrane, resulting in the cross-link reaction of the fatty-acid side chain. This could form transient pores in the cell membrane followed by depolarization and permeabilization of the cell membrane (34). This leads to the question why the first 10 s of treatment resulted in the absorbance peak intensity almost being reached, followed by a levelling off or decline over a prolonged time period, for both protein and DNA. It was shown that the aerobic degradation reaction started with an increase of the PAW treatment time (over 10 s). As a result of the existence of many highly reactive plasma species in the PAW (22, 35), the proteins and nucleic acid leaking out of the exposed cells decomposed quickly (36).

The temperature of the PAW increased with prolonged treatment time, and it reached equilibrium at 29°C after 20 min. The culture temperature of *S. mutans, A. viscosus* and *P. gingivalis* was 37°C. Thus, the inactivation effect of PAW was not caused by thermal effects. *Streptococcus mutans* and *A. viscosus* are considered as causative agents of dental caries, and they have the properties of acidogenicity and acid tolerance (37). However, the optimal pH for growth of *P. gingivalis* is pH 6.5–7.0 (38). The acidification of PAW is mainly a result of the generation of nitric acid, a hydrogen cation (H^+) and a superoxide anion ($O_2^-\cdot$) when exposed to an air environment (39), as represented by the following equation:

$$H_2O^+ + H_2O \rightarrow H^+(H_2O) + \cdot OH.$$

Consequently, when water reached a pH value of 3, growth of *S. mutans* and *A. viscosus* was not inhibited during the 120 s treatment time, while *P. gingivalis* growth was suppressed for the entire 120 s. However, Figure 2 shows that almost all *P. gingivalis* had been killed after 20 s of treatment with PAW, and so the impact of pH on *P. gingivalis* can be ignored. Therefore, it is concluded that a low pH is not the main reason for the disinfection effect following treatment with PAW. Weltmann speculated that acidity and reactive species are interconnected (40). A lower pH is more favourable for a reactive species to penetrate a cell membrane. On the other hand, the acidic conditions could accelerate chemical reactions, such as those induced by hydroperoxyl radicals ($HOO^{\cdot}$) because hydroperoxyl radicals have a high oxidizing power and will initiate the peroxidation of fatty acids in the cell membrane (20, 41). However, dental erosion could occur at low pH (42). Pontefract *et al.* (43) recommended that low-pH mouthrinses should not be considered for long-term (3 wk) or continuous use and never as prebrushing rinses. Considering that a low pH is detrimental for the teeth, future studies should try to increase the pH value of PAW to reduce the related side effects. The acidification of PAW is mainly a result of the working gas (air) and the PAW generation time, and changing the working gas or reducing the PAW generation time could raise the pH value (44). Ikawa *et al.* (41) applied sodium citrate buffer (pH = 6.5 ≈ 3.7) to neutralize the low pH, but the inhibitory effect decreased once the pH value exceeded pH 4.8 . Therefore, future studies should focus on balancing the inhibitory effect and the pH value.

Hydrogen peroxide is a strong oxidizer involved in the antimicrobial properties of PAW, especially in acidic environments. Naitali *et al.* (45) noted that 10 μM of acidified H_2O_2 is capable of reducing bacterial growth by up to 0.4 log. As shown in Fig. 5B, the concentration of H_2O_2 increased to 23.18 μM l^{-1} after 20 min. Thus, the H_2O_2 in PAW plays a significant role in the disinfection process:

$$\cdot OH + \cdot OH \rightarrow H_2O_2.$$

Furthermore, the speculated mechanism of sterilization of PAW is through ROS, especially free radicals such as O, ·OH, and H_2O_2, which are the most important germicidal agents. A lower pH value could enhance the effect of reactive species. These free radicals can damage cell-membrane integrity as well as cause protein and DNA leakage that consequently lead to bacterial cell death.

In this study, PAW was shown to be an effective disinfection agent for the three pathogenic species of oral bacteria. The active species caused changes to the bacterial cell membrane, resulting in leakage of intracellular proteins and DNA. All results suggest that PAW shows promise as a new type of mouthwash to kill or inhibit the growth of oral pathogenic bacteria. Future studies should focus on evaluating the biosafety of PAW on the normal oral tissue and the disinfection effects of PAW to biofilms rather than bacterial suspensions.

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Conflicts of interest – The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

References

1. Petersen PE. The World Oral Health Report 2003: continuous improvement of oral health in the 21st century – The approach of the WHO Global Oral Health Programme. *Community Dent Oral Epidemiol* 2003; **31**: 3–24.
2. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Periodontol* 2005; **43**: 5721–5722.
3. Sladek REJ, Filoche SK, Sissons CH, Stoffels E. Treatment of *Streptococcus mutans* biofilms with a nonthermal atmospheric plasma. *Lett Appl Microbiol* 2007; **45**: 318–323.
4. Yang B, Chen J, Yu Q, Li H, Lin MS, Mustapha A, Liang H, Wang Y. Oral bacterial deactivation using a low-temperature atmospheric argon plasma brush. *J Dent* 2011; **39**: 48–56.
5. Koban I, Holtfreter B, Hübner N-O, Matthes R, Sietmann R, Kindel E, Weltmann KD, Welk A, Kramer A, Kocher T. Antimicrobial efficacy of non-thermal plasma in comparison to chlorhexidine against dental biofilms on titanium discs in vitro – proof of principle experiment. *J Clin Periodontol* 2011; **38**: 956–965.
6. Koo HM, Hayacibara F, Schobel BD, Cury JA, Rosalen PL, Park YK. Inhibition of *Streptococcus mutans* biofilm accumulation and polysaccharide production by apigenin and *tt*-farnesol. *J Antimicrob Chemother* 2003; **52**: 782–789.
7. Ilie O, Van TAG, Van LMC. Numerical modelling of tooth enamel subsurface lesion formation induced by dental plaque. *Caries Res* 2014; **48**: 73–89.
8. Kulik EM, Lenkeit K, Chenaux S, Meyer J. Antimicrobial susceptibility of periodontopathogenic bacteria. *J Antimicrob Chemother* 2008; **61**: 1087–1091.
9. Andrian E, Grenier D, Rouabhia M. *Porphyromonas gingivalis*-epithelial cell interactions in periodontitis. *J Dent Res* 2006; **85**: 392–403.
10. Blascobaque V, Garidou L, Pomié C, Escoula Q, Loubieres P, Gall-David SL. Periodontitis induced by *Porphyromonas gingivalis* drives periodontal microbiota dysbiosis and insulin resistance via an impaired adaptive immune response. *Gut* 2016; **2**: 285–288.
11. Darveau RP, Tanner A, Page RC. The microbial challenge in periodontitis. *Periodontol 2000* 1997; **14**: 12–32.
12. Haas AN, Pannuti CM, Andrade AK, Escobar EC, Almeida ER, Costa FO. Mouthwashes for the control of supragingival biofilm and gingivitis in orthodontic patients: evidence-based recommendations for clinicians. *Braz Oral Res* 2014; **18**: 1–8.
13. Fatih Ö, Zeynep S, Zübeyde AP, Kürşat ER, Ülkü Ö, Orhan D. Effect of mouthrinse containing propolis on oral microorganisms and human gingival fibroblasts. *Eur J Orthod* 2007; **1**: 195–201.
14. Addy M. Chlorhexidine compared with other locally delivered anti-microbials. A short review. *J Clin Periodontol* 1986; **13**: 957–964.
15. Jones CG. Chlorhexidine: is it still the gold standard? *Periodontol 2000* 1997; **15**: 55–62.
16. Bassetti C, Kallenberger A. Influence of chlorhexidine rinsing on the healing of oral mucosa and osseous lesions. *J Clin Periodontol* 1980; **7**: 443–456.
17. Sheen S, Addy M. An in vitro evaluation of the availability of cetylpyridinium chloride and chlorhexidine in some commercially available mouthrinse products. *Br Dent J* 2003; **194**: 207–210.
18. Song L, Hou J, Van HC, Veeregowda DH, Busscher HJ, Sjollema J. Antimicrobials influence bond stiffness and detachment of oral bacteria. *J Dent Res* 2016; **95**: 793–799.
19. Pavlovich MJ, Ono T, Galleher C, Curtis B, Clark DS, Machala Z. Air spark-like plasma source for antimicrobial NOx generation. *J Phys D Appl Phys* 2014; **47**: 505202.
20. Liu ZC, Liu DX, Chen C, Li D, Yang AJ, Rong MZ, Chen HL, Kong MG. Physicochemical processes in the indirect interaction between surface air plasma and deionized water. *J Phys D Appl Phys* 2015; **48**: 495201.
21. Kamgang-Youbi G, Herry JM, Brisset JL, Bellon-Fontaine MN, Doubla A, Naïtali M. Impact on disinfection efficiency of cell load and of planktonic/adherent/detached state: case of *Hafnia alvei* inactivation by plasma activated water. *Appl Microbiol Biot* 2008; **81**: 449–457.
22. Kamgangyoubi G, Herry JM, Bellonfontaine MN, Brisset JL, Doublaa A, Naïtali M. Evidence of temporal postdischarge decontamination of bacteria by gliding electric discharges: application to *Hafnia alvei*. *Appl Environ Microbiol* 2007; **73**: 4791–4796.
23. Doubla A, Abdelmalek K, Khélifa Addou A, Brisset JL. Post-discharge plasma-chemical oxidation of Iron(II) complexes. *J Appl Electrochem* 2003; **33**: 73–77.
24. Ercan UK, Wang H, Ji HF, Fridman G, Brooks AD, Joshi SG. Nonequilibrium plasma activated antimicrobial solutions are broad-spectrum and retain their efficacies for extended period of time. *Plasma Process Polym* 2013; **10**: 544–555.
25. Li YL, Sun K, Ye GP, Liang YD, Pan H, Wang GM, Pan J, Zhang J, Fang J. Evaluation of cold plasma treatment and safety in disinfecting 3-week root canal *Enterococcus faecalis*, biofilm in vitro. *J Endodont* 2015; **41**: 1325–1330.
26. Pan J, Li YL, Liu CM, Tian Y, Yu S, Wang KL, Zhang J, Fang J. Investigation of cold atmospheric plasma-activated water for the dental unit waterline system contamination and safety evaluation in vitro. *Plasma Chem Plasma Process* 2017; **37**: 1–13.
27. Ma RN, Feng HQ, Liang YD, Zhang Q, Tian Y, Su B, Zhang J, Fang J. An evaluation of anti-oxidative protection for cells against atmospheric pressure cold plasma treatment. *J Phys D Appl Phys* 2013; **46**: 285401.
28. Murray CN, Riley JP. The solubility of gases in distilled water and sea water—II. Oxygen. *Deep Sea Res Oceanogr Abstr* 1969; **16**: 311–320.
29. Schoen MH, Freed JR. Prevention of dental disease: caries and periodontal disease. *Annu Rev Publ Health* 1981; **2**: 71–92.
30. Pavlovich MJ, Chang HW, Sakiyama Y, Clark DS, Graves DB. Ozone correlates with antibacterial effects from indirect air dielectric barrier discharge treatment of water. *J Phys D Appl Phys* 2013; **46**: 145202.
31. Seckbach J. *Origins: genesis, evolution and diversity of life*. Dordrecht, Netherlands: Kluwer Academic Publishers, 2004.
32. Cammack R, Attwood T, Campbell P, Parish H, Smith A, Vella F, Stirling J. *Oxford dictionary of biochemistry and molecular biology*. New York: Oxford University Press, 2006.
33. Chen CZ, Cooper SL. Interactions between dendrimer biocides and bacterial membranes. *Biomaterials* 2002; **23**: 3359–3368.
34. Zhang Q, Ma RN, Tian Y, Su B, Wang KL, Yu S, Zhang J, Fang J. Sterilization efficiency of a novel electrochemical disinfectant against *Staphylococcus aureus*. *Environ Sci Technol* 2016; **50**: 3184–3192.
35. Ma R, Feng H, Li F, Liang Y, Zhang Q, Zhu WD, Zhang J, Fang J. An evaluation of anti-oxidative protection for cells against atmospheric pressure cold plasma treatment. *Appl Phys Lett* 2012; **100**: 123701.
36. Laroussi M, Leipold F. Evaluation of the roles of reactive species, heat, and UV radiation in the inactivation of bacterial cells by air plasmas at atmospheric pressure. *Int J Mass Spectrom* 2004; **233**: 81–86.

37. Wei GX, Campagna AN, Bobek LA. Effect of MUC7 peptides on the growth of bacteria and on *Streptococcus mutans* biofilm. *J Antimicrob Chemother* 2006; **57**: 1100–1109.
38. Takahashi N, Schachtele CF. Effect of pH on the growth and proteolytic activity of *Porphyromonas gingivalis* and *Bacteroides intermedius*. *J Dent Res* 1990; **69**: 1266–1269.
39. Park G, Ryu YH, Hong YJ, Choi EH, Uhm HS. Cellular and molecular responses of Neurospora crassa to non-thermal plasma at atmospheric pressure. *Appl Phys Lett* 2012; **100**: 1–4.
40. Oehmigen K, Hähnel M, Brandenburg R, Wilke CH, Weltmann KD, Woedtke-Von TH. The role of acidification for antimicrobial activity of atmospheric pressure plasma in liquids. *Plasma Process Polym* 2010; **7**: 250–257.
41. Ikawa S, Kitano K, Hamaguchi S. Effects of pH on bacterial inactivation in aqueous solutions due to low-temperature atmospheric pressure plasma application. *Plasma Process Polym* 2010; **7**: 33–42.
42. Shaw L, Smith AJ. Dental erosion—the problem and some practical solutions. *Br Dent J* 1999; **186**: 115–118.
43. Pontefract H, Hughes J, Kemp K, Yates R, Newcombe RG, Addy M. The erosive effects of some mouthrinses on enamel. A study in situ. *J Clin Periodontol* 2001; **28**: 319–324.
44. Bai N, Sun P, Zhou HX, Wu HY, Wang RX, Liu FX, Zhu WD, Lopez JL, Zhang J, Fang J. Inactivation of *Staphylococcus aureus* in water by a cold, He/O_2 atmospheric pressure plasma microjet. *Plasma Process Polym* 2011; **8**: 424–431.
45. Naitali M, Kamgang-Youbi G, Herry JM, Bellon-Fontaine MN, Brisset JL. Combined effects of long-living chemical species during microbial inactivation using atmospheric plasma-treated water. *Appl Environ Microbiol* 2010; **76**: 7662–7664.