BMC Oral Health

RESEARCH

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Bactericidal effect of plasma-activated water generated by a novel super-potable plasma device as a novel antibacterial method: an in vitro study

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Abstract

Background A continuous risk from bacterial infection poses a major environmental and public health challenge. As an emerging strategy for inhibiting bacterial infections, plasma-activated water (PAW) has proved highly effective, environment-friendly, and non-drug resistant to many bacteria. However, the plasma device is too big and requires specialists to operate, which inevitably limits its real-life application. It has been confirmed that the bactericidal effect of traditional PAW would be significantly reduced in the organic-rich environments. To better resolve the limitations, a novel super-portable plasma device was applied, and the low concentration of H_2O_2 was added to enhance the bactericidal effect of PAW in the organ-rich environments.

Methods A super-portable plasma device, weighing only 1 kg, was applied to generate PAW. *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) and *Porphyromonas gingivalis* (*P. gingivalis*) were selected as the test bacteria. Colony-forming units (CFU) counting and bactericidal rate were employed to evaluate the bactericidal effect of PAW. Optical emission spectroscopy (OES) was used to identify the major reactive species in PAW. Different concentration of H_2O_2 was added to PAW (H_2O_2/PAW), and the pH, oxidation-reduction potential (ORP), conductivity, H_2O_2 and NO content of HP/PAW were measured to explore the bactericidal mechanisms. CCK-8 assay was used to assess the cytotoxicity of 0.1% H_2O_2/PAW on human periodontal ligament fibroblasts (hPDLFs).

Results The 20 mL of PAW generated by the novel super-portable plasma device achieved the bactericidal rate of 98.94% against *S. aureus* (t = 10 min) and 99.66% (t = 20 min). When the PAW was prepared with a volume of 20 mL, activation times of 6 min and 8 min, and the treatment time was 30 min, the bactericidal rates all exceeded 98% against *P. gingivalis*. The addition of 0.1% H_2O_2 significantly enhanced the antibacterial effect of PAW in the presence of Bovine Serum Albumin (BSA) ($p<0.05$). The addition of 0.1% H_2O_2 lowered the pH, increased the ORP and conductivity, and raised the content of H_2O_2 and NO in the solution. Cytotoxicity evaluation indicated that 0.1% H_2O_2/PAW had

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moderate cytotoxicity only after 20 min of preparation and 48 h of treatment, while other preparation and treatment times showed mild or no cytotoxicity.

Conclusions The PAW generated by the novel super-portable plasma device exhibited an excellent bactericidal effect. The addition of low concentrations of H_2O_2 to PAW, specifically 0.1% H_2O_2/PAW, maintained an effective antibacterial effect in complex environments in the presence of BSA with non-cytotoxicity.

Keywords Super-portable plasma device, Plasma-activated water, Antibacterial effect, Infection, H_2O_2

Introduction

Microorganisms are ubiquitous and encompass a wide range of species. Among the three most prevalent microorganisms (namely bacteria, fungi, and viruses), bacteria are regarded as the primary agents responsible for global public health concerns and economic burdens [1, 2]. A study published in The Lancet revealed that 7.7 million deaths in 2019 were attributable to 33 common bacterial infections, representing 13.6% of all global deaths, which makes bacterial infections the second leading cause of death globally, associated with one in eight deaths worldwide [3]. It is estimated that global economic losses due to antibiotic resistance could exceed $2 trillion, resulting in 28 million people being pushed into poverty by 2025 [3].

Numerous diseases, including oral diseases, are associated with bacterial infections. For example, *Porphyromonas gingivalis* (*P. gingivalis*) is the primary pathogen in periodontitis [4], *Streptococcus mutans* (*S. mutans*), and *Actinomyces viscosus* (*A. viscosus*) are the main pathogens in dental caries [5, 6]. *Staphylococcus aureus* (*S. aureus)* is linked to skin and soft tissue infections [7], *Streptococcus pneumoniae* (*S. pneumoniae*) can cause pneumonia [8], and *Neisseria meningitidis* (*N. meningitidis*) can lead to meningitis, and so on [9]. So it is important to take appropriate preventive and control measures to treat bacterial infections.

Antiseptics are widely used in most countries and are vital to meet quality and health standards. They are widely used extensively in disease prevention and control. For instance, the commonly used antibacterial mouthwashes in oral hygiene, such as chlorhexidine (CHX), are essential for controlling abnormal accumulation of oral plaque in some conditions, such as the disabled, patients in the ICU, and some patients who underwent oral surgeries [10]. However, the excessive or improper use of antiseptics can lead to many risks, such as promoting antibiotic resistance in bacteria and accelerating the spread of resistance genes. Saleem et al. demonstrated that chlorhexidine-resistant bacteria were also resistant to various antibiotics, including ampicillin, kanamycin, gentamicin, and tetracycline [11]. Besides that, CHX mouthwash can also result in dry mouth, altered taste sensations, and a discolored tongue. Furthermore, some antiseptics can produce residues that may pose potential harm to the environment and human health. Additionally, some antiseptics are irritating and corrosive, causing great irritation or even burns to the skin and mucous membranes with which they contact [12, 13].

Facing these limitations of traditional antiseptics, a new and efficient antibacterial method has been explored. Plasma-activated water (PAW), as an emerging technology, shows great potential in antibacterial areas [14, 15]. PAW is obtained by treating solutions with a plasma jet (PMJ), which has a wide range of applications in medicine, especially in the field of disinfection. Many studies have confirmed that the main biological effects of PAW are due to its rich content of reactive particles, such as reactive oxygen and nitrogen species (RONS) [16]. Our previous studies have found that PAW can effectively kill representative oral disease pathogens, such as *P. gingivalis*, *S. mutans*, and *A. viscosus*, making it a very promising mouthwash [17]. Plasma-activated microbubbles (PMB) combined with ultrasound were able to produce effective bactericidal effects against *Enterococcus faecalis* (*E. faecalis*), the main pathogen of refractory root canals [18]. In addition, it has been demonstrated that PAW exhibits a great bactericidal effect on *Hafnia alvei* (*H. alvei*), *E. coli*, *Saccharomyces cerevisiae* (*S. cerevisiae*), *S. aureus*, and *Candida albicans* (*C. albicans*) [19–23]. Moreover, many studies have shown that PAW is an antimicrobial agent that does not induce resistance or leave harmful substance residues [24, 25]. Therefore, as a green antimicrobial agent, PAW can play a significant role in various medical applications, including oral hygiene.

However, the traditional plasma device used in our previous studies was the typical dielectric barrier discharge (DBD), which had various drawbacks. For instance, the size of the traditional plasma devices was limited by the thickness of the dielectric barrier layer, which restricted the range and flexibility of their applications. They consumed a significant amount of energy and were not environmentally friendly. Additionally, their operation was relatively complex, requiring specialized technical personnel for operation and maintenance. All of these factors limited their application in many scenarios [17, 18]. We hope to propose a novel super-portable plasma generation device suitable for clinical use and special scenarios, such as outdoor and military operations. However, the antibacterial efficacy of the PAW produced by

this new plasma device remains uncertain. At the same time, we also found that the bactericidal effect of PAW was significantly reduced in the presence of organic matter such as Bovine Serum Albumin (BSA) [26]. However, in many clinical occasions, such as dentistry, various fluids, including blood, could significantly influence the antibacterial effect of PAW. Numerous studies have demonstrated that 3% H_2O_2 exhibits excellent antibacterial effects against various bacteria. However, there is currently no definitive conclusion on whether the addition of H_2O_2 improves the antibacterial efficacy of PAW in the presence of BSA.

The aim of this study is to propose a novel super-portable plasma device to meet the needs of clinical use and various special scenarios, and to explore the antibacterial effect and antibacterial mechanism of PAW and H_2O_2/PAW. To provide a clear overview of our experimental design, we have included a detailed flowchart (Supplementary Fig. 1).

Materials and methods

Bacteria and cell cultures

E. coli (ATCC 8099), *S. aureus* (ATCC 6538), and *P. gingivalis* (ATCC 33,277) were purchased from American Type Culture Collection (ATCC) and selected as the model organisms for inhibitory tests. *E. coli* and *S. aureus* were cultured in Luria-Bertani (LB) medium at 37 ℃ until they reached the logarithmic growth phase. *P. gingivalis* was cultured in Brain Heart Infusion (BHI) broth supplemented with hemin and vitamin K1, under anaerobic conditions (85% N_2, 10% H_2, and 5% CO_2) and subsequently grown aerobically at 37℃ until it reached the logarithmic growth phase. The bacterial concentrations were adjusted to 1×10^8 colony-forming units (CFU)/mL. Colonies were resuspended in phosphate-buffered saline (PBS) and then used within 2 h.

The primary human periodontal ligament fibroblasts (hPDLFs) (HUMICELLm001; obtained from iCell Bioscience, Inc., Shanghai, China) were cultured in α-MEME medium (Gibco, USA) supplemented with 10% fetal bovine serum (Solarbio, China) and 1% penicillin/streptomycin (Solarbio, China) under 5% CO_2 humidified atmosphere at 37℃ for the experiments. hPDLFs were sub-cultured using 0.25% trypsin-EDTA (Solarbio, China) when they reached 85-90% confluence. HPDLFs used for experiments were at the fourth passage while still maintaining their characteristic spindle morphology.

Plasma device, PAW, and H_2O_2/PAW generation

The traditional plasma device was a dielectric barrier discharge and powered by a direct current (DC) source. Owing to the thickness of the dielectric barrier layers, the traditional plasma device was large and weighed 40 kg, which made it inconvenient to move and carry. The working gas, Ar/O2(2 vol%), was supplied at a flow rate of 2 standard liters per minute (slm) and regulated by a flow controller. The operating current and voltage applied were 0.3 A and 3 kV, respectively. The novel plasma device (Fig. 1B) used in this experiment weighed about 1 kg and was only one-fifth the size of the traditional plasma devices [17]. PAW was generated at a frequency of 50–60 kHz, the output voltage of 30 kV, and the flow rate of working gas air was 5 L/min (Fig. 1C and E). The air used was ambient air, and its humidity was maintained at a relatively stable level throughout the experiments. The relative humidity ranged between 40% and 50%, with an average of approximately 45%. During the experiments, the laboratory temperature and ventilation conditions were also kept consistent to ensure the stability of the experimental environment.

As shown in Fig. 1D, PAW was produced by placing the plasma jet (PMJ) beneath the water surface, and the distance between PMJ and the liquid surface was 3 cm. Different volumes of sterile deionized water, 20 mL, 50 mL, and 100 mL, were activated by plasma for 10 min and 20 min to obtain PAW, which were referred to as 20-PAW, 50-PAW, and 100-PAW in this study, respectively. 20 mL of sterile deionized water was activated for different times, 2 min, 4 min, 6 min, and 8 min with plasma to obtain PAW, which were referred to as PAW-2, PAW-4, PAW-6, and PAW-8, respectively, in this study.

20 mL of 0.1% H_2O_2 or 3% H_2O_2 was activated by plasma for 20 min following the method described above to obtain 0.1% H_2O_2/PAW or 3% H_2O_2/PAW, respectively. In the evaluation of the cytotoxicity of 0.1% H_2O_2/PAW, 0.1% H_2O_2 was activated by plasma for different durations: 1 min, 5 min, 10 min, and 20 min. These treatments are referred to as 0.1% H_2O_2/PAW-1, 0.1% H_2O_2/PAW-5, 0.1% H_2O_2/PAW-10, and 0.1% H_2O_2/PAW-20, respectively.

Antibacterial procedure

To mimic the antibacterial process under realistic conditions and to investigate the influence of proteins on antimicrobial efficacy, 3% BSA (Beyotime biotechnology, China) was added. For each bacterial species, 1 mL of cultured bacterial suspension and 1 mL sterile deionized water or 3% BSA were added to 8 mL of different PAW or H_2O_2/PAW and treated for varying durations. Subsequently, 10-fold serial dilutions of 100 μL of the treated bacterial suspensions were immediately plated on an agar plate. The bacterial suspension treated with sterile deionized water was used as the negative control group (NC), while the suspension treated with 3% H_2O_2, which was widely used for antibacterial in clinical settings, was used as the positive control group (PC) [27]. The disinfection of bacteria was evaluated by counting the number of CFUs on a petri dish. Then the bacterial rate was

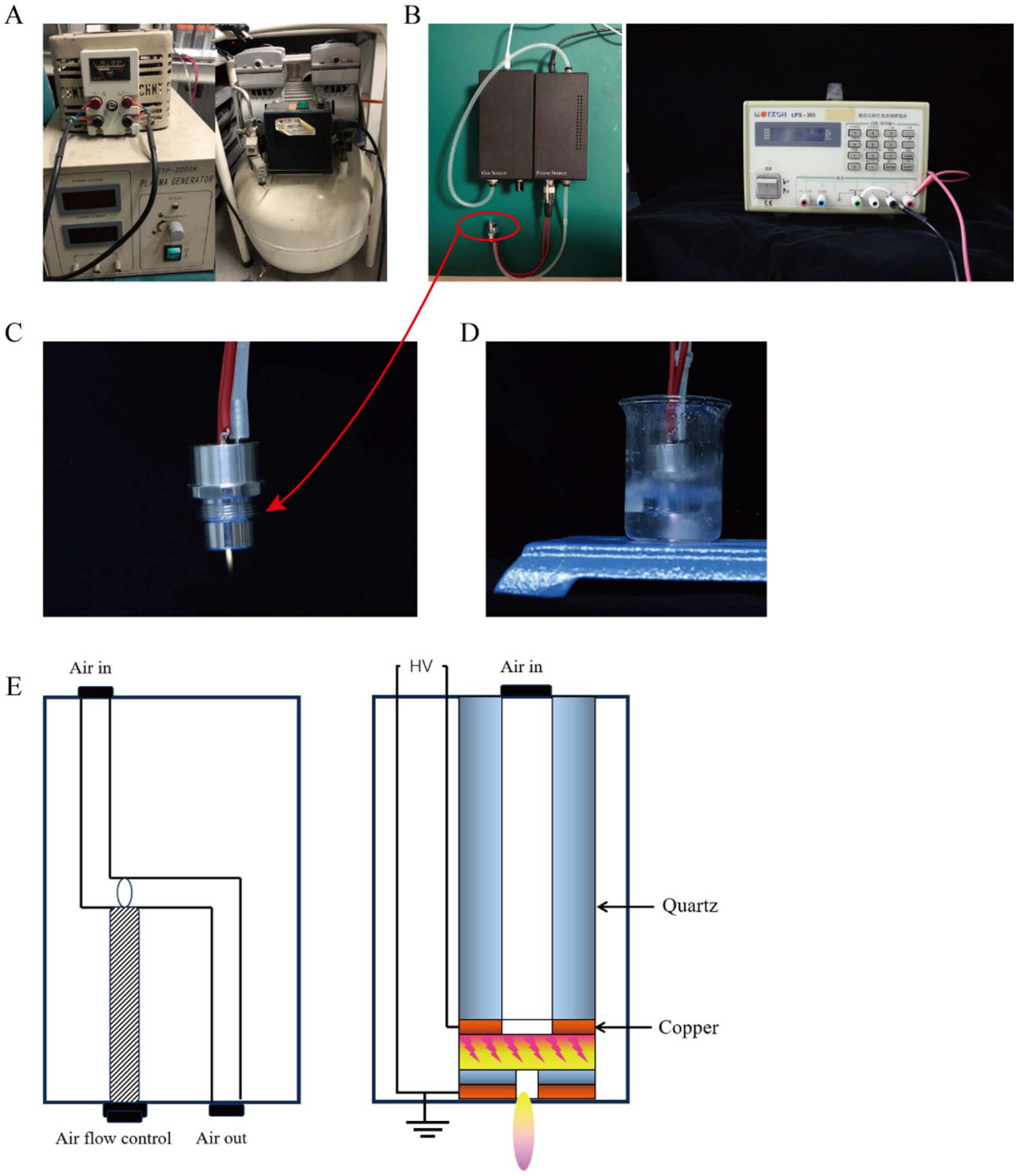

Fig. 1 Plasma generation device, image of PAW generation. (**A**) The photo of the traditional plasma device. (**B**) The photo of the new plasma device. (**C**) The photo of a plasma jet. (**D**) The photo of the PAW generation. (**E**) A schematic diagram of a plasma jet generated by the new plasma device. PAW, plasma-activated water

calculated using the formula below. Each experiment was performed at least three times for each condition.

$$\text{Bactericidal Rate} = \frac{(\text{CFU}_{\text{NC}} - \text{CFU}_{\text{treated}}}{\text{CFU}_{\text{NC}}} \times 100\%$$

Live/dead bacteria staining

In order to estimate the vitality of bacteria in suspension, we stained the suspension with LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, American). Syto9 permeates all bacterial membranes (green fluorescence), while propidium iodide (PI) only enters membrane-damaged cells, with their combination yielding red fluorescence. After different treatment times, the *S. aureus* suspension was washed with PBS. Subsequently, 1.5 μL of a Syto9 (3.34 mM stock in DMSO) and 1.5 μL PI (20 mM stock in DMSO) were added to 1 mL bacterial suspension, followed by incubation at 37 °C for 15 min in the dark. The samples were washed thoroughly to remove excess stain, and imaged using an inverted fluorescence microscope (OLYMPUS IX-730, Japan) equipped with appropriate excitation/emission filters for the two dyes.

Physical and chemical properties of PAW

To investigate the variations in pH, ORP, and conductivity in H_2O_2/PAW, 20 mL of sterilized deionized water or different concentrations of H_2O_2 was utilized to prepare H_2O_2/PAW, with the PMJ activation times and the treatment duration to *S. aureus* as the variables. The PMJ activation times were set at 5, 10, and 20 min to obtain H_2O_2/PAW, then the pH, conductivity, and ORP values of the solutions were measured using a C-600 multi-parameter water quality meter (Jiyi Technology Co., Ltd, China) immediately. While the PMJ activation time was 20 min and the treatment durations were established at 1, 3, 5, 10, and 20 min, after treatment, the pH, conductivity, and ORP values of the solutions were measured using a C-600 multi-parameter water quality meter immediately.

H_2O_2 and NO concentration measurement

The H_2O_2 concentration was assayed by a hydrogen peroxide assay kit following the manufacturer's instructions (Beyotime Biotechnology, China). According to the instructions, samples were added with reaction reagent at room temperature for 30 min and measured immediately with a spectrophotometer at a wavelength of 560 nm. The concentration of H_2O_2 was calculated according to a standard curve generated with a standard concentration of H_2O_2. The concentration of NO was measured by using a previously developed Griess reaction-dependent method (Beyotime Biotechnology, Beijing, China). The calibration curve was plotted by using $NaNO_2$ standard solutions for the calculation of the NO-released concentration according to the absorbance at 540 nm.

Optical emission spectroscopy

To identify the major excited reactive species in the employed plasma jet when it activated the sterile deionized water, OES was employed in the wavelength range of 200–1000 nm with an AvaSpec-2048-8 Fiber Optic Spectrometer (Avantes, USA). The plasma device was submerged in deionized water. Before the plasma was excited, the spectrum of the deionized water was detected using the spectrometer, and this spectral signal was used to set the baseline to zero. Then the plasma device was turned on for spectral detection, resulting in a pure plasma signal with the noise signal of the deionized water removed.

Measurement of cytotoxicity of 0.1% H_2O_2/PAW

According to the standard for in vitro cytotoxicity tests (ISO 10993-5), the cell viability rate above 70% is considered non-cytotoxic, 50–70% is classified as slightly cytotoxic, 30–50% is categorized as moderately cytotoxic, and below 30% is deemed severely cytotoxic. To evaluate the cytotoxicity of 0.1% H_2O_2/PAW, a CCK-8 assay was conducted to assess the viability of human periodontal ligament fibroblasts (hPDLFs). Initially, hPDLFs in the logarithmic growth phase were seeded at a density of 3×10^3 cells/well in 96-well plates with 100 μL of complete medium. 24 h post cell seeding, the medium was completely replaced with 100 μL 0.1% H_2O_2/PAW (PAW: DMEM = 1:1), then the cells were treated for 24 h, 48 h, and 72 h. Subsequently,

the culture medium was removed and replaced with 100 μL of CCK-8 working solution (90% complete culture medium with 10% CCK-8, vol%). The OD density at 450 nm was measured after 2 h of incubation in the culture incubator. The cytotoxicity was expressed as the percentage of cell viability relative to the control group, which did not receive 0.1% H_2O_2/PAW treatment.

Statistical analysis

All experiments were independently repeated three times. Statistical significance was calculated using SPSS version 22.0. An unpaired t-test was employed for the comparative analysis between the two groups. One-way analysis was used for comparisons among multiple groups, followed by Tukey's multiple comparisons test to identify inter-group differences. When comparing paired multiple groups, two-way analysis of variance (Two-way ANOVA) was applied, followed by Sidak's multiple comparisons test. The data were presented as the mean ± standard deviation (SD), and statistical significance was established at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Results

Bactericidal efficiency of PAW on *S. aureus* and *P. gingivalis*

As shown in Fig. 2A, there was a slight difference of 0.18% in the bactericidal rate between PAW generated by the traditional plasma device (Fig. 1A) and the novel one on *S. aureus* (Fig. 1B). The antimicrobial ability of PAW against *S. aureus* was presented in Fig. 2B. Compared to the NC, PAW significantly enhanced the bacterial efficiency against *S. aureus* ($p < 0.05$). The bactericidal efficacy was represented in a PAW treatment time-dependent way. With the longer time of PAW treatment, a higher bacterial reduction was achieved ($p < 0.05$). Moreover, the antibacterial efficiency also depended on the volume of plasma activation used for PAW generation, clearly indicating that higher inactivation potentials could be achieved with smaller plasma activation volumes. For the 10 min PAW treatment, the bactericidal rate significantly decreased from 99.8 to 96% as the plasma activation volume increased from 20 to 100 mL ($p < 0.05$). Because 20-PAW demonstrated the strongest bactericidal efficiency, 20 mL of sterile deionized water was used to generate PAW in the following experiments. To further explore the antibacterial effect of PAW, *S. aureus* was used to perform a Live/DEAD staining assay using Syto9 and PI stains. Figure 2C showed that *S. aureus* treated with PAW for 10 min and 20 min exhibited strong red fluorescence, indicating a strong bactericidal effect of PAW.

P. gingivalis is a Gram-negative bacterium that plays a vital role in the pathogenesis of periodontitis and is highly destructive. As shown in Fig. 2D, PAW exhibited excellent antibacterial effects against *P. gingivalis* compared to NC ($p < 0.05$). At the treatment time of 1 min, the bactericidal rate gradually increased as the preparation time of PAW was extended ($p < 0.05$). Therefore, increasing the preparation time of PAW could also enhance its bactericidal efficacy. Additionally, if the treatment time of PAW was extended, a good bactericidal effect could still be achieved even with a shorter preparation time.

0.1% H_2O_2 enhanced the bactericidal efficiency of PAW on *S. aureus*

The influence of BSA on the disinfection efficacy was also studied. *S. aureus* was used as the test bacterium. As shown in Fig. 3A, the antimicrobial efficiency of PAW was significantly reduced in the presence of BSA. To address this challenge, different concentrations of H_2O_2 were used to produce H_2O_2/PAW. In the presence of BSA, the addition of 0.1% H_2O_2 to PAW to form 0.1% H_2O_2/PAW resulted in a significantly higher antibacterial effect compared to the PAW + BSA group. Moreover, when the treatment time was less than 20 min, the antibacterial effect of 0.1% H_2O_2/PAW was also significantly

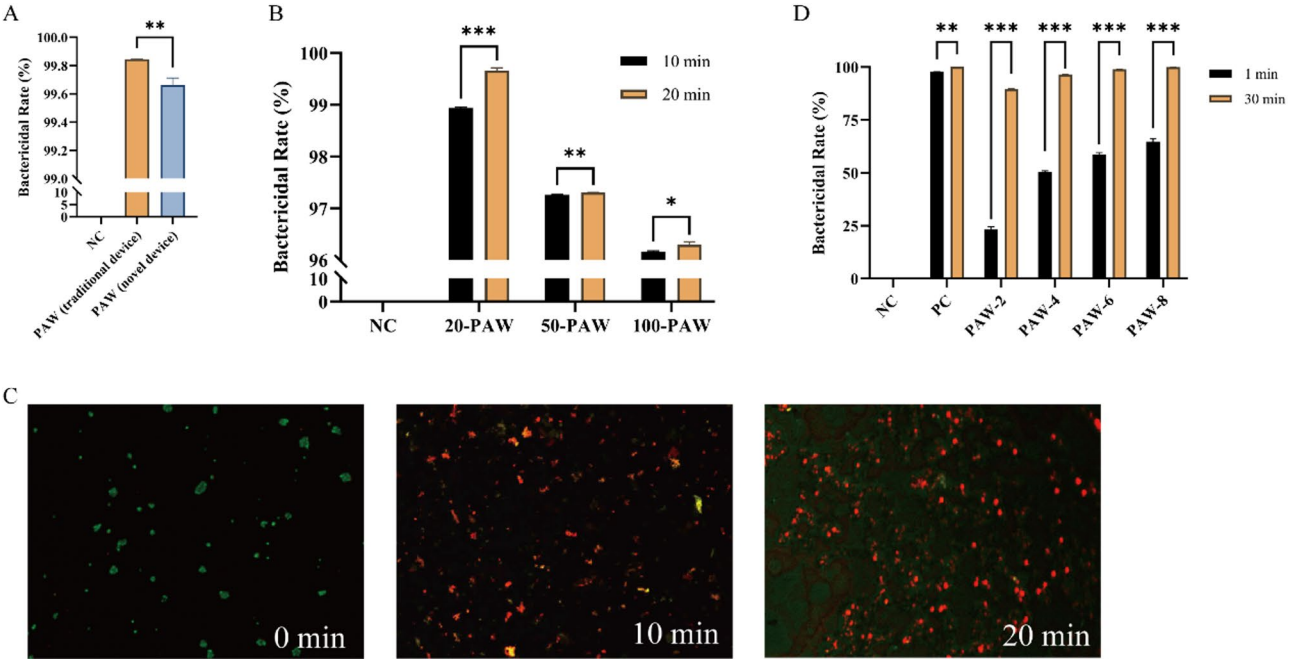

Fig. 2 The bactericidal efficiency of PAW. (**A**) The disinfection efficiency of PAW generated by the new device and the traditional device against *S. aureus*. (**B**) The bactericidal efficiency of 20-PAW, 50-PAW, and 100-PAW for treatment times of 10 min and 20 min against *S. aureus*. (**C**) Syto9 and PI stains of *S. aureus* treated by 20-PAW. (**D**) The bactericidal efficiency of PAW on *P. gingivalis* with different preparation and treatment times. The data are expressed as the means ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NC, the negative control group; PC, the positive control group; 20-PAW, 50-PAW and 100-PAW, 20 mL, 50 mL and 100 mL of sterile deionized water were activated by PMJ for 20 min to obtain PAW respectively; PAW-2, PAW-4, PAW-6, PAW-8, 20 mL of sterile deionized water were activated by PMJ for 2 min, 4 min, 6 min, 8 min to obtain PAW respectively

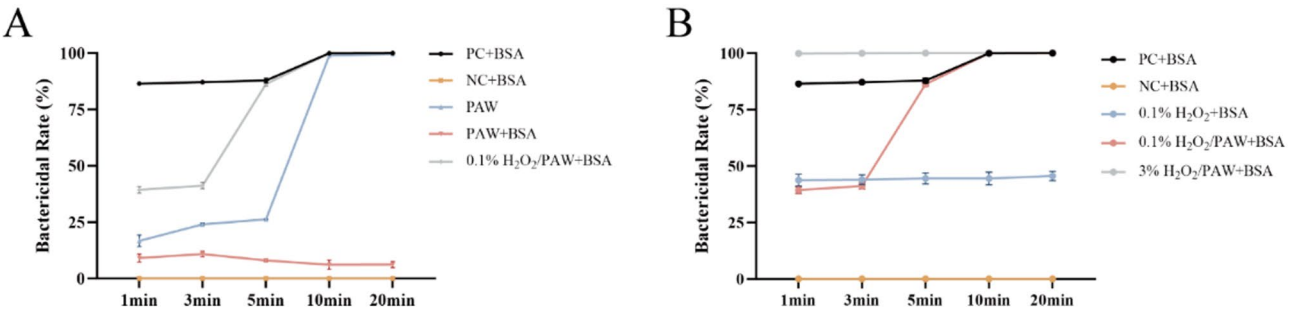

Fig. 3 H_2O_2 enhanced the disinfection efficiency of PAW against *S. aureus*. (**A**) The disinfection effect of PAW with or without BSA. (**B**) The disinfection efficiency of different concentrations of H_2O_2/PAW. The data are expressed as the mean ± SD. BSA, Bovine Serum Albumin; 0.1% H_2O_2/PAW and 3% H_2O_2/PAW, 20 mL of 0.1% H_2O_2 or 3% H_2O_2 were activated by PMJ for 20 min, respectively

higher than that of the PAW group. As shown in Fig. 3B, 3% H_2O_2/PAW demonstrated a stronger antimicrobial effect than 3% H_2O_2 alone ($p < 0.05$). 0.1% H_2O_2 showed limited antimicrobial efficiency compared to the negative group, but 0.1% H_2O_2/PAW exhibited excellent antimicrobial efficacy. It was speculated that there may be a synergistic effect of H_2O_2 and PAW, which could enhance the bactericidal capacity of PAW. Although the antibacterial effect of 3% H_2O_2 /PAW was significantly stronger than that of 0.1% H_2O_2/PAW in the short term, when the treatment time was 20 min or longer, the antibacterial effects of the two groups were comparable. Due to the irritant nature of 3% H_2O_2 on human mucous, it was recommended to use 0.1% H_2O_2/PAW as a substitute.

Bactericidal effect of H_2O_2/PAW on *E. coli*

We also explored the antibacterial effects of different concentrations of H_2O_2/ PAW on *E. coli* in the presence of BSA, with a treatment duration of 20 min. As shown in Fig. 4, the antibacterial efficacy of the 0.1% H_2O_2/PAW group did not show a statistically significant difference compared to that of the 3% H_2O_2/PAW group and the PC group. However, the antibacterial effect of the 0.1% H_2O_2/PAW group was significantly greater than that of the 0.1% H_2O_2 group ($p < 0.001$).

Analysis by ORP, pH, conductivity

The pH, ORP, and conductivity were measured to explore the antibacterial mechanisms of H_2O_2/PAW. It was observed that the addition of 0.1% H_2O_2 lowered the solution pH while increasing ORP and conductivity values. As shown in Fig. 5A, the pH value gradually decreased as the PAW preparation time increased. Additionally, with the same preparation time, the pH of the solution added with BSA was higher compared to that without BSA. Figure 5B showed that with the same preparation time ($t = 20$ min), the longer treatment time resulted in a lower pH value. Although 3% H_2O_2 also exhibits acidity, when mixed with PAW, the pH of the mixture was significantly lower. This confirmed the results of the previous experiments, indicating that the bactericidal efficiency of PAW could be significantly enhanced by adding H_2O_2. Figure 5C demonstrated that the ORP value of the solution increased as the preparation time increased. Additionally, a higher concentration of H_2O_2/PAW, with the same preparation time, led to a higher ORP value. The addition of BSA resulted in a decrease in ORP compared to the group without BSA. Figure 5D illustrated that regardless of the treatment duration, the ORP value of the 3% H_2O_2/PAW + BSA group was consistently higher than that of the 3% H_2O_2 + BSA group. Furthermore, during the treatment time of 1 to 10 min, the ORP values for both groups gradually increased, followed by a decline in ORP values during the 10 to 20 min treatment period, suggesting that the bactericidal effect of H_2O_2/PAW peaked between 10 and 20 min. The evidence for the presence of ions in the PAW was measured using a portable conductivity meter. Figure 5E showed that the conductivity of H_2O_2/PAW considerably increased as the plasma activation time and H_2O_2/PAW concentration increased. Figure 5F revealed that the conductivity of the 3% H_2O_2/PAW + BSA group was significantly higher than that of the 3% H_2O_2 + BSA group, regardless of the treatment duration. Notably, the conductivity of the 3% H_2O_2/PAW + BSA group peaked at approximately 10 min.

H_2O_2 and NO content in H_2O_2/PAW

It is reported that there was a large amount of reactive species, such as OH:, H^-, and NO_3^-, generated in water through plasma jet irradiation. The levels of H_2O_2 and NO in different solutions were tested. Figure 6A and B showed that H_2O_2 and NO were produced in the PAW. The concentration of H_2O_2 and NO in 0.1% H_2O_2/PAW and 3% H_2O_2/PAW was significantly higher than that in PAW ($p < 0.001$). Furthermore, the concentrations of H_2O_2 and NO in 0.1% H_2O_2/PAW and 3% H_2O_2/PAW were higher than those in 0.1% H_2O_2 and 3% H_2O_2, respectively ($p < 0.001$). The higher concentrations of H_2O_2 added to PAW, the higher levels of H_2O_2 and NO in the solution.

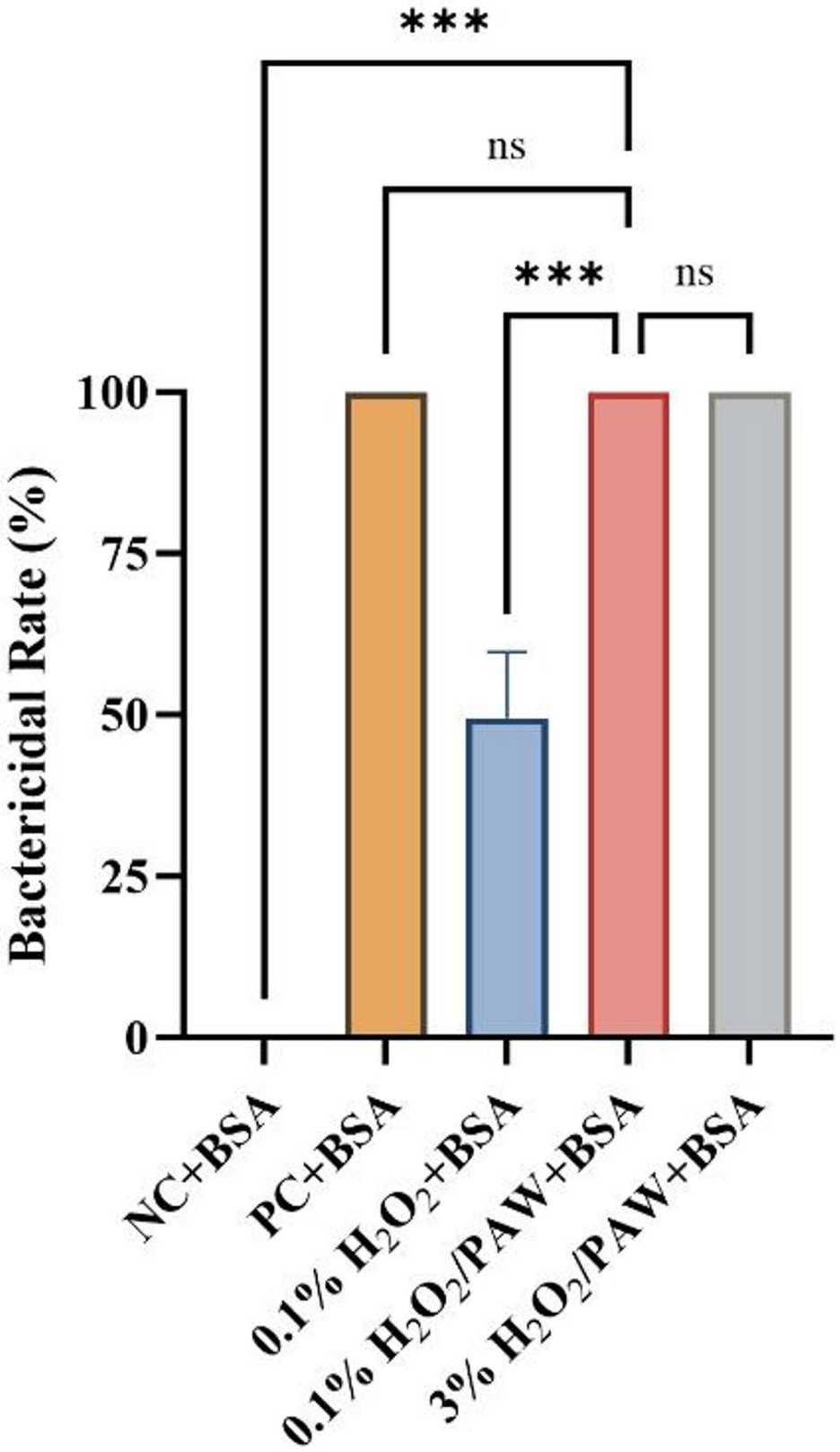

Fig. 4 The disinfection efficiency of H_2O_2/PAW against *E. coli*. The data are expressed as the means ± SD; *** $p < 0.001$

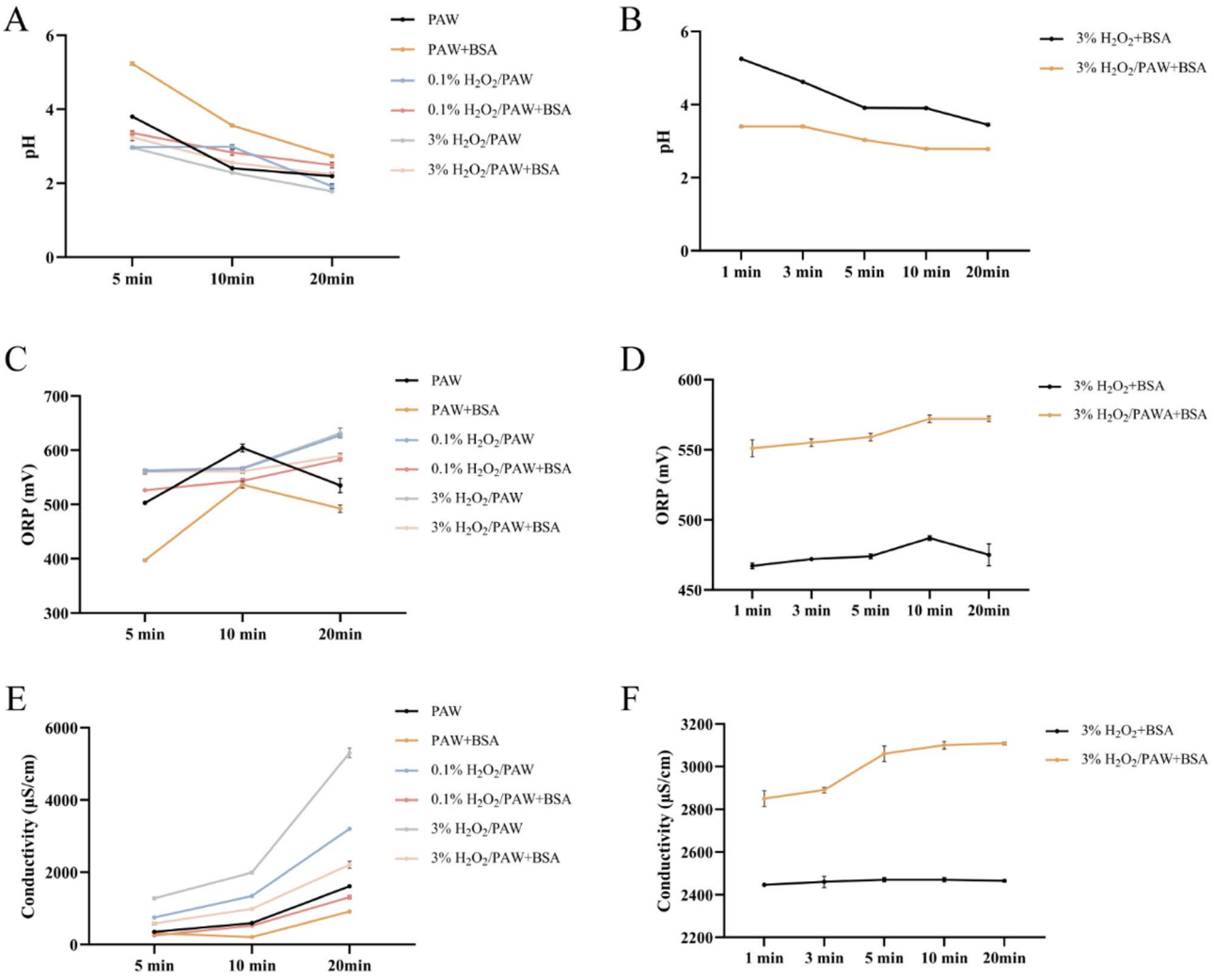

Fig. 5 Changes in physicochemical properties of H_2O_2/PAW. (**A**), (**C**) and (**E**) Changes of pH, ORP, and conductivity value of H_2O_2/PAW in different activation times, respectively. (**B**), (**D**) and (**F**) Changes of pH, ORP, and conductivity value of H_2O_2/PAW in different treatment times for 20 min of PMJ activation time. The data are expressed as the means ± SD

OES analysis

The OES spectra of the employed plasma jet when it activated the sterile deionized water was evaluated to identify the major active species. Figure 7 presented the emission spectrum of plasma within the range of 200 to 1000 nm. N2 second positive system ((N_2 (C→B)) emission peaks (300 to 445 nm), and N_2^+ first negative system (N_2^+) emission peaks (380 to 520 nm) and N_2^+ first positive system ((N_2^+(A→B)) emission peaks (545 to 755 nm) were dected [28–30]. Additionally, NOγ emission peaks (200 nm to 300 nm) and strong ·OH emission peaks (306.8 nm) were detected. The significant O peaks were observed near 787.3 nm and 844.5 nm, which were primarily produced by the dissociation of O_2 [29].

The cytotoxicity of 0.1% H_2O_2/PAW in hPDLFs

As shown in Fig. 8, the cell viability of the group (20 min preparation and 72 h treatment) was below 50%, indicating moderate cytotoxicity. The cytotoxicity of the other groups was either mild or non-cytotoxic.

Discussion

Numerous diseases, such as periodontitis and dental caries, are infectious bacterial diseases that can significantly affect individuals' health and quality of life [31, 32]. Therefore, it is crucial to implement effective measures to control bacterial infections. While currently available antiseptics demonstrate considerable efficacy, they are accompanied by several significant limitations [33, 34]. A major concern is the development of antiseptic resistance, primarily driven by the overuse of these agents, which poses a substantial challenge in both clinical and public health settings [35, 36]. In addition to antiseptic resistance, there are some side effects. CHX mouthwash, commonly used for oral hygiene, can lead to tongue discoloration, dental staining, and abnormal taste

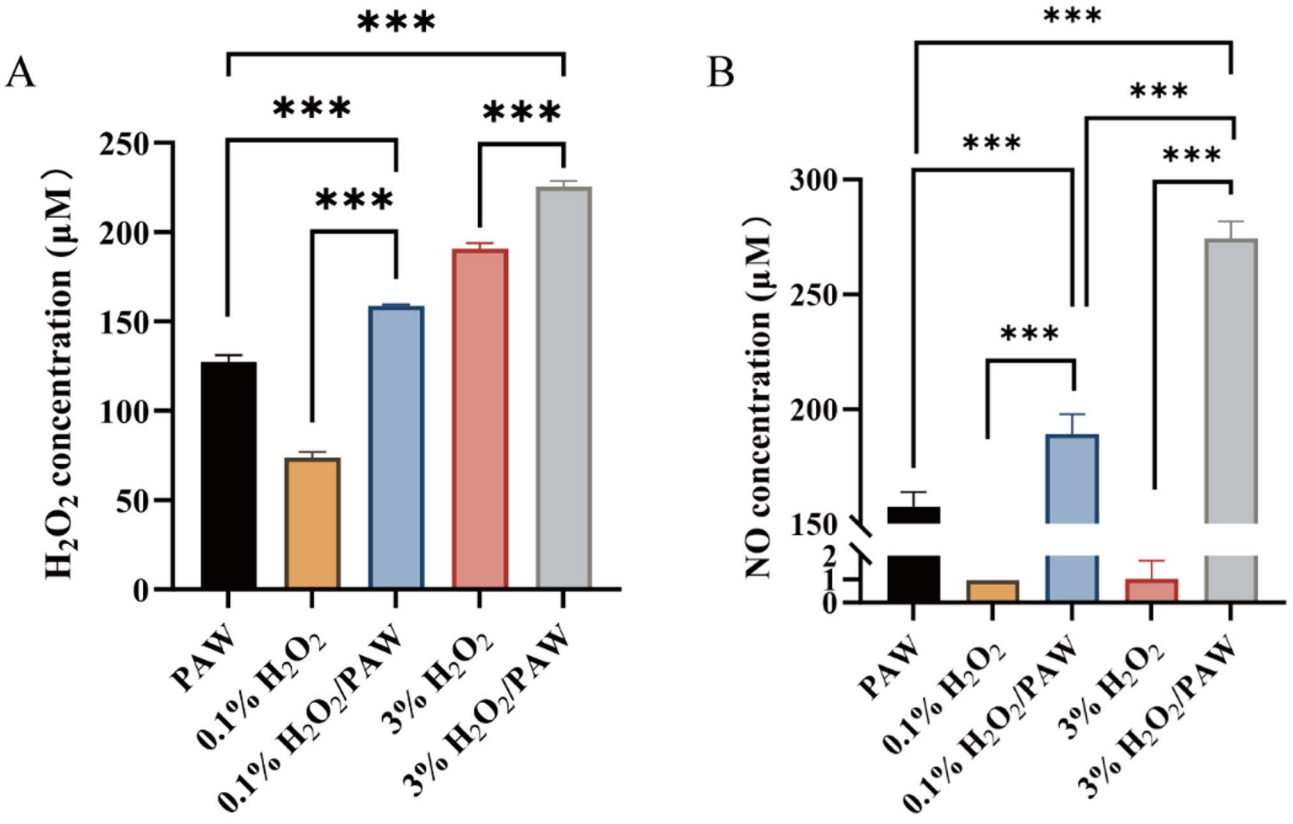

Fig. 6 H_2O_2 (**A**) and NO (**B**) concentration in H_2O_2/PAW. The data are expressed as the means ± SD; ***$p < 0.001$

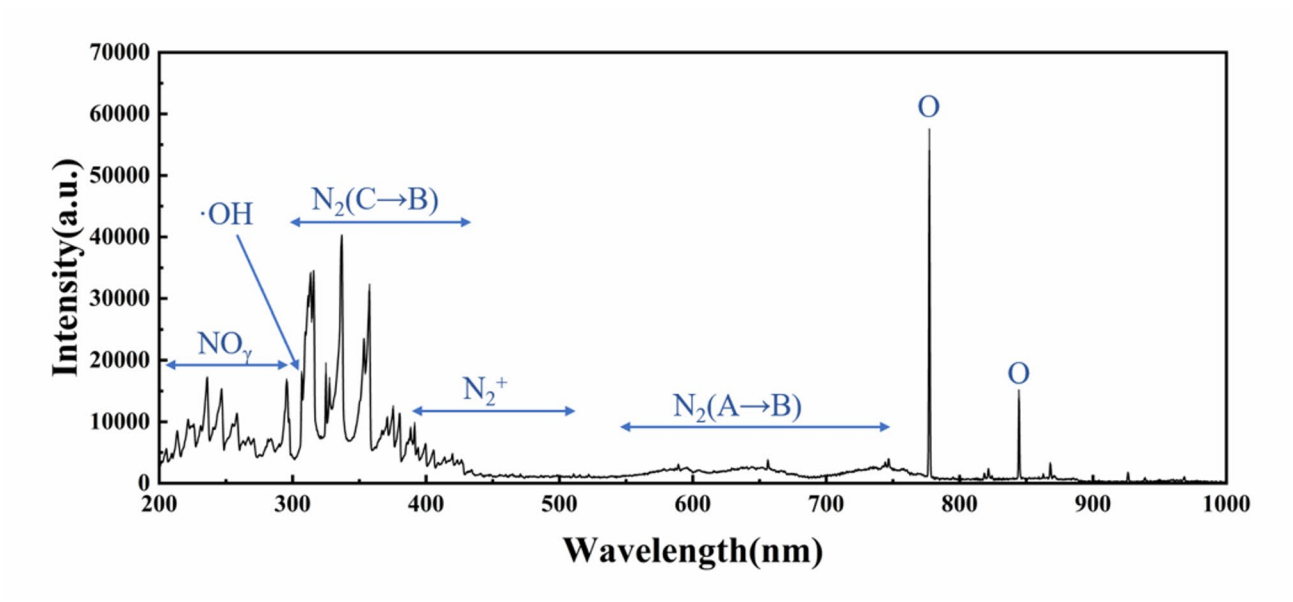

Fig. 7 Optical emission spectrum of PAW ranging from 200 to 1000 nm operated in water

[37]. These drawbacks highlight the urgent need for the development and promotion of novel antiseptics. Non-thermal plasma has been extensively used to inactivate biological species [38], and PAW has been proven to have a pronounced disinfection effect [39].

The plasma device used in our previous studies, as well as in the research of others, has revealed that its structural complexity often results in large sizes, high operational difficulty, and limited portability, which hinders its clinical applicability, especially in specialized scenarios

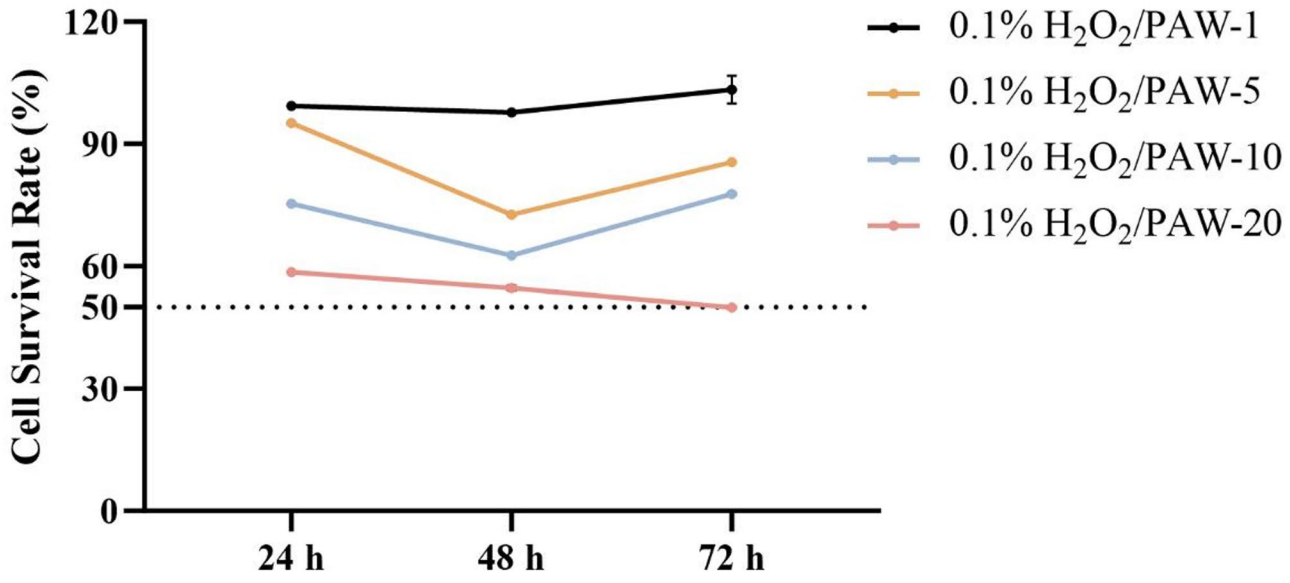

Fig. 8 The cytotoxicity of 0.1% H_2O_2/PAW with different preparation and treatment times on human periodontal ligament fibroblasts. The data are expressed as the means ± SD

such as outdoor or military activities [17, 18]. To address these challenges, we have developed a novel plasma activation device that is compact, lightweight, and user-friendly, making it highly suitable for clinical use and other specific applications. As shown in Fig. 2A, the PAW produced by the novel device exhibited significant antibacterial efficacy against *S. aureus*, with a bactericidal rate that was only 0.18% lower than that produced by the traditional device. This minor difference highlights the potential of our new device to provide a practical and effective solution for antimicrobial needs in various settings. *P. gingivalis* is one of the primary pathogens in periodontitis and plays a significant role in the development and progression of periodontitis. Therefore, *P. gingivalis* was also selected as the test bacterium to evaluate the antibacterial effect of PAW generated by the novel plasma device. The results showed that the antibacterial efficacy of PAW was closely related to both the preparation and treatment times. When the treatment time was 1 min, the longer preparation time of PAW led to a better antibacterial effect. However, when the treatment time was extended to 30 min, the antibacterial efficacy became relatively consistent when the preparation time of PAW was 4 min or longer. This suggested that PAW had the potential to be used as a mouthwash for its antibacterial properties. Nevertheless, further in-depth investigations are needed to explore its antibacterial properties against a broader range of pathogenic bacteria and to determine the optimal treatment and preparation times.

Traditional antiseptics, such as CHX, exert their bactericidal effects by disrupting bacterial cell membranes and inhibiting biofilm formation. However, this specific mechanism can lead to the selection of resistant strains [40]. In contrast, the antibacterial efficacy of PAW is primarily attributed to the generation of reactive species, including hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), nitrate (NO_3^-), and so on [41]. The bactericidal effect of RONS is attributed to their high reactivity, enabling them to interact effectively with polysaccharides, lipids, proteins, and nucleic acid molecules in bacteria and biofilms, thereby damaging bacterial structures and causing cellular damage [25]. The non-specific mode of action of PAW is a critical factor in its ability to avoid antibiotic resistance [42]. PAW employs a broad spectrum of reactive species that can target various cellular components. Recent research demonstrated that PAW not only matched but often exceeded the antibacterial efficacy of traditional agents like chlorhexidine and triclosan. These findings suggest that PAW could serve as a more effective alternative in combating resistant bacterial infections.

However, the current research on the antibacterial properties of PAW is mostly conducted under relatively ideal conditions in absence of organic matter, whereas the oral cavity and body are the places where contain a large amount of organic matter. Previous studies have also indicated that the presence of organic matter could considerably reduce the bactericidal effect of PAW [26]. Our results, as shown in Fig. 3A, further validated that the antibacterial efficacy of PAW was greatly diminished in the presence of BSA. This reduction is likely due to BSA acting as a non-specific antioxidant, which can consume RONS around it. At the same time, the physical barrier created by BSA can reduce the direct contact between PAW and bacteria, thereby decreasing the bactericidal effect of PAW.

To improve the applicability of PAW in actual clinical applications, we have tried to add enhancers to improve its antibacterial effect and found that the addition of a super-low concentration of H_2O_2 could significantly improve the antibacterial ability of PAW in the presence of BSA, as shown in Fig. 3A. *E. coli* is a model organism used in bactericidal experiments and belongs to the category of Gram-negative bacteria. Under certain circumstances, it can pose a threat to health and has been associated with the occurrence and development of oral diseases [43, 44]. Therefore, the antibacterial effect of H_2O_2/PAW on *E. coli* was also explored in the presence of BSA. Figure 4 showed that 0.1% H_2O_2/PAW exhibited significant antibacterial activity against *E. coli*. This indicated that 0.1% H_2O_2/PAW maintains robust antibacterial activity even in the presence of organic matter, providing support for the development of a novel antibacterial method. We hypothesize that H_2O_2, as a well-known strong oxidant, could undergo redox reactions with the sulfhydryl groups of BSA, leading to the formation of thiolactic acid or disulfide bonds. This process likely prevents the interference effect of BSA on the antibacterial activity of PAW. Some studies have also found that the presence of free radicals can affect the antioxidant properties of serum albumin [45].

Although H_2O_2 itself possesses antibacterial properties, our research findings indicated that H_2O_2/PAW exhibited significant synergistic antibacterial effects. Although hydrogen peroxide (H_2O_2) itself possesses antibacterial properties, our research findings indicated that H_2O_2/PAW exhibited significant synergistic antibacterial effects. This observation is consistent with previous studies that have shown that plasma-activated H_2O_2 solutions have superior antibacterial efficacy compared to PAW solutions alone [46, 47]. This phenomenon may be attributed to the generation of high-energy particles (P in R1) during the plasma activation process. After the addition of H_2O_2, free electrons are more likely to react with H_2O_2 (R1-R2), thereby enhancing the bactericidal effect. Meanwhile, our experiments demonstrated that the longer the preparation and treatment time were correlated with the more pronounced the bactericidal effect of H_2O_2/PAW. This means that in practical applications, the preparation and treatment time of H_2O_2/PAW can be adjusted according to actual needs to achieve the optimal bactericidal effect.

$$H_2O_2 + P \rightarrow \cdot OH + \cdot H + P \quad (R1)$$

$$e^- + H_2O_2 \rightarrow e^- + 2 \cdot OH \quad (R2)$$

The values of pH, ORP, and conductivity are critical for evaluating the antibacterial efficacy and exploring the mechanisms of PAW. Numerous studies have demonstrated that under the optimal pH, PAW is more likely to generate reactive oxygen species (ROS) and other active substances, which exert strong oxidative effects on bacteria, thereby achieving bactericidal outcomes [48]. ORP is regarded as an important factor influencing microbial inhibition. A high ORP can damage the outer and inner membranes [49]. Thus, it is of great significance to explore ORP changes of PAW for monitoring bactericidal efficacy. Conductivity value reflects the concentration of ions, free radicals, and so on in the solution. Higher conductivity often indicates a higher presence of active substances, which are associated with enhanced antibacterial effects [50]. Our experimental results supported these views. Due to the plasma activation, substances such as H_2O_2 and NO were produced in the solution, leading to a gradual increase in ORP and conductivity, and a decrease in the pH value. This trend indicated a consistent relationship between these parameters and the antibacterial efficacy of H_2O_2/PAW. Moreover, our results also indicated that BSA could change the properties of PAW by reducing the ion concentration in the solution, especially active substances with strong oxidizing power, such as RONS and hydrogen ions, thereby reducing the bactericidal efficiency of PAW. We can avoid the impact of BSA by adding H_2O_2. The experiments demonstrated that this addition not only increased the ion concentration in the solution but also underwent redox reactions with BSA, reducing the pH of the solution while increasing the concentration of active substances such as RONS in the solution, thereby increasing conductivity and ORP.

As shown in Fig. 6A and B, we observed that the H_2O_2 and NO content in the 0.1% H_2O_2/PAW and 3% H_2O_2/PAW groups was significantly higher than that in the PAW group; this observation is consistent with previous studies [46, 47]. This may be likely due to the interaction between H_2O_2 and the reactive species in PAW, such as hydroxyl radicals and nitric oxide, which play a crucial role in the antibacterial process. This trend that the NO content in the H_2O_2/PAW groups was significantly higher than that in the PAW group may be attributed to the high-energy electrons in the plasma jet, which can react with nitrogen-containing substances present in the solution to produce NO. Additionally, when the plasma jet interacts with solution, H_2O_2 molecules may participate in a series of chemical reactions, generating other reactive species such as OH·. These active substances further promote the generation of NO, resulting in a higher concentration of NO in the solution. Moreover, under acidic conditions, H_2O_2 as a reactant may facilitate the reaction between a greater number of H particles and the reactive nitrogen species produced by PAW, leading to increased NO formation. This interplay between H_2O_2 and PAW highlights the complex mechanisms involved

in the generation of reactive nitrogen species and underscores the potential of utilizing PAW in combination with hydrogen peroxide to enhance the production of beneficial reactive species for antimicrobial applications [46, 47].

The results from OES, shown in Fig. 7, have revealed that the atomic oxygen and excited nitrogen species generated in the plasma gas can readily convert into various RONS. These include ·OH, singlet oxygen, H_2O_2, and NO, all of which are known for their high reactivity. This finding underscores the presence of a significant number of active particles in the plasma gas produced by air plasma discharge, which is essential for the subsequent generation of PAW with this plasma gas [30]. The formation of these reactive species is closely related to the antibacterial efficacy of PAW. The ROS and RNS generated in the gaseous phase can enhance the antibacterial properties of PAW by promoting the formation of additional free radicals when dissolved in solution. This synergy between the gaseous and liquid phases highlights the potential of utilizing plasma technology to optimize the antimicrobial effects of PAW, particularly in clinical and environmental applications.

The cytotoxicity is equally important when assessing the antimicrobial properties. Our results indicated that the cytotoxicity of H_2O_2/PAW varied under different preparation and application conditions. In most cases, H_2O_2/PAW exhibited mild or no cytotoxicity, providing reassurance for its use in clinical and other occasions. This indicated that as long as users properly control the activation and treatment time, its non-cytotoxicity can be ensured.

However, this experiment still has certain limitations. The types of bacteria designed for the antibacterial performance of H_2O_2/PAW in this experiment are not numerous, and the experiment only explains the antibacterial mechanism of H_2O_2/PAW in the presence of BSA from the perspective of changes in physicochemical properties, without further in-depth exploration. The bactericidal efficacy of H_2O_2/PAW is influenced by various factors, including preparation time, exposure time, and the type of bacteria. Therefore, it is essential to select appropriate parameters based on different application scenarios. Moreover, we have preliminarily assessed the biocompatibility of H_2O_2/PAW using the CCK-8 assay, which is mainly based on cell proliferation and toxicity analysis. This method only reflects the cytotoxicity at the cellular level. To obtain a more comprehensive evaluation of the biosafety of H_2O_2/PAW, further studies are needed from multiple aspects. It is hoped that more in-depth exploration can be carried out in the future.

Conclusion

In conclusion, we have developed a super-portable plasma excitation device for the preparation of plasma, suitable for clinical use and various complex environments, such as outdoor settings and military applications. While achieving the portability and ease of operation of the plasma device, PAW produced by this device demonstrated excellent antibacterial effects against two common pathogens *S. aureus* and *P. gingivalis*. By adding a low concentration of H_2O_2, 0.1% H_2O_2/PAW maintained an effective antibacterial effect in organic-rich environments against *S. aureus* and *E. Coli*. By comprehensively considering physical and chemical properties, biological effects, and cytotoxicity, 0.1% H_2O_2/PAW has the potential to become an important antiseptic in medical settings, such as dentistry and other fields.

Abbreviations

PAW	Plasma-activated water
S. aureus	*Staphylococcus aureus*
E.coli	*Escherichia coli*
P.gingivalis	*Porphyromonas gingivalis*
BSA	Bovine Serum Albumin
ORP	Oxidation-reduction potential
CFU	Colony-forming units
H_2O_2	Hydrogen peroxide
CHX	Chlorhexidine
DWLUs	Dental water tubule units
RONS	Reactive oxygen and nitrogen species
S.mutans	*Streptococcus mutans*
A.viscosus	*Actinomyces viscosus*
S.pneumoniae	*Streptococcus pneumoniae*
N.meningitidis	*Neisseria meningitidis*
LB	Luria-Bertani
BHI	Brain Heart Infusion
NO	Nitric oxide
O_3	Ozone
NO_3^-	Nitrate
NO_2^-	Nitrite
OH	Hydroxyl radicals
$^1O_2$	Singlet linear oxygen
O_2^-	Superoxide
PMJ	Plasma jet
HPLCs	Human periodontal ligament fibroblasts
$ONOO^1$	Peroxynitrite
OES	Optical emission spectroscopy

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12903-025-06217-6.

Supplementary Material 1

Supplementary Material 2

Acknowledgements

The authors are highly thankful engineer of Kai Zhang and Kaile Wang of Peking University for providing technological assistance in improving the plasma device in this research project.

Author contributions

Conception and design of the study: Zuomin Wang, Ruixue Wang and Yinglong Li; Acquisition of data: Yu Liu, Xin Yu and Fang Zhang; Analysis of data: Yu Liu, Fang Zhang and Ruonan Ma; Drafting manuscript: Yu Liu, Yu Xing,

Yinglong Li and Ruixue Wang; Revising manuscript: Zuomin Wang, Yinglong Li, Ruonan Ma and Ruixue Wang. All authors have read and approved the final manuscript.

Funding
This study was supported by the R&D Program of Beijing Municipal Education Commission (KM202410025013) and Beijing Nova Programme Interdisciplinary Cooperation Project (20230484449).

Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
No applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 11 October 2024 / Accepted: 20 May 2025
Published online: 06 June 2025

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