Journal of Dental Sciences 17 (2022) 459–467

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Original Article

Comparisons of the killing effect of direct current partially mediated by reactive oxygen species on *Porphyromonas gingivalis* and *Prevotella intermedia* in planktonic state and biofilm state — an *in vitro* study

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Received 28 July 2021; Final revision received 30 July 2021
Available online 25 August 2021

KEYWORDS
Direct current;
Porphyromonas gingivalis;
Prevotella intermedia;
Biofilm;
Reactive oxygen species;
Killing effect

Abstract *Background/purpose:* Bacterial biofilms formed on the surface of tissues and biomaterials are major causes of chronic infections in humans. Among them, *Porphyromonas gingivalis* (*P. gingivalis*) and *Prevotella intermedia* (*P. intermedia*) are anaerobic pathogens causing dental infections associated with periodontitis. In this study, we evaluated the killing effect and underlying mechanisms of direct current (DC) as an antimicrobial method *in vitro*. *Materials and methods:* We chose *P. gingivalis* and *P. intermedia* in different states to make comparisons of the killing effect of DC. By viable bacteria counting, fluorescent live/dead staining, reactive oxygen species (ROS) assay, addition of ROS scavenger DMTU and mRNA expression assay of ROS scavenging genes, the role of ROS in the killing effect was explored. *Results:* The planktonic and biofilm states of two bacteria could be effectively killed by low-intensity DC. For the killing effect of 1000 μA DC, there were significant differences whether on planktonic *P. gingivalis* and *P. intermedia* (mean killing values: 2.40 vs 2.62 $\log_{10}$ CFU/mL) or on biofilm state of those (mean killing values: 0.63 vs 0.98 $\log_{10}$ CFU/mL). 1000 μA DC greatly induced ROS production and the mRNA expression of ROS scavenging genes. DMTU could partially decrease the killing values of DC and downregulate corresponding gene's expression. *Conclusion:* 1000 μA DC can kill *P. gingivalis* and *P. intermedia* in two states by promoting

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https://doi.org/10.1016/j.jds.2021.07.025

overproduction of ROS, and *P. intermedia* is more sensitive to DC than *P. gingivalis*. These findings indicate low-intensity DC may be a promising approach in treating periodontal infections.

Introduction

Periodontitis, one of the most prevalent human diseases in the world, has been reported that approximately more than 50% of Chinese adults suffered from it and 30% had severe periodontitis among them.[1] Periodontitis is initiated by pathogenic bacteria in subgingival plaque biofilms, of which one of the most important members is the Gram-negative anaerobe *Porphyromonas gingivalis* (*P. gingivalis*) and *Prevotella intermedia* (*P. intermedia*).[2–4] As the major periodontal pathogen, *P. gingivalis* and *P. intermedia* are also associated with various systemic diseases such as respiratory infection, rheumatoid arthritis and atherosclerosis.[5,6] Moreover, clinical *P. gingivalis* and *P. intermedia* strains have been shown to be resistant to multiple antibiotics including amoxicillin, metronidazole and so on.[7–9] And biofilm itself can be more resistant to antibiotics compared to their planktonic conditions.[10] Correspondingly, it is necessary to develop alternative therapeutic approaches for controlling infections associated with *P. gingivalis and P. intermedia*.

Previous studies have reported the antibacterial effect of direct current (DC) and electrical field *in vitro* against many planktonic microbes.[11,12] And there was a phenomenon that 2–7 days stimulation of low-intensity electric current substantially reduced the quantity of viable cells in biofilms, which was defined as "electricidal effect".[13] Nowadays, electricidal effect has been confirmed in various bacterial biofilm models.[14,15] However, the underlying mechanisms of DC in killing the planktonic bacteria or bacteria biofilms were lack of awareness and remained to be elucidated.[11,12,16–18] To our knowledge, there is no studies on the effect of DC on *P. intermedia*, and little is known about the changes and role of intracellular ROS when DC plays out in *P. gingivalis* and *P. intermedia*.

In this study, we aimed to study and compare the killing effect of DC on the planktonic state and biofilm state of *P. gingivalis* and *P. intermedia*. On the basis, we further examined the role of ROS in the killing effect, which may obtain a better understanding of the mechanism underlying the antibacterial effect of DC on *P. gingivalis* and *P. intermedia*.

Materials and methods

Bacteria strain, culture conditions and DC-stimulator apparatus

P. gingivalis ATCC 33277 and *P. intermedia* ATCC 25611 was routinely incubated at 37 °C in an anaerobic chamber, containing an atmosphere of 80% N_2 and 20% CO_2. And brain–heart infusion broth (BHIS) was supplemented with 5 μg/mL hemin and 1 μg/mL vitamin K1 as liquid media. The solid media of BHIS blood agar was obtained after supplementing the liquid media with 20 mg/mL brain–heart infusion agar (Oxoid Inc., Ogdensburg, NY, USA) and 50 μL/mL sterile defibrinated sheep blood. Gram staining and 16S rDNA identification were performed to confirm pure culture status of *P. gingivalis* and *P. intermedia* cells.

The DC-stimulator apparatus (KuShi Medical Technology Co.Ltd., Shanghai, China) is shown in Fig. 1A, which is composed of three key components: one 5 voltage lithium battery, two carbon electrodes and one DC output chip which can control constant current intensity ranging from 0 to 1000 μA in whole circuit. For the carbon electrodes, anode and cathode were kept a 3 mm's fixed distance and inserted into the bacteria solution, whose total effective contact area with liquid medium was 30 mm^2. And the schematic diagram of DC stimulation to two bacteria is shown in Fig. 1B.

The formation and harvest of *P. gingivalis* and *P. intermedia* biofilms

P. gingivalis or P. intermedia suspension were adjusted to optical density (OD) at 600 nm of about 0.1 (10^8 CFU/mL) and placed on 24-well plates purchased from Corning (Corning Inc., New York, NY, USA) for 3 days to form biofilms. And the biofilms in the bottom of each well were harvested by sonication (100 W) for 1 min and mechanical scrape with sterile pointed probe and suspended with liquid medium.

Colony form units (CFU) assay

CFU assay was performed to quantify viable cells of *P. gingivalis or P. intermedia* in planktonic or biofilm states. After the treated bacteria were transferred to liquid medium and serially diluted, the samples were plated on blood agar for further counting by using easyspiral Pro® (Interscience, St. Nom La Bretèche, France).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability staining

MTT assay was used to measure the metabolic activity of viable *P. gingivalis or P. intermedia* in biofilms. The treated bacteria biofilms were stained in 0.5 mg/mL MTT and incubated for 2 h at room temperature. Subsequently dimethylsulfoxide was added in each well for 15 min,

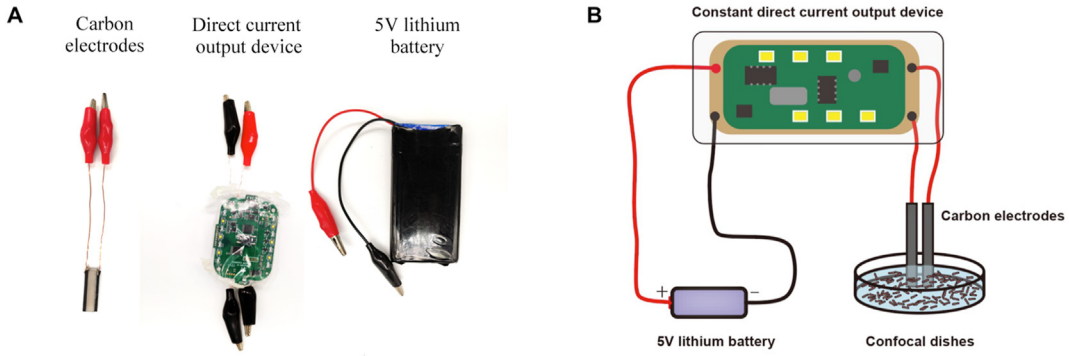

Figure 1 Direct current (DC) apparatus outputting constant DC ranging from 0 to 1000 μA (A) Three key components of DC apparatus (B) Schematic diagram of DC apparatus.

100 μL stained solution were measured in 96-well plates (Corning) at 490 nm.

Confocal laser scanning microscopy (CLSM) observation

The treated *P. gingivalis* and *P. intermedia* in planktonic state or biofilm state were stained with LIVE/DEAD® Baclight ™ bacterial viability kit (Invitrogen, Waltham, MA, USA) for 20 min. Then the stained bacteria were observed using CLSM (Leica Microsystems, Mannheim, Germany). Wavelengths of 488 nm and 520 nm were set as excitation and emission wavelengths, respectively. And the biofilms were scanned from the bottom to the top, corresponding Z-section was 0.3 μm apart.

Reactive oxygen species (ROS) assay

After collecting the treated *P. gingivalis* and *P. intermedia* in planktonic state or biofilms state, total intracellular ROS in bacteria were measured by GENMED oxidative stress-activated fluorescence assay kit (Genmed Scientifics Inc., Shanghai, China). 200 μL stained bacteria solution was measured in the form of relative fluorescence intensity (RFU) under the excitation wavelength of 490 nm and emission wavelength of 530 nm using the Enspire microplate reader (PerkinElmer, Rodgau, Germany).

The killing effect of DC with different intensities on *P. gingivalis* and *P. intermedia* in planktonic state and biofilm state

2 mL 10^8 CFU/mL planktonic bacterial solutions in 24-well plates were stimulated by 0, 10, 100 and 1000 μA DC for 1 h, 6 h and 12 h under anaerobic and light-resistant conditions. And the viable bacteria were calculated by CFU counting.

The *P. gingivalis* and *P. intermedia* biofilms were stimulated by 0, 10, 100 and 1000 μA DC for 24 h and 72 h under anaerobic and light-resistant conditions. The treated biofilms were stained by MTT assay.

ROS mediated the killing effect of 1000 μA DC on *P. gingivalis* and *P. intermedia* in planktonic state and biofilm state

The preformed biofilms were stimulated by 1000 μA DC alone or 1000 μA DC combined with *N*, *N*-dimethylthiourea (DMTU) (Sigma—Aldrich, St Louis, MO, USA) for 12 h. The concentration of DMTU for the planktonic state and biofilm state were 10 mM and 20 mM, respectively. Afterwards, the treated planktonic bacteria or biofilms were observed by ROS assay, CFU counting, CLSM, and the following genes expression assay.

Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The expression of intracellular ROS related genes in *P. gingivalis* and *P. intermedia* was analyzed by q-PCR. Total RNA of bacteria was extracted using the SV total RNA isolation kit (Promega, Fitchburg, WI, USA). cDNA was synthesized with a Reverse Transcription System (Toyobo, Osaka, Japan) and detected using Starlighter SYBR Green qPCR Mix (Foreverstar Biotech, Beijing, China) in 7500 Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). The q-PCR was carried out for 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min 16S rDNA was served as the inference control. The genes and corresponding primers sequences used for q-PCR were synthesized commercially (Ruibiotech, Beijing, China) and shown as follows: For *P. gingivalis*, Superoxide dismutase Fe—Mn (sod): forward (TAAGCACCTGAAGACCTA) and reverse (CGGCATTGTTGAAGATAC). Ferrous iron transport protein B (feoB): forward (ACTCAACGGTGTCATTCT) and reverse (ACGGTCAGCATCAGTATC). Hydrogen peroxide-inducible genes activator (OxyR): forward (CGAGGAAGAGCAGCAATC) and reverse (ACACGAGGCAGGAGATAG). 16S rDNA: forward (TGTAGATGACTGATGGTGAAA) and reverse (ACTGTTAGCAACTACCGATGT). For *P. intermedia*, feoB: forward (CTTCCGATGATGAGTTGTT) and reverse (CGCAATACCAATGAGATAGA). OxyR: forward (CATTGCTTTCGCGAACAACT) and reverse (TCAAGCGCAAGTTCGGGAAT). 16S rDNA: forward (TATCGCGTATCCAACCTTCC) and reverse (TCAATCCTGCACGCTACTTG).

Statistical analysis

The data were presented as mean ± SD, and the viable bacterial amount were converted to logarithms. Comparisons between two or multiple groups were made with the Student's t-test or One-way ANOVA, in which post-hoc analysis was performed with the Tukey test. Data were analyzed by GraphPad Prism 8.4 software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant at a p value of <0.05.

Results

The killing effect of DC with different intensities on planktonic and biofilm states of *P. gingivalis* and *P. intermedia*

For the planktonic *P. gingivalis*, compared to 0 μA group, the viable bacteria counting showed the number of viable bacteria was significantly decreased when treated with 10 μA DC for 12 h, 100 μA DC for 6 h and 12 h and 1000 μA DC for 1 h, 6 h and 12 h (Fig. 2A). For *P. gingivalis* biofilms, the MTT assay showed the viable bacteria metabolic activity was significantly decreased when treated with 10 μA DC or 100 μA DC for 72 h, 1000 μA DC for 24 h and 72 h compared to 0 μA group (Fig. 2B).

For the planktonic *P. intermedia*, compared to 0 μA group, the viable bacteria counting showed the number of viable bacteria was significantly decreased when treated with 10 μA DC for 12 h, 100 μA DC or 1000 μA DC for 1 h, 6 h and 12 h (Fig. 2C). For *P. intermedia* biofilms, the MTT assay showed the viable bacteria metabolic activity was significantly decreased when treated with 100 μA DC for 72 h or 1000 μA DC for 24 h and 72 h compared to 0 μA group (Fig. 2D).

Of these, under the same conditions of stimulation time, the amount of killing planktonic bacteria increased as the intensity of DC increased, and vice versa. The higher the DC intensity, the longer the stimulation time, the lower is the metabolic activity of biofilms.

The promotion of ROS mediated the killing effect of DC on planktonic and biofilm states of *P. gingivalis* and *P. intermedia*

For the planktonic *P. gingivalis* and *P. intermedia*, 1000 μA DC exponentially increased the intracellular ROS content (Fig. 3A and E) combined with significant upregulation of ROS scavenging genes, including sod in *P. gingivalis*, feoB and OxyR in *P. gingivalis* and *P. intermedia* (Fig. 3C and G). It also caused a viable bacteria reduction of 2.40 $\log_{10}$ CFU/mL and 2.62 $\log_{10}$ CFU/mL compared to control,

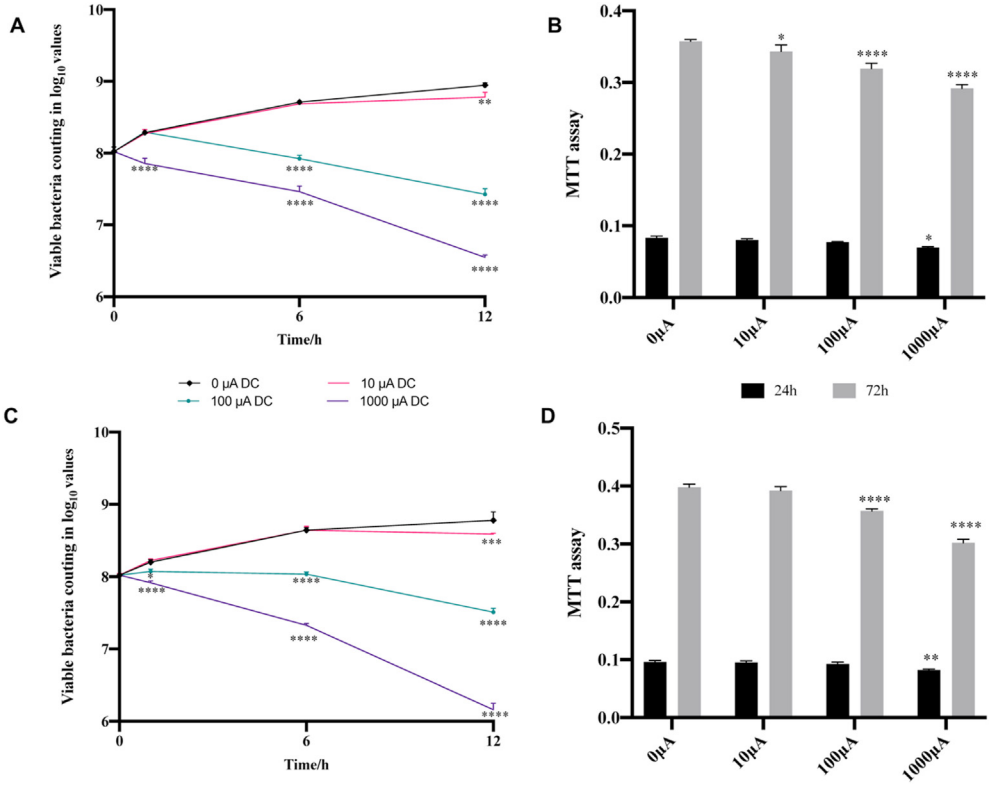

Figure 2 The killing effect of direct current (DC) with different intensities (0, 10, 100 and 1000 μA) on *P. gingivalis* and *P. intermedia* in planktonic state and biofilm state. Colony form units counting in the planktonic *P. gingivalis* (A) and *P. intermedia* (C) after exposed to DC for 1 h, 6 h and 12 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability staining in *P. gingivalis* biofilms (B) *and P. intermedia* biofilms (D) after treated by DC for 24 h and 72 h *, **, ***, **** denote significant difference of $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively.

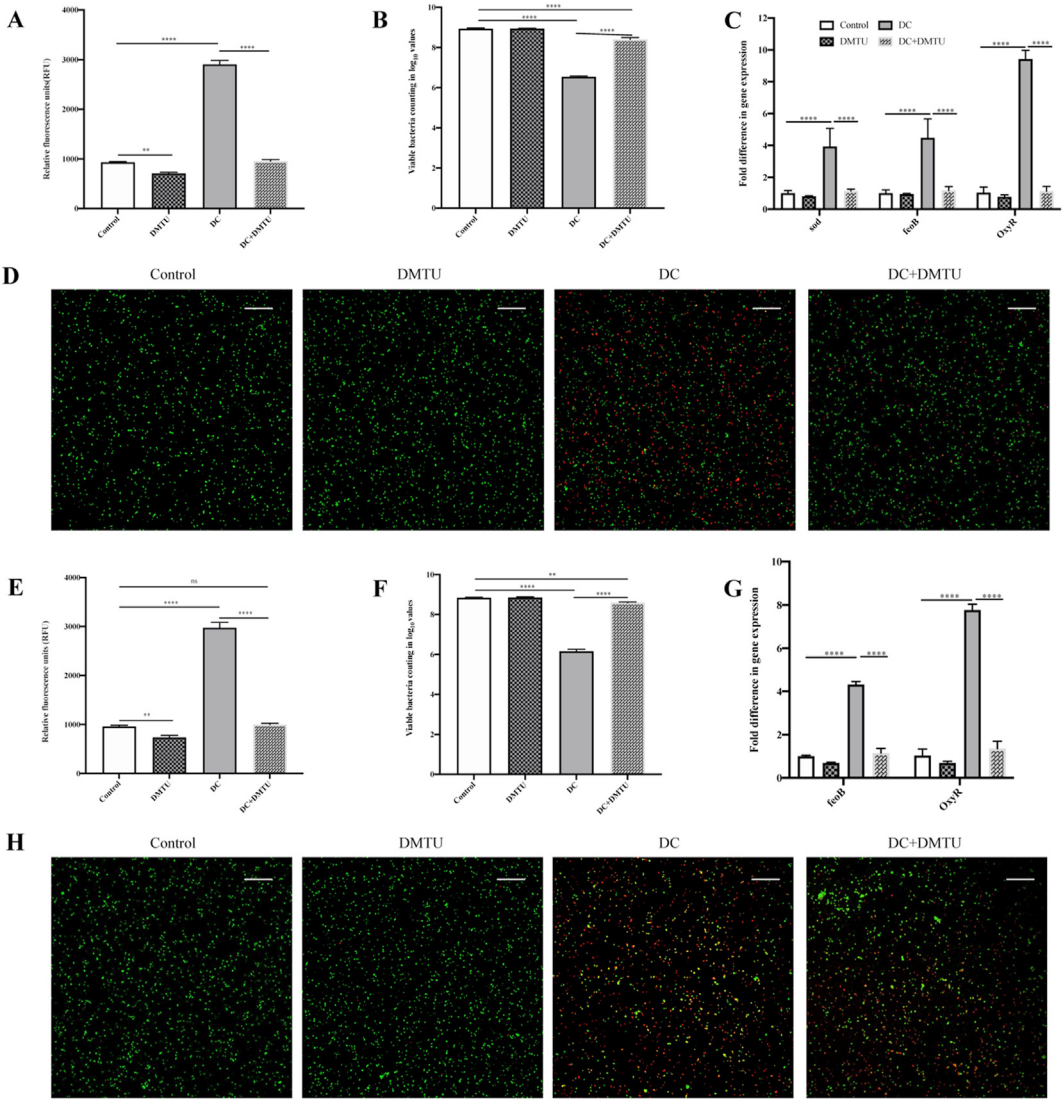

Figure 3 The killing effect of 1000 μA direct current (DC) on the planktonic *P. gingivalis* and *P. intermedia*. Bacterium were treated by 0 μA DC and 1000 μA DC combined with or without 10 mM N, *N*-dimethylthiourea (DMTU), and subject to analyses of ROS level in the form of relative fluorescence unit (RFU) (A in *P. gingivalis*, E in *P. intermedia*), CFU counting (B in *P. gingivalis*, F in *P. intermedia*), expression of ROS related genes (C in *P. gingivalis*, G in *P. intermedia*) and confocal laser scanning microscopy (CLSM), scale bar = 10 μm (D in *P. gingivalis*, H in *P. intermedia*),. *, **, ***, **** denoted significant difference of $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively.

respectively (Fig. 3B and F). Under the same size of fluorescence view, the DC-treatment group showed higher proportion of dead bacteria stained red than the control group (Fig. 3D and H). The addition of DMTU led to the reduction of intracellular ROS content whether alone or combined with the treatment of DC (Fig. 3C and G). When treated by 1000 μA DC with 10 mM DMTU, the killing viable bacteria amount shown large decreases in both planktonic *P. gingivalis* and planktonic *P. intermedia*, down to 0.53 $\log_{10}$ CFU/mL and 0.25 $\log_{10}$ CFU/mL, respectively. The fluorescence view further confirmed the results, DC combined DMTU group showed higher proportion of viable bacteria than the DC alone group and dead bacteria than the control group (Fig. 3D and H).

For *P. gingivalis* and *P. intermedia* biofilms, after exposed to 1000 μA DC, the level of ROS and the mRNA expression of ROS scavenging genes both showed significant increases (Fig. 4A and E), and the killing effect of DC reached 0.63 $\log_{10}$ CFU/mL and 0.98 $\log_{10}$ CFU/mL compared to the controls, respectively (Fig. 4B and F). 20 mM DMTU significantly decreased ROS contents in biofilms than untreated controls, but it had no significant effect on the viable bacteria amount and the mRNA expression level of sod, feoB and OxyR. Moreover, DMTU weakened the killing efficacy of 1000 μA DC whether on *P. gingivalis* or *P. intermedia*, which decreased to 0.20 $\log_{10}$ CFU/mL and 0.42 $\log_{10}$ CFU/mL, respectively (Fig. 4B and F). This observation was also confirmed by CLSM view.

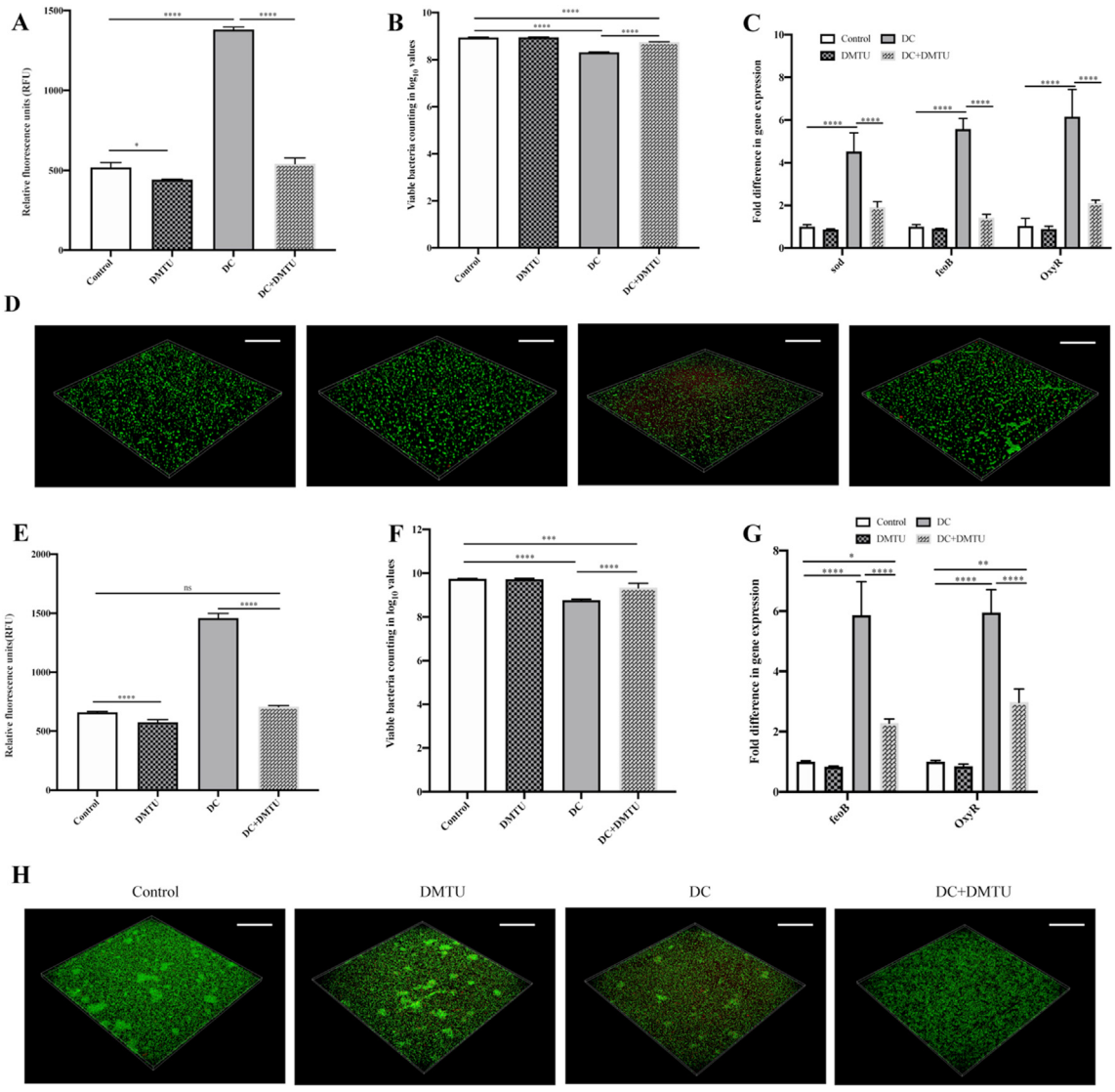

Figure 4 The killing effect of 1000 μA direct current (DC) on the *P. gingivalis* and *P. intermedia* biofilms. The biofilms were treated by 0 μA DC and 1000 μA DC combined with or without 20 mM DMTU, and subject to analyses of ROS level in the form of RFU (A in *P. gingivalis*, E in *P. intermedia*), CFU counting (B in *P. gingivalis*, F in *P. intermedia*), expression of ROS related genes (C in *P. gingivalis*, G in *P. intermedia*) and CLSM, scale bar = 50 μm (D in *P. gingivalis*, H in *P. intermedia*). *, **, ***, **** denoted significant difference of $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively.

Whether for *P. gingivalis* biofilms or *P. intermedia* biofilms, DC combined DMTU group showed higher proportion of viable bacteria than the DC alone group (Fig. 4D and H).

Notably, regardless of *P. gingivalis* or *P. intermedia*, when combined with 1000 μA DC, 10 mM DMTU applied in planktonic state and 20 mM DMTU applied in the biofilm state were able to significantly downregulate the expression of ROS scavenging genes compared with the DC alone group, to the level close to the untreated controls (Fig. 4C and G).

Comparisons of the killing values of 1000 μA DC on different bacteria in different states

Whether for *P. gingivalis* or for *P. intermedia*, compared to the planktonic state, the killing effect of 1000 μA DC on biofilm state was weaker significantly (Fig. 5A). Whether in planktonic state or in biofilm state, compared to *P. gingivalis*, the killing effects of 1000 μA DC on *P. intermedia* significantly was stronger (Fig. 5A).

Discussion

The present study firstly showed that constant DC using carbon electrodes had killing effect on planktonic and biofilm states of *P. intermedia* and planktonic *P. gingivalis* ranging from 10 μA to 1000 μA. In this study, we found the 1000 μA DC showed the highest killing activity in *P. gingivalis* and *P. intermedia*, and it was also able to lead the overproduction of ROS in both planktonic state and biofilm state. After the addition of ROS scavenger (ie, DMTU), the level of ROS decreased, and the killing effect of DC showed

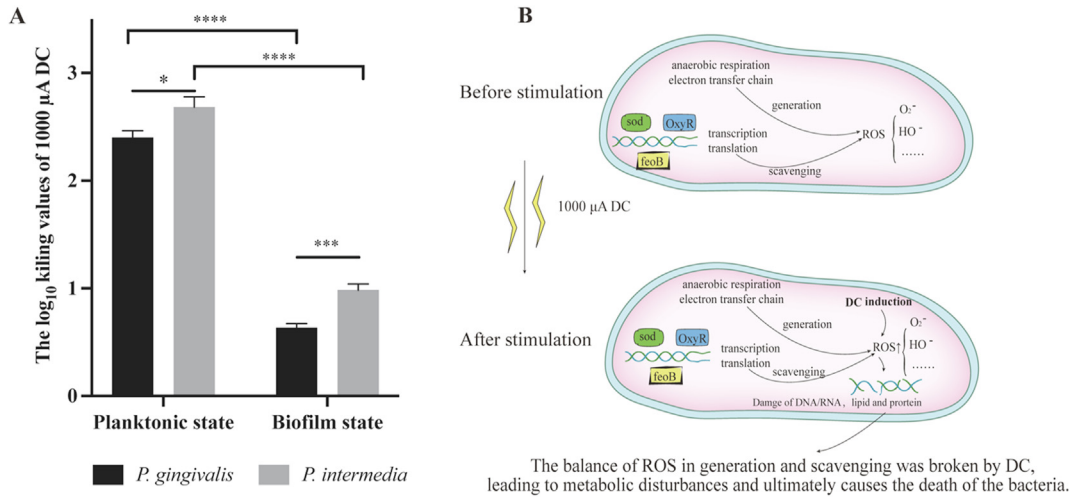

Figure 5 Comparisons of the killing values of 1000 μA direct current (DC) on different bacteria in planktonic state and biofilm state (A) and the mechanism diagram of possible DC-induced bacteria death based on the experimental results (B). *, **, ***, **** denoted significant difference of $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively.

partial reduction. These results may suggest oxidative stress caused by DC may mediate the lethal effects on *P. gingivalis* and *P. intermedia*.

In planktonic *P. gingivalis* and *P. intermedia*, we found the intensity dependence and time dependence in the killing effect of DC, which was consistent with previous studies on other microorganisms.[14,19] Besides, 1000 μA DC for 12 h was confirmed the existence of electricidal effect in *P. gingivalis* and *P. intermedia* biofilms, and it resulted in more logarithmic killing (> 2.0 $\log_{10}$ CFU/mL bacteria kill) in planktonic bacteria than that (< 1.0 $\log_{10}$ CFU/mL bacteria kill) of bacteria biofilms whether for *P. gingivalis* or *P. intermedia*, which were in accordance with the data in prior research on other bacteria species. The mature biofilm is composed of bacteria and extracellular matrix. The existence of extracellular matrix offers protection to bacteria inside, which can prevent the penetration of antibiotics, quench highly reactive oxygen complexes and increased the adhesion force of bacteria.[20,21] And we suggest the components of extracellular matrix in bacteria biofilms may protect it from the killing effect of DC. Besides, the underlying bacteria in biofilms either grow slowly or are in dormancy,[22] which may result in low sensitivity and high resistance to external stimulation (e.g., DC).

Normally, obligately anaerobic respiration occurs in *P. gingivalis* and *P. intermedia*, and ROS were mainly produced in the respiratory electron transport chain and the cytoplasm. And bacteria could produce corresponding enzymes to remove excess ROS by redox metabolism and establish the intracellular redox balance.[23] If the redox balance is broken, the overproduced ROS can damage important biological macromolecules such as lipids, proteins, RNA and DNA, thus leading to widespread cellular damage and cell death.[23,24] It has been assumed that DC could induce ROS production and result in bacterial death in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms.[25,26] Similarly, in addition to planktonic bacteria, we found increased intracellular ROS in bacteria biofilms following exposure to 1000 μA DC for 12 h. To further confirm the role of ROS in DC-associated cell death to *P. gingivalis* and *P. intermedia*, we examined the gene expression of sod, feoB and OxyR, which play an important role in balancing the overproduction of ROS.[27,28] We found that DC treatment markedly upregulated sod, feoB and OxyR in *P. gingivalis*, OxyR and feoB in *P. intermedia* biofilms. Importantly, as a ROS scavenger, DMTU could partially weaken the killing efficacy of DC on *P. gingivalis* and *P. intermedia*. Besides, it reversed the upregulation of ROS related genes whether in planktonic state or biofilm state. Hereby, oxidation stress promoted by DC reveals a potential mechanism underlying the killing effect (The possible mechanism diagram of DC-induced bacteria death is shown in Fig. 5B).

Interestingly, whether in planktonic state or biofilm state, the killing effect of DC treatment on *P. intermedia* was stronger than *P. gingivalis*. The possible reasons for this will be discussed below. In the ROS balance system of bacteria, there are two key redox regulators.[29] One redox regulator is the OxyR system sensitive to hydrogen peroxide, which can control the corresponding genes expression of glutathione reductase, NADPH-dependent hydroperoxide reductase and so on. These enzymes are mainly used to remove excessive intracellular hydrogen peroxide in bacteria.[30,31] Another transcriptional regulator is the secondary SoxRS system, which can activate the genes expression of sod, feoB and glucose-6-phosphate dehydrogenase in response to excessive superoxide anion. In the SoxRS system of *P. gingivalis* ATCC 33277, sod gene encodes superoxide dismutase Fe–Mn, which is the first line to against ROS and plays the crucial role in removing superoxide anion.[32,33] However, in the encoding genes of *P. intermedia* ATCC 25611, there are not sod gene,[34] and this means the key ROS scavenging enzymes may be less in *P. intermedia*, and the ability of *P. intermedia* to remove excessive ROS may be weaker than *P. gingivalis*. From this perspective, it could explain why the sensitivity of *P. intermedia* to DC is higher compared to *P. gingivalis*.

In conclusion, this study has demonstrated that DC can kill *P. gingivalis* and *P. intermedia* whether in planktonic state or biofilm state. In contrast to *P. gingivalis*, *P. intermedia* were more sensitive to DC. And the induction of ROS production may critically account for the killing effect of DC against such two periodontal pathogens. The maximum harmless levels of DC that human body can perceive and sustain on hand are around 1000 μA and 6000 μA, respectively.[35] Considering the future clinical application, the DC intensity range in our study was safe in humans. However, it still needs more animal studies and clinical trials to further prove the antibacterial effect of low-intensity DC. Hence, our findings suggest that low-intensity DC is a promising approach for controlling *P. gingivalis* or *P. intermedia* biofilms. In the future, development of clinically suitable DC devices may indicate to effective management of dental infections.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

The authors are grateful to Lilian Li (KuShi Medical Technology Co., Ltd., Shanghai, China) for technical assistance. The work was supported partly by the National Natural Science Foundation of China (No. 81700975) to Peng Li.

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