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Direct current exerts electricidal and bioelectric effects on *Porphyromonas gingivalis* biofilms partially via promoting oxidative stress and antibiotic transport

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Low electric current can inhibit certain microbial biofilms and enhance the efficacy of antimicrobials against them. This study investigated the electricidal and bioelectric effects of direct current (DC) against *Porphyromonas gingivalis* biofilms as well as the underlying mechanisms. Here, we firstly showed that DC significantly suppressed biofilm formation of *P. gingivalis* in time- and intensity-dependent manners, and markedly inhibited preformed *P. gingivalis* biofilms. Moreover, DC enhanced the killing efficacy of metronidazole (MTZ) and amoxicillin with clavulanate potassium (AMC) against the biofilms. Notably, DC-treated biofilms displayed upregulated intracellular ROS and expression of ROS related genes (sod, feoB, and oxyR) as well as porin gene. Interestingly, DC-induced killing of biofilms was partially reversed by ROS scavenger N-dimethylthiourea (DMTU), and the synergistic effect of DC with MTZ/AMC was weakened by small interfering RNA of porin gene (si-Porin). In conclusion, DC can exert electricidal and bioelectric effects against *P. gingivalis* biofilms partially via promotion of oxidative stress and antibiotic transport, which offers a promising approach for effective management of periodontitis.

Keywords: direct current, *Porphyromonas gingivalis*, biofilms, electricidal effect, bioelectric effect

Introduction

Periodontitis is the major cause of tooth loss in adults worldwide with huge socio-economic burden (Kassebaum *et al.*, 2014; Jin *et al.*, 2016). It is induced by plaque biofilms colonizing on the surfaces of oral cavity and periodontal niches (Sedlacek and Walker, 2007; Maisonneuve and Gerdes, 2014). Biofilms can be up to 1,000 to 1,500 times more resistant to antibiotics compared to their planktonic counterparts, making biofilm-related infections extremely difficult to tackle (Gu *et al.*, 2019). *Porphyromonas gingivalis*, as a keystone periodontal pathogen, plays a key role in the development and progression of periodontitis (Hajishengallis *et al.*, 2012). It has been demonstrated that adjunctive use of antibiotics such as metronidazole (MTZ) and amoxicillin can reduce the levels of periodontal pathogens, and it offers additional benefits to periodontal treatment outcomes (Belibasakis and Thurnheer, 2014; Kassebaum *et al.*, 2014). Nevertheless, our recent study has discovered that *P. gingivalis* can form persisters to withstand lethal treatment of MTZ, probably responsible for the persistence of periodontal infections (Li *et al.*, 2018). Moreover, the efficacy of comprehensive periodontal therapy is compromised in patients with persistent infection of *P. gingivalis* and other pathogens (Colombo *et al.*, 2012). Therefore, it is imperative to develop novel therapeutic approaches for effective control of *P. gingivalis* associated biofilm infections.

Since the antibacterial effects of electric current was observed during wound healing, various studies have demonstrated electric current can substantially inhibit microbial biofilms, which is defined as "electricidal effect" (del Pozo *et al.*, 2008, 2009b). Besides, electric current is capable of enhancing the activity of antimicrobials against biofilms, which is termed the "bioelectric effect" (Blenkinsopp *et al.*, 1992; Zhang *et al.*, 2014; Lasserre *et al.*, 2016). Multiple hypotheses have been proposed to account for electricidal effect, including disruption of bacterial membrane integrity as well as generation of chlorine, hypochlorous acid, oxygen and reactive oxygen species (ROS). And it was proposed that bioelectric effect may result from bacterial autolysis, increased delivery of oxygen, disruption of biofilm matrix charges, and increased penetration of antimicrobial agents due to contraction and expansion of biofilms. However, the exact mechanisms underlying electric current on biofilms remain to be elucidated (Sandvik *et al.*, 2013; Brinkman *et al.*, 2016).

The discovery of electricidal and bioelectric effects indeed provides a promising approach to control biofilms with increased antimicrobial resistance, including oral/periodontal plaque biofilms (del Pozo *et al.*, 2008; Freebairn *et al.*, 2013; Hari *et al.*, 2018). Currently, the effects of direct current (DC) on the adhesion and formation of *P. gingivalis* biofilms are not clear, and it remains unknown whether DC has synergistic effect with MTZ and amoxicillin-clavulanate potassium (AMC) on *P. gingivalis* biofilms. Hence, the aim of this study

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was to investigate the electricidal and bioelectric effects of DC on *P. gingivalis* biofilms and the underlying mechanisms.

Materials and Methods

Electric apparatus

The laboratory electric apparatus (Shanghai Kushi medical technology Co.) is shown in Fig. 1A. It contained three parts: one 5-V lithium battery supply, one DC power output delivering 10 to 1,000 μA constant DC and two sterile carbon electrodes. Besides, anode and cathode were designed small enough to be easily placed in 24-well polystyrene plates (Corning) and confocal dishes (NEST) (20 mm diameter).

Bacteria strain, culture conditions, and biofilm formation

Porphyromonas gingivalis (ATCC 33277) was cultured on brain-heart infusion (BHI, OXIFID) with 5% (v/v) sheep blood

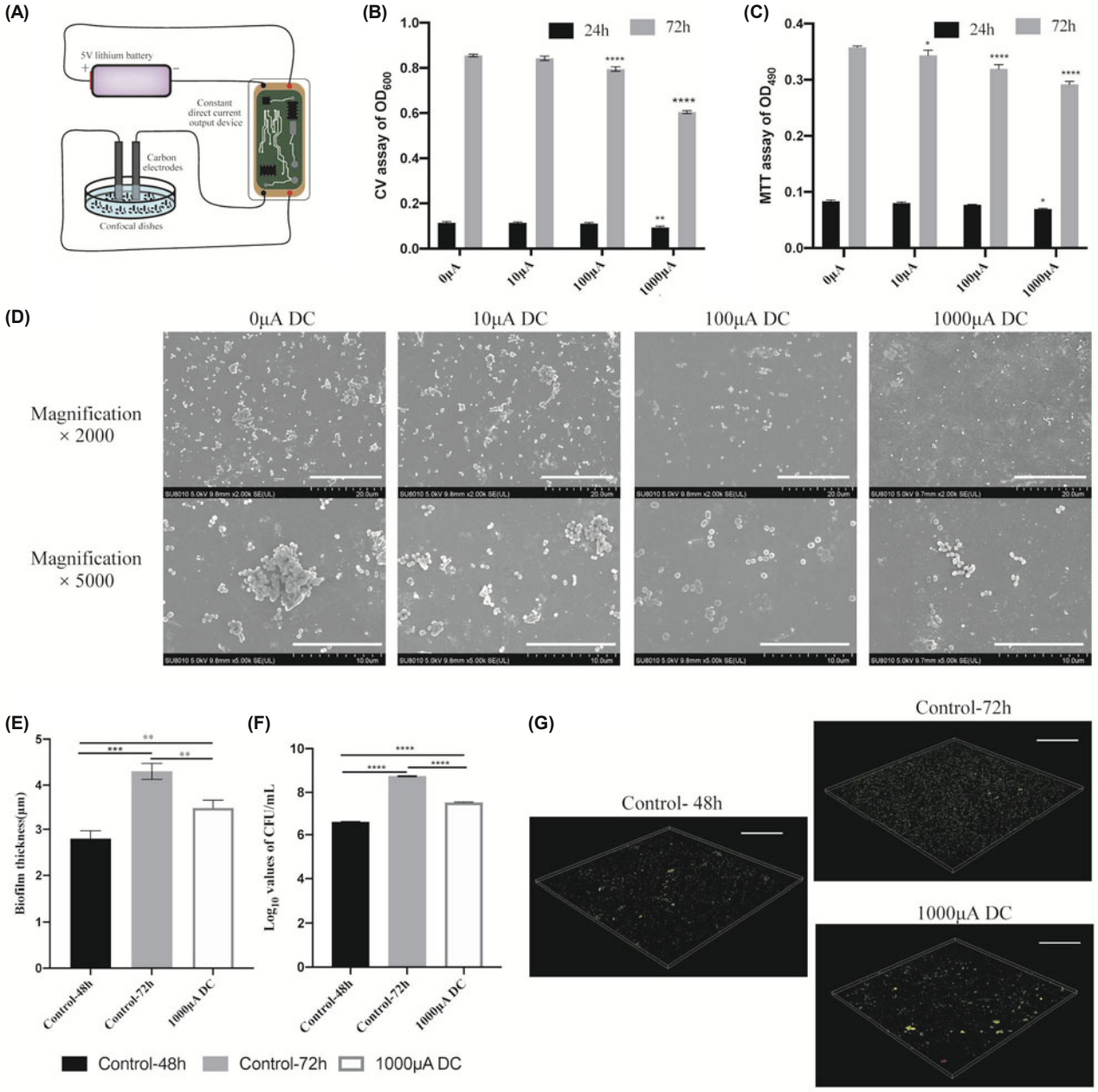

Fig. 1. The inhibitory effect of DC on *P. gingivalis* biofilms formation. Crystal violet staining (B) and MTT assay (C) showing intensity- and time-dependent inhibition of *P. gingivalis* biofilm formation by DC (0, 10, 100, and 1,000 μA) treatments for 24 and 72 h. (D) Scanning electron microscopy (SEM) images of *P. gingivalis* biofilms treated with or without 1,000 μA DC for 24 h on silicon wafer at magnifications of × 2,000 (Scale bar = 20 μm) and × 5,000 (Scale bar = 10 μm). Biofilm thickness (E), colony form units (CFU) counting (F) and CLSM images (G) (Scale bar = 50 μm) in the 48 h-*P. gingivalis* biofilms treated with or without 1,000 μA DC for 24 h. Live cells are stained green and dead cells are red-colored in the CLSM images. *, **, ***, **** denote significant difference of $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively.

agar plates anaerobically (37°C, 80% N_2 and 20% CO_2). Planktonic *P. gingivalis* was grown in BHI broth supplemented with 5 mg/L hemin (Sigma) and 1 mg/L vitamin K1 (Hopebio). The bacteria were incubated to logarithmic phase and adjusted to 10^8 colony-forming units (CFU)/ml.

To form *P. gingivalis* biofilms, 2 ml standardized bacteria suspension was added in each well of 24-well polystyrene plates (Corning) unless otherwise specified and incubated for 72 h.

Antibiotic susceptibility

The susceptibility of planktonic *P. gingivalis* to MTZ and AMC (Sigma) was determined using broth microdilution method. Serial 2-fold dilutions of antibiotics were prepared in 96-well plates and bacteria suspensions were inoculated to a final concentration of 10^6 CFU/ml. After incubation at 37°C for 48 h, the minimal inhibitory concentration (MIC) was defined as the lowest concentration of each antibiotic that prevented visible bacterial growth.

Scanning electron microscopy (SEM) observation

Porphyromonas gingivalis biofilms were formed on silicon wafers (Zhejiang Lijing Silicon Material Co.) as aforementioned and fixed with 4% (v/v) glutaraldehyde for 30 min followed by dehydration with serial ethanol at 37°C. Subsequently, the samples were coated with platinum and observed using a SEM instrument (Hitachi).

Confocal laser scanning microscopy (CLSM) observation

Porphyromonas gingivalis biofilms were formed on glass-bottomed confocal peri dishes (NEST) (20 mm diameter) as aforementioned and stained with the LIVE/DEAD® Baclight™ bacterial viability kit (Invitrogen) for 20 min. Next, the samples were observed using a CLSM system (Leica Microsystems) and the maximum biofilm thickness was measured by converting the z-stack (at 0.3 μm intervals) images.

Quantification of *P. gingivalis* biofilms

Porphyromonas gingivalis biofilms were stained with 200 μl of 0.1% (w/v) crystal violet (CV) (Solaribio) for 10 min. Next, the unbound CV solution was gently removed. The biofilms were washed once with PBS and dried for 30 min at 37°C. 100 μl 95% ethanol (v/v) was then added and OD values at 600 nm were measured.

The metabolic activity of *P. gingivalis* biofilms was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining. The biofilms were treated with 0.5 g/L MTT and incubated for 2 h. Subsequently, dimethylsulfoxide (DMSO) was added to each well and shaken for 15 min. OD values at 490 nm were measured.

CFU counting was performed to quantify viable cells in *P. gingivalis* biofilms. *P. gingivalis* biofilms were harvested by sonication (100 W, 1 min) and mechanical scrape. The samples were serially 10-fold diluted and plated on blood agar plates for counting CFU using easyspiral Pro® (Interscience).

Effect of DC on *P. gingivalis* biofilm formation

To investigate the effect of DC on biofilm formation of *P. gingivalis*, 2 ml bacterial suspension (10^8 CFU/ml) was inoculated in each well of 24-well plates and stimulated by 10, 100 and 1,000 μA DC for 24 or 72 h under anaerobic and light-resistant condition. Afterwards, the DC-treated biofilms and untreated controls were analyzed by CV staining, MTT assay and SEM.

The *P. gingivalis* biofilms formed on confocal dishes for 48 h were treated with or without 1,000 μA DC for 24 h. The biofilms thickness was calculated by CLSM and CFU counting were recorded.

Combined effect of DC with MTZ or AMC on preformed *P. gingivalis* biofilms

The performed *P. gingivalis* biofilms for 72 h were stimulated by 1,000 μA DC alone or in combination with 16 μg/ml MTZ or 16 μg/ml AMC for 12 h. The treated biofilms were analyzed by CLSM, CFU counting and the following gene expression assay.

ROS assay

The performed *P. gingivalis* biofilms for 72 h were stimulated by 0 μA DC, 1,000 μA DC alone or in combination with 10 mM N, N-dimethylthiourea (DMTU) for 12 h. The treated biofilms are collected to measure the intracellular ROS contents by the GENMED oxidative stress-activated fluorescence assay kit (Genmed scientifics). Two hundred μl stained biofilm solution in black 96-well plates (Corning) was measured in the form of relative fluorescence intensity under the excitation wavelength of 490 nm and emission wavelength of 530 nm by microplate reader (Enspire).

Silencing of porin gene

The siRNA of porin gene (si-Porin) was synthesized commercially (Ruibiotech Ltd.) with the following sequences: forward:

Table 1. List of genes and corresponding primers sequence

Gene symbol	Genes discription	Abbreviation in the study	Forward sequence of primer (5′-3′)	Reverse sequence of primer (5′-3′)
sod	Superoxide dismutase Fe-Mn	sod	TAAGCACCTGAAGACCTA	CGGCATTGTTGAAGATAC
feoB	Ferrous iron transport protein B	feoB	ACTCAACGGTGTCATTCT	ACGGTCAGCATCAGTATC
PGN_RS01750	Hydrogen peroxide-inducible genes activator	oxyR	CGAGGAAGAGCAGCAATC	ACACGAGGCAGGAGATAG
PGN_RS04100	porin	porin gene	CACTTCCTTCCGCACTTG	GATGGTAGTTGGAGACAGTTG
PGN_RS03415	Efflux RND transporter permease subunit	RND-TPS	GACGATAAGACAGAAGGAGAT	TGATTGGCAGACGAAGAA
PGN_RS07350	Efflux RND transporter periplasmic adaptor subunit	RND-TPA	CAGGATTCGCTCTACAGG	CACGTTCAGGTCTTCCAC
PGN_RS01015	16S ribosomal RNA	16S	TGTAGATGACTGATGGTGAAA	ACTGTTAGCAACTACCGATGT

5′-GCAGAUGGAGGAACAGUUATT-3′, reverse: 5′-UAA CUGUUCCUCCAUCUGCTT-3′, and it was introduced into *P. gingivalis* cells in biofilms by liposome-mediated transfection. The preformed biofilms cultured for 72 h were transfected with 10 nM si-Porin for 12 h. Subsequently, the biofilms were treated by 1,000 μA DC alone or in combination with 16 μg/ml MTZ or 16 μg/ml AMC. The treated biofilms were subject to CFU counting, CLSM and the following gene expression assay.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

The expression of intracellular ROS related genes and porin gene in *P. gingivalis* biofilms was analyzed by qRT-PCR. Total RNA was extracted from bacterial samples using the SV total RNA isolation kit (Promega). cDNA was synthesized using the Reverse Transcription System (Toyobo) and qRT-PCR was performed using Starlighter SYBR Green qPCR Mix (Foreverstar biotech co.) in 7500 Real-Time PCR System (Applied Biosystems). 16S rRNA gene was used as the internal reference. The primers used for qRT-PCR were synthesized commercially (Ruibiotech) and listed as follows: superoxide dismutase Fe-Mn (sod), ferrous iron transport protein B (feoB), hydrogen peroxide-inducible genes activator (oxyR), porin, efflux RND transporter periplasmic adaptor subunit (RND-TPS) and efflux RND transporter periplasmic adaptor subunit (RND-TPA). The primer sequences are shown in Table 1. The relative expression levels of selected genes were determined by the comparative Ct method.

Statistical analysis

The data were presented as mean ± SD and analyzed by *t*-test and one-way ANOVA using GraphPad Prism 8.4 software (GraphPad Software Inc). A p-value < 0.05 was considered statistically significant.

Results

DC inhibited biofilm formation of *P. gingivalis*

CV staining showed that biofilm formation of *P. gingivalis* was significantly inhibited when treated with 100 μA DC for

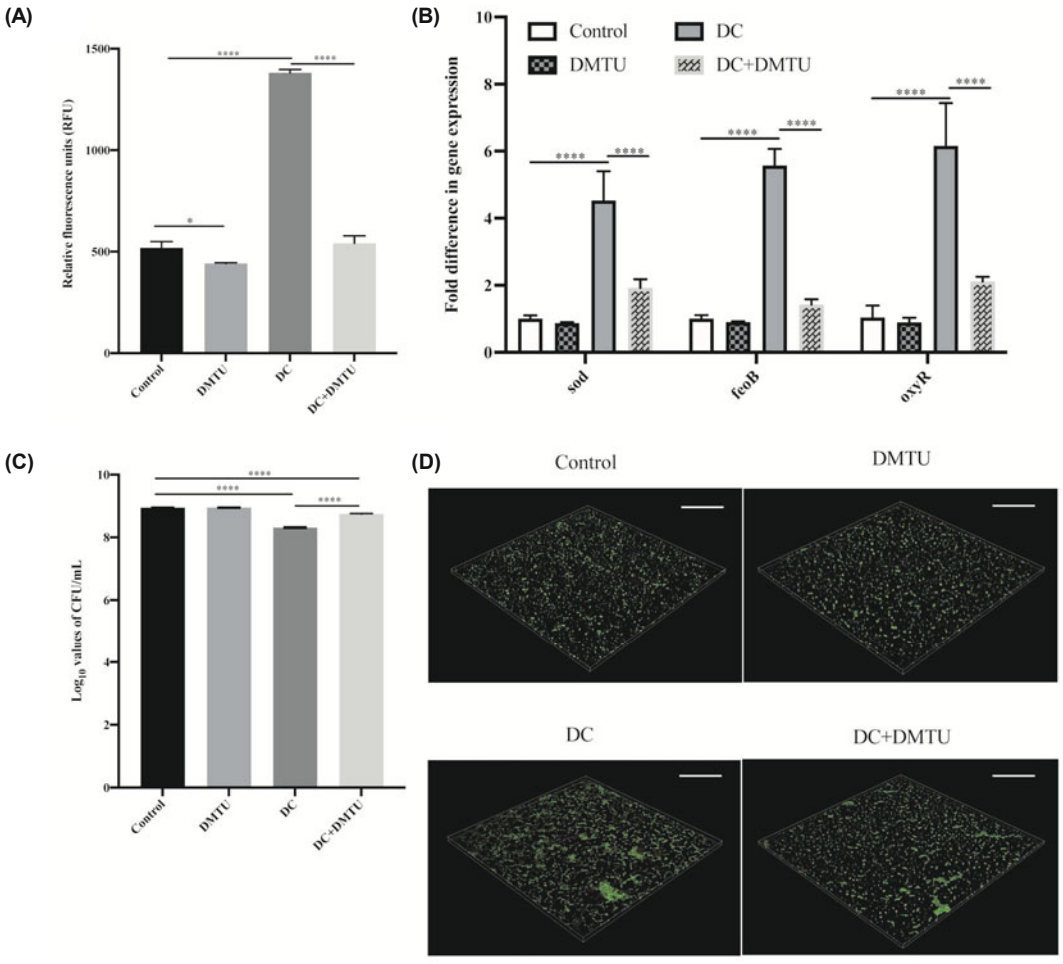

Fig. 2. The role of ROS induced by DC on *P. gingivalis* biofilms. The biofilms were treated by 0 μA DC (Control), 10 mM N,N'-Dimethylthiourea (DMTU), 1,000 μA DC combined with or without 10 mM DMTU. (A) ROS level in the form of relative fluorescence units (RFU). The expression of ROS related genes (B), CFU counting (C) and CLSM images (D) (Scale bar = 50 μm). *, **, ***, **** denote significant difference of $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively.

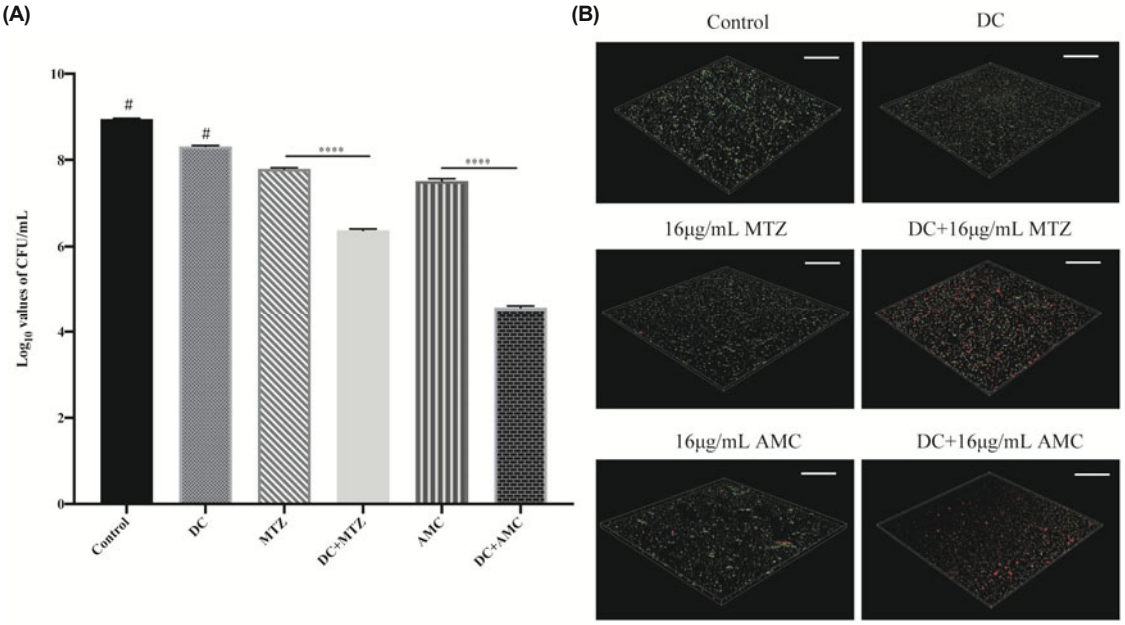

Fig. 3. The synergistic effect of DC with MTZ/AMC on *P. gingivalis* biofilms. The biofilms were treated by 0 μA DC, 16 μg/ml MTZ, 16 μg/ml AMC, and 1,000 μA DC combined with or without 16 μg/ml MTZ or 16 μg/ml AMC for 12 h. And they were subjected to analyses of CFU counting (A), CLSM images (B) (Scale bar = 50 μm) and the expression of antibiotic transport related genes including RND efflux genes (C) and porin gene (D). *, **, ***, **** denote significant difference of $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively. # denotes significant difference of p-value < 0.0001 compared with all other groups.

72 h or 1,000 μA DC for 24 and 72 h (Fig. 1B). After 72 h DC treatment, the metabolic activity of adherent *P. gingivalis* decreased gradually with the increase of DC intensity from 10 to 1,000 μA (Fig. 1C). However, when the stimulation time shortened to 24 h, the metabolic activity of *P. gingivalis* biofilms was only inhibited by DC treatment at 1,000 μA (Fig. 1C). Herein, 1,000 μA DC was selected for subsequent assays. SEM showed the DC treatment at 1,000 μA for 24 h markedly suppressed the bacteria density of sessile *P. gingivalis* with reference to the untreated controls (Fig. 1D).

Moreover, to investigate whether DC can inhibit biofilm maturation, 48 h-old *P. gingivalis* biofilms were prepared and treated with DC at 1,000 μA for 24 h. CLSM analysis showed that the thickness and density of *P. gingivalis* biofilms further increased after another 24 h culture and partially inhibited by the DC treatment (Fig. 1E and G). As evidenced by CFU

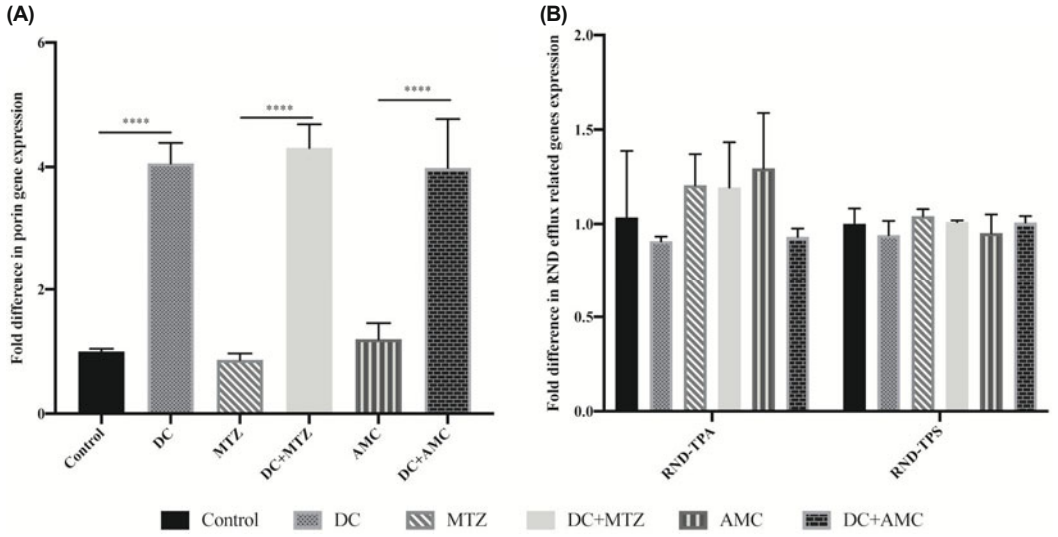

Fig. 4. The expression of antibiotic transport related genes including RND efflux genes and porin gene of *P. gingivalis* biofilms. The biofilms were treated by 0 μA DC, 16 μg/ml MTZ, 16 μg/ml AMC, and 1,000 μA DC combined with or without 16 μg/ml MTZ or 16 μg/ml AMC for 12 h. And the expression of RND-TPS gene, RND-TPA gene (A) and porin gene (B) were measured. *, **, ***, **** denote significant difference of $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively.

counting, the number of viable cells in the DC treated biofilms was significantly lower than the 72 h-controls (Fig. 1F).

ROS was involved in the electricidal effect of DC on *P. gingivalis* biofilms

The level of ROS in DC-treated *P. gingivalis* biofilms increased significantly compared to untreated controls (Fig. 2A). DMTU could slightly reduce the ROS level in the untreated controls and markedly inhibit the augmented ROS production in the DC-treated biofilms. No significant difference was found between the biofilms treated by DC combined with DMTU and untreated controls (Fig. 2A).

Notably, DC treatment led to significant upregulation of sod, feoB and oxyR in *P. gingivalis* biofilms, which were abrogated by DMTU (Fig. 2B). And there was no significant difference between the biofilms treated by DC combined with DMTU and untreated controls (Fig. 2B).

The number of viable cells in *P. gingivalis* biofilms was greatly reduced by DC treatment and not affected by DMTU (Fig. 2C). Compared to DC treatment alone, there was a significant increase of 0.43 $\log_{10}$ CFU/ml in the biofilms treated by DC and DMTU, while the CFU was still lower than that of untreated controls (Fig. 2C). LIVE/DEAD staining and CLSM further confirmed these results (Fig. 2D).

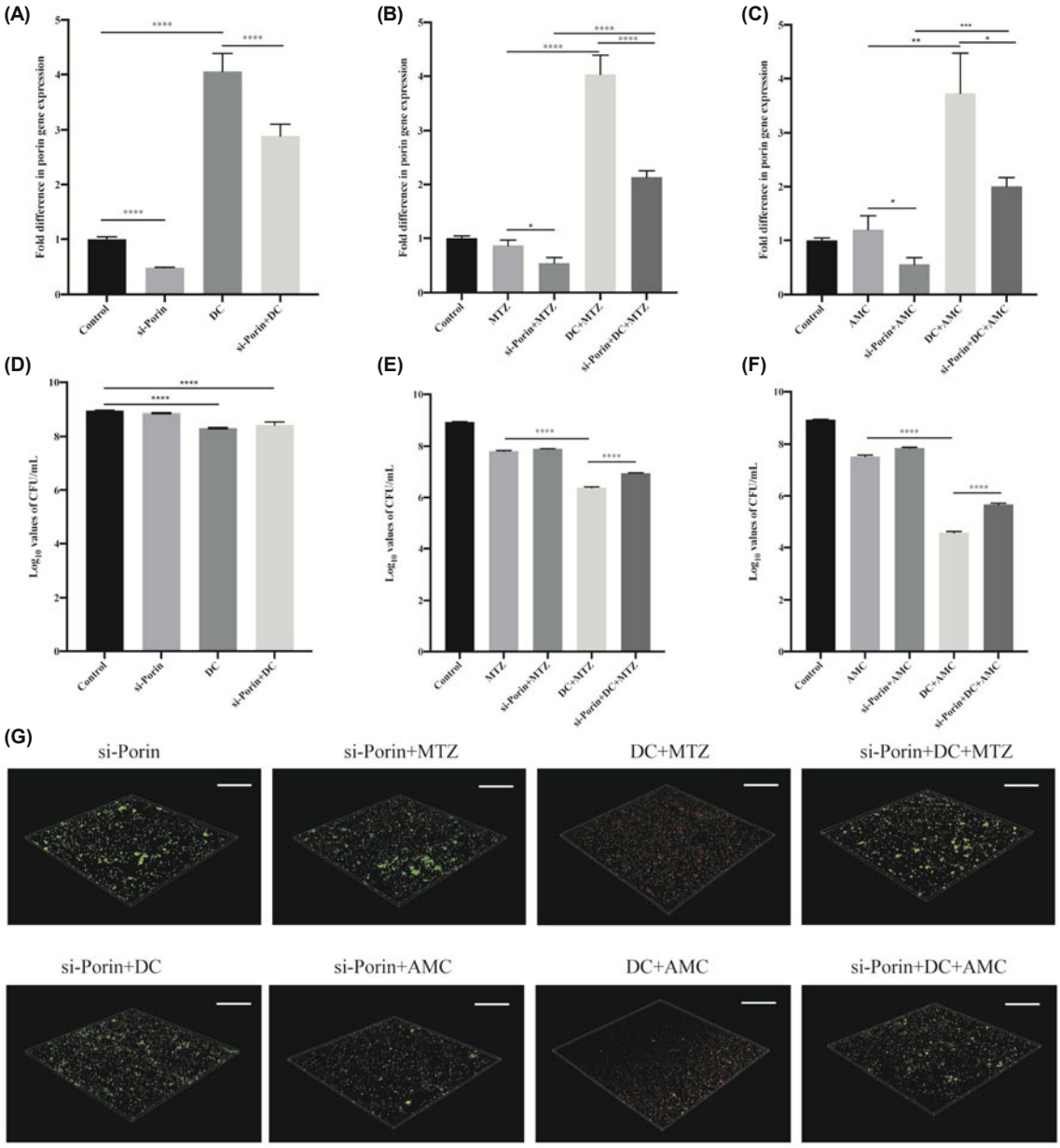

Fig. 5. The effect of small interfering RNA of porin gene (si-Porin) on *P. gingivalis* biofilms treated by DC (1,000 μA) combined with or without 16 μg/ml MTZ/AMC for 12 h. The gene silencing efficacy of si-Porin (A), the relative expression of porin gene after treated with 1,000 μA DC, si-Porin and MTZ/AMC (B and C), CFU counting (D, E, and F) and CLSM images (G) of the biofilms are shown. Scale bar = 50 μm. *, **, ***, **** denote significant difference of $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively.

DC had synergistic effect with MTZ or AMC against *P. gingivalis* biofilms

The MICs of MTZ and AMC against planktonic *P. gingivalis* were 0.063 and 0.03 μg/ml, respectively. The viable bacteria cells in untreated biofilms reached a mean of 8.95 $\log_{10}$ CFU/ml and treatment with 1,000 μA DC, 16 μg/ml MTZ and 16 μg/ml AMC resulted in a $\log_{10}$ reduction of 0.63, 1.14, and 1.42, respectively (Fig. 4A). Notably, there was 1.43 $\log_{10}$ reduction of CFU in the biofilms treated with DC and MTZ compared to those treated with MTZ alone (Fig. 3A). Similarly, 2.95 $\log_{10}$ reduction of CFU was observed in the biofilms treated with DC and AMC in comparison to AMC alone (Fig. 3A). CLSM images showed that there were more dead cells (stained in red) in *P. gingivalis* biofilms after treatment by 1,000 μA DC together with MTZ or AMC compared to MTZ or AMC alone (Fig. 3B).

The role of porin in the bioelectric effect of DC on *P. gingivalis* biofilms

To explore the role of antibiotic transport related genes in the bioelectric effect of DC, this study examined the genes expression of porin, RND-TPS and RND-TPA in *P. gingivalis* biofilms. The mRNA expression of porin gene was not altered by MTZ or AMC treatment alone (Fig. 4A). However, the DC treatment significantly enhanced the expression of porin in the biofilms (Fig. 4A). The expression of RND-TPS and RND-TPA showed no significant difference among the biofilms treated by DC, MTZ, AMC, or DC combined with MTZ/AMC (Fig. 4B).

Next, 10 nM si-Porin was used to treat the *P. gingivalis* biofilms and its silencing efficiency reached 51% (Fig. 5A). Moreover, si-Porin treatment significantly inhibited the increased expression of porin induced by DC as well as DC combined with MTZ/AMC (Fig. 5B and C).

As revealed by CFU counting, the si-Porin treatment per se had no significant inhibitory effect on the *P. gingivalis* biofilms, meanwhile it did not affect the killing efficacy of DC (Fig. 5D). Interestingly, the si-Porin treatment sightly reversed the killing efficacy of MTZ and AMC, whereas markedly increased the levels of viable cells in the biofilms treated with DC in combination with MTZ/AMC (Fig. 5E and F). The findings were further confirmed by CLSM (Fig. 5G).

Discussion

The present study firstly showed that constant DC could inhibit biofilm formation of *P. gingivalis* in time- and intensity-dependent manners, which is consistent with previous observations on other microorganisms (del Pozo *et al.*, 2009a; Zhang *et al.*, 2014). It has been suggested that the maximum harmless levels of DC that human body can perceive and sustain on hand are around 1,000 μA and 6,000 μA, respectively (Dalziel, 1961; Prasad *et al.*, 2010). In this study, we found 1,000 μA DC was able to inhibit the formation and maturation of *P. gingivalis* biofilms by decreasing cell density and thickness of the biofilms. It was reported that the levels of MTZ and amoxicillin in gingival crevicular fluid were 13 μg/ml and 14 μg/ml respectively after administration with 500 mg MTZ/amoxicillin 2–3 times daily for at least 2 days (Tenenbaum *et al.*, 1997; Pähkla *et al.*, 2005). This study demonstrated that the killing efficacy of 16 μg/ml MTZ/AMC on *P. gingivalis* biofilms was enhanced significantly by 1000 μA DC. Taken together, the findings indicate that DC is a potential approach for effective control of *P. gingivalis*-associated biofilm infections. Due to the current lack of well-designed DC devices suitable for oral use in animals and humans, further studies are required to screen the optimum parameters of DC for controlling oral/periodontal biofilms.

It was reported that DC could induce ROS production and result in the death of bacteria in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms (Brinkman *et al.*, 2016; Niepa *et al.*, 2016). Similarly, we found increased intracellular ROS in *P. gingivalis* biofilms following exposure to 1,000 μA DC for 12 h. ROS, including superoxide, hydrogen peroxide, hydroxyl radical, and other oxygen- or nitrogen-based reactive species, can damage macromolecules and lead to widespread cellular damage and cell death (Dixon and Stockwell, 2014). Hence, we hypothesized that ROS production mediates the DC-associated cell death in *P. gingivalis* biofilms. To confirm the hypothesis, we further examined the gene expression of sod, oxyR, and feoB, which play an important role in balancing overproduction of ROS (Wu *et al.*, 2008; Sestok *et al.*, 2018). We found that DC treatment markedly upregulated the expression of sod, oxyR, and feoB in *P. gingivalis* biofilms. Notably, DMTU, as a ROS scavenger, could reverse the upregulation of ROS related genes and partially lessen the killing efficacy of DC on *P. gingivalis* biofilms (Ramakrishnan *et al.*, 2016). Hereby, the ability of DC to induce ROS production represents an important mechanism of action of inhibiting *P. gingivalis* biofilms. In the data not shown, we also found similar phenomenon in the planktonic *P. gingivalis*. The same DC treatment resulted in more killing (mean 2.40 log10 CFU/ml reduction) in planktonic bacteria than that in biofilms, which may suggest that *P. gingivalis* biofilms may be more resistant to DC than its planktonic cells.

To uncover the mechanisms underlying the bioelectric effect of DC, the expression of antibiotic transport related genes was examined. The competition and interaction between drug efflux pumps and outer membrane channels regulate the entry of antibiotics into cellular targets and determine the stable concentration of antibiotics in bacteria cells, thereby affecting their antibiotic susceptibility (Klobucar *et al.*, 2020). The genes for RND pump, the major drug efflux pump in *P. gingivalis,* include RND-TPS and RND-TPA (Inoue *et al.*, 2015), and we found that their expression showed no significant changes in the DC-treated biofilms. This suggests that drug efflux pumps are probably not implicated in the bioelectric effect of DC. Interestingly, there was significant upregulation of porin gene in the DC-treated *P. gingivalis* biofilms with or without MTZ/AMC treatments, and its expression was not affected by MTZ/AMC as well as DMTU. Importantly, silencing of porin by siRNA considerably weakened the synergistic effect of DC with MTZ/AMC. Porin belongs to one of outer membrane channels superfamily and involves the passive transport of small molecule drugs such as MTZ and AMC (Naito *et al.*, 2008; Tomás *et al.*, 2010). The mutational loss or decrease of porin would cause antibiotic resistance, especially for hydrophilic antibiotics (Tomás *et al.*, 2010;

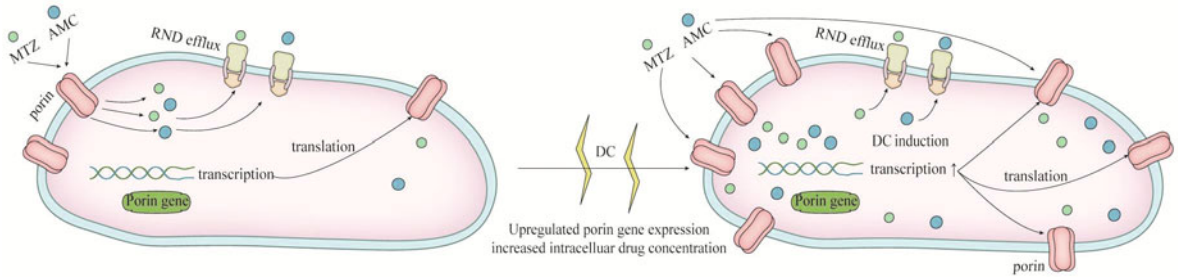

Fig. 6. **The schematic diagram of potential mechanism of bioelectric effect.**

Veloo *et al.*, 2012). A previous study showed DC led to aggregation of intracellular proteins and increased permeabilization of antibiotics to bacteria cells (Niepa *et al.*, 2016). Therefore, DC may induce the production of channel protein porin in *P. gingivalis* biofilms and enhance the permeabilization of MTZ/AMC (Fig. 6). Besides, we also found 1,000 μA DC had synergistic effect with the combination of MTZ and AMC, which caused more synergistic killing (mean 2.86 $\log_{10}$ CFU/ml) than that of 1,000 μA DC combined with MTZ or AMC (Unshown data). Interestingly, the synergistic effect of DC with AMC seemed to be superior to that with MTZ. This may be explained by the higher hydrophilicity of AMC, which is easier to diffuse into biofilm cells via porin channel than the relatively lower hydrophilic MTZ.

This study had several limitations. Although we found DC exerted electricidal and bioelectric effects on *Porphyromonas gingivalis* biofilms and revealed the potential underlying indirect mechanisms from the gene level, there is still lack of experiments from the protein level or direct pathways to confirm such mechanisms. In addition, due to the lack of mature and commercial first antibody of porin, we are unable to go one step further.

In summary, this study has demonstrated that DC can inhibit the biofilm formation and disrupt the preformed biofilms of *P. gingivalis*, and it has provided the first evidence on the synergistic effect of DC with MTZ/AMC in inhibition of *P. gingivalis* biofilms. The induction of ROS production and promotion of antibiotic transport via porin channel may critically account for the electricidal and bioelectric effects of DC against *P. gingivalis* biofilms. The current findings suggest that DC is a promising therapeutic approach for controlling oral/periodontal biofilms and development of clinically suitable DC devices may contribute to effective management of periodontal diseases.

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Conflict of Interest

The authors declared that they have no conflict of interest.

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