Original article

Effects of D-arginine on *Porphyromonas gingivalis* biofilm

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Abstract: *Porphyromonas gingivalis* (*P. gingivalis*) is one of the major pathogenic bacteria of periodontitis or peri-implantitis. *P. gingivalis* tends to attach to the implant's neck with the formation of biofilm, leading to peri-implantitis. D-arginine has been shown to have a potential antimicrobial role. In this study, *P. gingivalis* was cultured in Brain Heart Infusion broth together with D-arginine. After 3 days (inhibition) or 6 days (dissociation), these were characterized using crystal violet (CV) staining for the biofilm, extracellular polysaccharide (EPS) production from the biofilm, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for biofilm activation. Furthermore, the *P. gingivalis* biofilm was observed by scanning electron microscopy (SEM). D-arginine effectively reduced biomass accumulation and promoted dissociation at concentrations of ≥ 50 mM and 100 mM, respectively. Through CV staining, D-arginine concentrations of EPS production from the biofilm for inhibition and dissociation effects was ≥ 50 mM and 100 mM, respectively. In addition, D-arginine affected biofilm activation for the corresponding concentrations: ≥ 60 mM for inhibition and ≥ 90 mM for dispersal. Under SEM observation, D-arginine changed the *P. gingivalis* biofilm structure in relatively high concentrations for inhibition or dissociation, respectively. The authors concluded that D-arginine could inhibit the formation of *P. gingivalis* biofilm and promote the dissociation of *P. gingivalis* biofilm.

Keywords: D-arginine, dissociation, inhibition, *P. gingivalis* biofilm

Introduction

Oral biofilm, or dental plaque, is a thin film that comprises organized, complex microorganisms that adhere to oral tissues [1]. In addition to oral tissues, biofilms adhere naturally to the surface of dental materials in biologic environments, such as implants and restorations. Biofilm is associated with common oral conditions, such as dental caries and gingivitis. Poor oral hygiene potentiates exaggerated biofilm deposition, gingival inflammation, alveolar bone resorption, tooth loosening, or peri-implantitis [2], which mainly accounts for loss of teeth or implants [3]. The use of dental implants for restoring missing teeth has shown highly satisfactory results. Patients with missing teeth or edentulous jaws prefer replacements using implants [4]. Dental implants allow for restoration without damaging the adjacent teeth and the rehabilitation of masticatory function [5], thereby improving the quality of a patient's life [6]. However, dental implants exposed to an oral environment do provide a potential surface for acquired pellicle and attracted bacterium to form a biofilm [7].

Biofilm microorganisms secrete matrixes and metabolites to stimulate immune response [8]. Considering the microbiology of the oral biofilm, *P. gingivalis* is one of the major pathogenic bacteria associated with the development of gingivitis, periodontitis, and peri-implantitis. The structure and virulence factors of *P. gingivalis* play key roles in the damage of the periodontium, bone loss, and loosening of implants [9]. Thus, the removal

of *P. gingivalis* biofilms appears to be a prerequisite in the therapy of peri-implantitis.

Presently, effective approaches for the removal of biofilms mainly include mechanical and chemical methods, as well as photodynamic therapy (PDT). Because implants need a rough surface for osseointegration, the use of mechanical measures, such as periodontal scaling [10], dental floss, or rinsing may not be a feasible option [11]. Chemical plaque control, such as the use of chlorhexidine mouthwashes, has an effective role in controlling microorganisms including *Streptococcus mutans* [12] and *Candida albicans* [13] and periodontal pathogens such as *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* [14]. Although chlorhexidine has an effective action against oral bacteria, its long-term use can lead to unwanted effects, such as altered sensations in taste, discoloration of the oral mucosa/tongue/gingiva [15], imbalances of the oral flora, and the development of drug resistance [16]. PDT is a reformatory antimicrobial method. It decontaminates the implant surface and activates the regenerative procedures [17]. However, PDT is unable to fully eradicate these biofilms [18], and may cause a photosensitive reaction to the skin and mucosa. Therefore, presently, there is no effective treatment modality to completely eliminate biofilms on the implant surface and fully prevent peri-implantitis.

Recently, research on D-amino acids has been a hotspot for study. The amino acids that make up the majority of life on earth are L-amino acids, which are enantiotopic chiral molecules of D-amino acids. Two chiral molecules imagined with each other have similar physical and chemical characteristics, but may have adverse biological properties [19]. D-amino acids can potentially affect the strength, amount, and composition of the bacterial peptidoglycan [20], and reduce the production of lipoprotein in the extracellular matrix [21]. In addition, researchers have found that D-tryptophan, D-leucine, D-methionine, and D-tyrosine may inhibit *Bacillus subtilis* biofilm formation and facilitate the triggering of biofilm disassembly, the functions of which could be counteracted by their corresponding L-amino acids [22]. Arginine residue dissolution from L-arginine occurs after hydrolysis participates in the formation of the arginine-specific cysteine proteinase (Arg-gingipain) of *P. gingivalis* [23]. Gingipain can activate the production of reactive oxygen species and induce peroxidation to induce the inflammation and destruction of periodontal tissues [24]. Thus, the aim of the present study was to establish the *P. gingivalis* biofilm and investigate the effects of D-arginine in inhibiting and disassembling the *P. gingivalis* biofilm. The present data provides theoretical information regarding the inhibition and dissociation of *P. gingivalis* biofilms *in vitro*.

Materials and Methods

Bacterial culture and reagents

The present study used the *P. gingivalis* strain (ATCC 33277) grown on Brain Heart Infusion (BHI) (Qing Dao Hope Bio-Technology Co, Ltd, Qing Dao, P. R. China) broth supplemented by menadione (0.5 µg/mL) and hemin (5 mg/mL). After culturing in anaerobic conditions (5% CO₂, 10% H₂, and 85% N₂) at 37°C for 5 to 7 days, the bacterial strains were grown to the log phase (optical density [OD]: 0.8 to 1.0 at 600 nm). Then, the cultures were inoculated into fresh BHI broth supplemented by menadione and hemin. As previously reported, the biofilm formed by bacterial colonies at 10⁸ CFU/mL exhibited a tightly connected and homogeneous structure [25]. Consequently, for all assays in the present study, bacteria in

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Fig. 1 Growth curves of *P. gingivalis* under different D-arginine concentrations. (A) 10 mM to 70 mM; (B) 80 mM to 130 mM: at a D-arginine concentration ≥ 80 mM, *P. gingivalis* growth was inhibited.

the logarithmic phase were diluted to 10^8 CFU/mL with bacterial culture medium using a spectrophotometer.

D-arginine (BOMEI Biotechnology Co, Ltd, Hefei, P. R. China) was diluted in distilled water and stored at room temperature. Based on these preliminary experiments, the concentrations of D-arginine for inhibiting biofilm formation ranged from 0 to 80 mM, while for triggering biofilm disassembly, concentrations ranged from 60 to 130 mM. Experiments with distilled water and no added D-arginine were used as the control group.

Bacterial growth

The bacterial strains were harvested from the BHI liquid culture medium anaerobically at 37°C , and diluted to 10^8 CFU/mL using freshly prepared culture medium based on the OD 600 value. *P. gingivalis* and D-arginine (up to 130 mM) were added to centrifugal tubes at a ratio of 3:1 and cultured in an anaerobic condition at 37°C for 72 h. Simultaneously, the OD 600 value was measured every 4 h using a UV/Vis spectroscopic microplate reader (Bole Life Medical Products Co, Ltd, Shanghai, P. R. China), and the corresponding bacterial growth curve was plotted.

Biofilm determination

Crystal violet (CV) staining was used to analyze the *P. gingivalis* biofilm. The diluted bacteria inoculum (10^8 CFU/mL) was added to 96-well plates. Variable concentrations of D-arginine (0 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, and 80 mM) were added to each well. After 72 h, the liquid was carefully pipetted out of each well, leaving behind a layer of *P. gingivalis* biofilm at the bottom of the well. Each well was washed using phosphate-buffered saline (PBS) at a pH of 7.4 (Boster Biological Technology Ltd, Wuhan, P. R. China), and gently air-dried for 20 min. Following fixation in paraformaldehyde (3.7%) for 15 min, CV was added to each well for 20 min. Then the biofilms were washed with PBS and decolorization was performed with ethanol (95% v/v). Finally, the ethanol from each well was transferred to another sterile 96-well plate, and the biofilm biomass was evaluated using a microplate reader at OD 600 nm. For disassembling the biofilms, the bacteria inoculum was added into 96-well plates to allow the biofilm to form for 72 h, followed by the replacement of a new BHI liquid culture accompanied by the D-arginine solution in variable concentrations (0 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 110 mM, 120 mM, and 130 mM). This was cultured for 72 h, and all evaluations were performed using exactly the same methods as described above.

Extracellular polysaccharide (EPS) measurement

Bacteria EPS was tested through the phenol-sulfuric acid method. Briefly, the mixture of bacteria inoculum and D-arginine solution was cultured for 3 days, and the biofilm was washed with PBS, followed by washing with distilled water (40 μL), 6% phenol solution (40 μL diluted in distilled water), and sulfuric acid (97%) after air-drying for 20 min. Finally, the plate was incubated for 20 min at room temperature. The quantity of polysaccharides in the biofilm was determined by measuring the absorbance at OD 490 nm. For triggering the biofilm disassembly, the nutrient solution was renewed after 72 h, and D-arginine was added for the follow-up steps using the same methods described above.

MTT assay

To measure the effect of D-arginine on *P. gingivalis* biofilm activation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was carried out. The 24-well plate with 3 days' worth of biofilm and D-arginine solution was rinsed with PBS, followed by the addition of MTT to each well for 4 h in the dark. Then, the supernatant was removed, 1 mL of dimethyl sulfoxide was added into the well, and the plate was shaken at room temperature for 20 min. The activation of bacteria biofilm was assessed at OD 540 nm. With regard to the scattering biofilm, the biofilm was formed for 3 days following exactly the same process.

Scanning electron microscopic (SEM) analysis

The 3-day biofilm for inhibition and 6-day biofilm for scatter were washed three times using PBS, fixed in glutaraldehyde 2.5% at 4°C overnight, and dehydrated by gradient alcohol solutions (30% to 100%). After completely drying, the biofilm was mounted and coated using a gold-sputter coater, and observed under the SEM (Hitachi High-Tech International Trade Co, Ltd., Shanghai, P. R. China).

Statistical analysis

Data analyses were performed using SPSS (version 19, IBM, New York, USA). Comparisons among groups were assessed using analysis of variance. In addition, Dunnett's *t*-test was used to compare each test group with the control group. A *P*-value of <0.05 was considered statistically significant. All experiments were performed in triplicate, and mean values were used.

Results

Bacteria growth

The growth curves of *P. gingivalis* under different D-arginine concentrations were achieved by measuring the OD 600 nm values every 4 h (Fig. 1A, B). When the D-arginine concentration was ≥ 80 mM, there was an obvious inhibitory effect on *P. gingivalis* growth (Fig. 1B). Furthermore, the logarithmic growth period of *P. gingivalis* began at approximately 12 to 16 h, but was delayed with the increase in D-arginine concentration (Fig. 1A).

Biofilm determination

The *P. gingivalis* biofilm was determined by CV staining (Fig. 2). For inhibiting the biofilm formation, no significant difference was observed for D-arginine ≤ 40 mM (Fig. 2A). However, the biofilm exhibited a significant reduction when D-arginine was ≥ 50 mM ($P < 0.05$). Furthermore, the increase in D-arginine concentration led to more significant inhibitory effects (Fig. 2B). With regard to the scatter biofilm, biofilm growth under high concentrations of D-arginine (≥ 90 mM) revealed a tendency of dissociation, while a significant difference was observed when the D-arginine concentration was ≥ 100 mM ($P < 0.01$, Fig. 2B). Therefore, depending on the D-arginine concentration, a dual effect, including the inhibition and dissociation of the *P. gingivalis* biofilm, was observed.

EPS measurement

Polysaccharide is one of the important metabolites that play a critical role

Fig. 2 CV staining for *P. gingivalis* biofilms. (A) D-arginine concentration ≥ 50 mM inhibited the *P. gingivalis* biofilm formation; (B) To dissociate biofilms, at least 100 mM of D-arginine was needed to achieve the result.

Fig. 3 EPS measurement of *P. gingivalis* biofilms. (A) With a D-arginine concentration of ≥ 50 mM, the production of EPS in the *P. gingivalis* biofilm was inhibited; (B) For the dissociation of biofilms, the difference in EPS production was found at a D-arginine concentration of ≥ 100 mM.

Fig. 4 MTT assay. (A) The activation of *P. gingivalis* biofilm was inhibited under a D-arginine concentration of ≥ 60 mM; (B) The concentration threshold was increased to 90 mM for dissociation.

in bacteria survival. In the present study, EPS measurement was carried out using the phenol-sulfuric-acid method. Under the circumstance of biofilm inhibition (Fig. 3A), the production of EPS was significantly inhibited when the concentration of D-arginine was ≥ 50 mM ($P < 0.05$). For dissociation situations, the efficient D-arginine concentration to reduce EPS production was ≥ 100 mM ($P < 0.05$). The other groups did not exhibit any significant difference (Fig. 3B).

MTT assay

The present study used the MTT assay to assess the effects of D-arginine concentration on *P. gingivalis* biofilm viability. The inhibitory effect clearly appeared at a D-arginine concentration of ≥ 60 mM ($P < 0.05$, Fig. 4A). Although the biofilm had a strong drug-resistance ability, increasing the D-arginine concentration to ≥ 90 mM significantly reduced the viability of

the bacteria biofilm ($P < 0.05$, Fig. 4B). These results imply that D-arginine has the potential to inhibit the bacterial viability of *P. gingivalis* in both free and biofilm forms.

SEM

The morphological features of the *P. gingivalis* biofilm were observed under SEM (Fig. 5). The D-arginine affected the *P. gingivalis* biofilm formation, especially when the concentration increased to ≥ 50 mM, in which there was a tendency to inhibit biofilm formation (Fig. 5F). In addition, the biofilm structure changed from multiple layers to a single layer by increasing the concentration. In terms of dissociation, D-arginine also had an effective function on the *P. gingivalis* biofilm. Although these biofilms were formed through a 3-day culture, a tendency of changing structures after adding D-arginine at high concentrations (≥ 90 mM) demonstrated the

Fig. 5 SEM images showing the morphology of *P. gingivalis* biofilms at different D-arginine concentrations ($\times 5k$, $\times 30k$). A through I for inhibition and J through R for dissociation: A, 0 mM; B, 10 mM; C, 20 mM; D, 30 mM; E, 40 mM; F, 50 mM; G, 60 mM; H, 70 mM; I, 80 mM; J, 0 mM; K, 60 mM; L, 70 mM; M, 80 mM; N, 90 mM; O, 100 mM; P, 110 mM; Q, 120 mM; R, 130 mM

promotion of *P. gingivalis* biofilm dissociation (Fig. 5N).

Furthermore, when the drug concentration was ≥ 90 mM, the destruction of bacterial structures was observed. Furthermore, it appeared that the cell wall dissolved, and the content flowed out under the conditions of relatively high concentrations (Fig. 5N-R). These observations indicate that D-arginine has the potential to effectively control the *P. gingivalis* biofilm.

Discussion

The present study attempted to investigate the effects of D-arginine in inhibiting and disassembling the biofilm of *P. gingivalis* *in vitro*. For this purpose, *P. gingivalis* was cultured with variable concentrations of D-arginine, and the inhibitory effects were evaluated using CV, MTT, and SEM analysis. These present results revealed that D-arginine effectively reduced biofilm accumulation (at ≥ 50 mM) and promoted dissociation (100 mM).

Bacterial plaque biofilms can induce the progressive destruction of the periodontium for teeth [26] or peri-implantitis for implants [27]. As one of the main pathogenic bacteria, *P. gingivalis* can attach to the implant neck and secrete polysaccharides and enzymes that promote biofilm formation [7] and the manipulation of the host defense [28]. The inhibition of bacterial plaque and its corresponding secretions enhances its immune defense response to resist invasion [29]. Nevertheless, an immune response that lacks targeting may lead to soft-tissue swelling, alveolar resorption, and loosening of the implant. These inflammatory cytokines are elevated in pathological tissues when compared with healthy tissues [30,31].

Therefore, plaque control and the avoidance of an immune inflammatory response to periodontal tissues are effective ways to prevent periodontitis or peri-implantitis.

At present, there are many approaches for removing biofilms [32,33], and seldom do many of these have ideal effects. A number of antimicrobial peptides have been reported to be effective against oral infections [34]. Compared to antimicrobials, D-amino acid has exhibited good antibacterial results with fewer side effects, and avoids only oral flora imbalance and bacterial drug resistance [16]. Sarı et al. [35] exploited the complex from the D-glycine, D-alanine, and Schiff base, which exhibited significant activity against both gram-positive and -negative bacteria. Defensins peptides have been shown to have both antibacterial and antifungal properties, and have been proposed for implant and periodontal membrane preventive coatings [36]. In addition, the single application of D-amino acid has also exhibited antibacterial action [37,38]. In contrast, Sarkar et al did not observe any inhibitory effects on *Staphylococcus aureus* [39]. Such contrary effects may be associated with the different experimental conditions and bacterial strains because the specific effects of D-amino acid are not similar for all types of bacterium. However, the results of a previous study revealed the potential role of the antibacterial effect on *P. gingivalis* [25]. On the basis of these extended preliminary experiments, D-arginine was selected as the target amino acid, considering that the arginine residue may have a potential role in antimicrobial property [40]. Arginine residue participates in the composition of Arg-gingipain of *P. gingivalis*, and this was explored using its chiral isomer.

In the present study, the D-arginine concentration was determined on the basis of the bacteria growth curve, and the concentration range of biofilm inhibition and splitting was estimated. The *P. gingivalis* biofilm was examined by CV staining. It was revealed that D-arginine could reduce membrane biofouling and promote biofilm scatter when the value was ≥ 50 mM and ≥ 100 mM, respectively, and this effect was strengthened with the increase in D-arginine concentration. However, CV staining cannot distinguish between live and dead cells. Therefore, these results included both cells, and an EPS examination was required for further confirmation. The survival of bacteria is based on the secretion of EPS, and bacterial cells are surrounded by polysaccharides, which form the biofilm. Thus, EPS is an essential part for biofilm formation. To determine the secretory functions of *P. gingivalis*, EPS in the biofilm was measured through phenol-sulfuric acid. In the process of biofilm formation, D-arginine reduced EPS secretion at a higher concentration (≥ 50 mM), suggesting that D-arginine has an inhibitory effect on the EPS secretion of *P. gingivalis*. For biofilm dispersion, this also worked when the concentration was ≥ 100 mM. Although the correlation between EPS and biofilm formation could not be determined, D-arginine disturbed the *P. gingivalis* biofilm formation by affecting EPS secretion, or through the effect of the EPS secretion. The disturbance of the *P. gingivalis* biofilm formation confirms that D-arginine could inhibit *P. gingivalis* biofilm formation and promote dissociation by CV and EPS assay. Furthermore, the MTT assay was carried out to ensure the effect of D-arginine on *P. gingivalis* activation. Like the tendencies before, the activation of *P. gingivalis* was affected when the drug was at a relatively high concentration (≥ 60 mM for inhibition and ≥ 90 mM for dispersion).

To observe the morphology and structure of *P. gingivalis* biofilm, SEM images were exploited. In the inhibition assay, there were no obvious differences at concentrations ≤ 20 mM, but the biofilm changed under the concentration of ≥ 30 mM. A D-arginine concentration that reached 60 mM revealed that the connection between cells was lost, and led to a scattered distribution. In addition, a higher concentration was found in the dissociation group when compared with the inhibition group, which demonstrates that microbial biofilms have an increased resistance toward antibacterial agents when compared with free-form bacteria [41]. Although bacteria exists either in free-form or biofilm-form, and may be transformed between these two forms, planktonic bacteria are comparatively easier to remove because of the low defense capability [42]. Moreover, in the dissociation group, with the 3-day growth, the layer number and secretion were significantly greater than the inhibition group. When the concentration was ≥ 90 mM, a tendency of dispersion was observed, while at a concentration of 100 mM, the biofilm became sparse.

Interestingly, in a high-concentration environment (90 mM), both the biofilm shape and bacteria morphology changed. However, the cell walls were destroyed, and the content flowed out. This may be a toxic dose for

P. gingivalis when exposed to a high D-arginine concentration, suggesting that D-arginine can be used as an ideal agent to resist *P. gingivalis* invasion. All of these findings reflect the sensitive response of *P. gingivalis* to D-arginine, and that this achieved an inhibitory result on *P. gingivalis* biofilm accumulation, and in accelerating its dissociation. However, there was no evidence that D-arginine interfered with the balance of the normal oral flora in the present study. Therefore, further investigations are required to explore the potential of D-arginine for pathogenic bacteria of periodontitis and peri-implantitis.

The present study was the first to investigate the effects of D-arginine on the major pathogenic bacterium of periodontitis or peri-implantitis (*P. gingivalis*), in view of the adverse biological properties of the enantiotopic chiral molecule. These results indicate that D-arginine can be used as an ideal drug to control biofilm—not only for prevention, but also for treatment. However, the dose for treatment is higher than that for prevention, and there are more patients who need treatment than prevention, which signifies more drug risks. In addition, *in vitro* and *in vivo* cell studies need to be conducted to determine drug toxicity. Moreover, understanding the mechanisms of D-arginine effects on *P. gingivalis* biofilm needs further investigation in order to provide theoretical support for further clinical applications.

In conclusion, the present *in vitro* study revealed that D-arginine inhibits the formation of *P. gingivalis* biofilm, and promotes the dissociation of *P. gingivalis* biofilm.

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Conflict of interest

All authors declare no conflict of interest in this work.

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