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 (disassociation), these were characterized using crystal violet (CV) staining and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bro- 

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 biological environments, such as implants and restorations. Biofilm is associated with common oral conditions, such as dental caries and gingivitis. Dental hygienists potentiate the growth of enterococcal organisms in biofilm deposition, gingival inflammation, alveolar bone resorption, tooth loosening, or peri-implantitis [2], 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 enantiomeric molecular pairs of d-amino acids. Two chiral molecules are 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-phenylalanine, and d-tyrosine 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 reduce peroxidation to induce the inflammation and destruction of periodontal tissues [24].

Thus, the aim of the present study was to establish the inhibitory effects of d-arginine in inhibiting and disassembling the P. gingivalis biofilm. The present study 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] of 0.8 to 1.0 (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 10^6 CFU/mL exhibited a tightly connected and homogeneous structure [25]. Consequently, for all assays in the present study, bacteria in
the logarithmic phase were diluted to 10^8 CFU/mL with bacterial culture medium using a spectrophotometer.

\( \text{d-arginine} \) (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 \( \text{d-arginine} \) 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 \( \text{d-arginine} \) 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. \( P. \text{gingivalis} \) and \( \text{d-arginine} \) (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 spectrophotometer. After completely drying, the biofilm was mounted and coated using a gold-sputter coater (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 \( P. \text{gingivalis} \) biofilm. The diluted bacteria inoculum (10^6 CFU/mL) was added to 96-well plates. Variable concentrations of \( \text{d-arginine} \) (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 \( P. \text{gingivalis} \) 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 bacterial 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 \( \text{d-arginine} \) solution in variable concentrations (0 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 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 \( \text{d-arginine} \) 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 \( \text{d-arginine} \) was added for the follow-up steps using the same methods described above.

**MTT assay**

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

**Biofilm determination**

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

**EPS measurement**

Polysaccharide is one of the important metabolites that play a critical role
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 \( \text{d-arginine} \) was \( \geq 50 \text{ mM} \) \((P < 0.05)\). For dissociation situations, the efficient \( \text{d-arginine} \) concentration to reduce EPS production was \( \geq 100 \text{ mM} \) \((P < 0.05)\). 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 \( \text{d-arginine} \) concentration on \( P. \text{gingivalis} \) biofilm viability. The inhibitory effect clearly appeared at a \( \text{d-arginine} \) concentration of \( \geq 60 \text{ mM} \) \((P < 0.05)\). For dissociation situations, the effective \( \text{d-arginine} \) concentration to reduce biofilm viability was \( \geq 90 \text{ mM} \) \((P < 0.05)\). These results imply that \( \text{d-arginine} \) has the potential to inhibit the bacterial viability of \( P. \text{gingivalis} \) in both free and biofilm forms.

**SEM**

The morphological features of the \( P. \text{gingivalis} \) biofilm were observed under SEM (Fig. 5). The \( \text{d-arginine} \) affected the \( P. \text{gingivalis} \) biofilm formation, especially when the concentration increased to \( \geq 50 \text{ mM} \), 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, \( \text{d-arginine} \) also had an effective function on the \( P. \text{gingivalis} \) biofilm. Although these biofilms were formed through a 3-day culture, a tendency of changing structures after adding \( \text{d-arginine} \) at high concentrations \( \geq 90 \text{ mM} \) demonstrated the
The present study attempted to investigate the effects of \textit{d}-arginine in inhibiting and disassembling the biofilm of \textit{P. gingivalis}. To observe the morphology and structure of \textit{P. gingivalis} biofilm, this study revealed the potential role of the antibacterial effect on \textit{P. gingivalis} [25]. On the basis of these extended preliminary experiments, \textit{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 \textit{P. gingivalis}, and this was explored using its chiral isomer.

In the present study, the \textit{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 \textit{P. gingivalis} biofilm was examined by CV staining. It was revealed that \textit{d}-arginine could reduce membrane biofouling and promote biofilm scatter when the value was $\geq 50$ mM and $\geq 100$ mM, respectively, and this effect was strengthened with the increase in \textit{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 \textit{P. gingivalis}, EPS in the biofilm was measured through phenol-sulfuric acid. In the process of biofilm formation, \textit{d}-arginine reduced EPS secretion at a higher concentration ($\geq 50$ mM), suggesting that \textit{d}-arginine has an inhibitory effect on the EPS secretion of \textit{P. gingivalis}. For biofilm disper-
sion, this also worked when the concentration was $\geq 100$ mM. Although the correlation between EPS and biofilm formation could not be determined, \textit{d}-arginine disturbed the \textit{P. gingivalis} biofilm formation by affecting EPS secretion, or through the effect of the EPS secretion. The disturbance of the \textit{P. gingivalis} biofilm formation confirms that \textit{d}-arginine could inhibit \textit{P. gingivalis} biofilm formation and promote dissociation by CV and EPS assay. Furthermore, the MTT assay was carried out to ensure the effect of \textit{d}-arginine on \textit{P. gingivalis} activation. Like the tendencies before, the activation of \textit{P. gingivalis} was affected when the drug was at a relatively high concentration ($\geq 60$ mM for inhibition and $\geq 90$ mM for dispersion).

To observe the morphology and structure of \textit{P. gingivalis} biofilm, SEM images were explored. In the inhibition assay, there were no obvious differences at concentrations $\leq 20$ mM, but the biofilm changed under the concentration of $\geq 50$ mM. A \textit{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 $\geq 90$ 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 promotion of \textit{P. gingivalis} biofilm dissociation (Fig. 5N).

### Discussion

The present study attempted to investigate the effects of \textit{d}-arginine in inhibiting and disassembling the biofilm of \textit{P. gingivalis} in vitro. For this purpose, \textit{P. gingivalis} was cultured with variable concentrations of \textit{d}-arginine, and the inhibitory effects were evaluated using CV, MTT, and SEM analysis. These present results revealed that \textit{d}-arginine effectively reduced biofilm accumulation (at $\geq 50$ 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, \textit{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, \textit{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 \textit{d}-glycine, \textit{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 \textit{d}-amino acid has also exhibited antibacterial action [37,38]. In contrast, Sarkar et al. did not observe any inhibitory effects on \textit{Staphylococcus aureus} [39]. Such contrary effects may be associated with the different experimental conditions and bacterial strains because the specific effects of \textit{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 \textit{P. gingivalis} [25].

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{Inhibition} & \textbf{Dissociation} \\
\hline
A & J \\
B & K \\
C & L \\
D & M \\
E & N \\
F & O \\
G & P \\
H & Q \\
I & R \\
\hline
\end{tabular}
\caption{SEM images showing the morphology of \textit{P. gingivalis} biofilms at different \textit{d}-arginine concentrations ($\geq 5k$, $\geq 30k$). 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, 90 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.}
\end{table}
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 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 enantiomeric chiral molecular. 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 research 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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