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Evaluation of antimicrobial peptide LL-37 for treatment of *Staphylococcus aureus* biofilm on titanium plate

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Abstract

The antimicrobial peptide LL-37 belongs to the cathelicidin family and is one of the few human bactericidal peptides with potent antistaphylococcal activity. *Staphylococcus aureus* is one of the main infection bacteria in orthopedic implant therapy. Biofilm formation after bacterial infection brings more and more severe test for clinical antiinfection treatment.

However, there are few studies on LL-37 in *S. aureus* infection of prosthesis. In this work, addition to research the antibacterial activity and the inhibitory effect on bacterial adhesion of LL-37, an in vitro model of *S. aureus* biofilm formation on titanium alloy surface was established to observe the inhibitory effect of LL-37.

The results showed that LL-37 has a strong antibacterial effect on *S. aureus* in vitro, and the minimum inhibitory concentration (MIC) is about 0.62 μM. Moreover, LL-37 has significant impact on the adhesion of *S. aureus* when the concentration ≥0.16 μM and significant anti-staphylococcal biofilm effects on static biofilm models at the concentration of 0.31 to 10 μM. Additionally, LL-37 at 5 μM had a significant destructive effect on *S. aureus* biofilm ($P < .05$) that formed on the titanium alloy surface.

This study further confirmed the role of LL-37 in the process of *S. aureus* infection, including antimicrobial activities, inhibition of bacterial adhesion, and inhibition of mature biofilm. LL-37 can significantly destroy the stable biofilm structure on the titanium alloy surface in vitro, which may provide a new way for refractory infection caused by *S. aureus* in titanium alloy prosthesis infection.

Abbreviations: MIC = minimum inhibitory concentration, PBS = phosphate-buffered saline, TSB = tryptic soy broth.

Keywords: antimicrobial peptide, biofilm, LL-37, *Staphylococcus aureus*, titanium plate

1. Introduction

Antimicrobial peptides are a class of active antigens induced by the biological immune system, which can resist the infection of a variety of pathogens and are an important part of the innate immune system of multicellular organisms.[1] Compared with traditional antibiotics, antimicrobial peptides have the advantages of broad antibacterial spectrum and faster bactericidal rates.[2,3] With the emergence of antibiotic-resistant strains in large numbers, the application of antimicrobial peptides as a new antibacterial agent in clinical treatment has aroused many scholars' interest, also provided a new idea and means for the treatment of clinical infection.[4–9] Clinically, 80% of human bacterial infections are in the form of biofilm. Bacterial biofilm appears in the form of bacterial community, showing high resistance to antibiotics.[10] According to statistics, bacteria in biofilms can be 100 to 1000 times more resistant to antibiotics than their planktonic state.[11–13] Bacterial biofilm is an organized group of bacteria formed by attaching multiple bacteria to non-biological or biological surfaces, secreting a polymer matrix and wrapping itself in it. Once bacteria are colonized on the surface of human life tissues (heart, lung, intestine, urethra, etc.) or medical biomaterials (artificial heart valves, artificial joints, implanted catheters, etc.), bacterial biofilm infection may be caused.[14] As a new antibacterial agent, antimicrobial peptides play a unique advantage in the process of inhibiting and killing bacterial biofilm.[15]

Antimicrobial peptide LL-37 is the only member of cathelicidins family found in human body. LL-37 is expressed and secreted in a variety of epithelial cells, immune cells, body fluids, trauma secretions, etc. in the human body. It can exert antimicrobial activity, participate in immune regulation of the body, and promote wound repair. It is an active small molecule peptide with multiple functions.[16] Studies have shown that LL-37 has a broad antibacterial spectrum, such as antibacterial, antifungal, antiviral, antiprotozoal and other antimicrobial

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effects.[17–20] The antibacterial activity of LL-37 is higher than that of traditional antibiotics, and its minimum inhibitory concentration (MIC) is similar to that of fluoroquinolones such as ofloxacin and ciprofloxacin, but higher than that of norfloxacin and enoxacin.[21] Existing studies have shown that LL-37, as an endogenous antibiotic not only has a good sterilization effect on planktonic *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and other pathogens, also has inhibitory activity against *S. aureus*, *Staphylococcus epidermidis*, *P. aeruginosa*, *Acinetobacter baumannii* in the form of biofilm.[22–25]

In orthopedic implants, titanium alloy (Ti6A14V) materials are widely used. Biomedical titanium alloy materials are a class of functional structural materials applied in biomedical engineering, often applied in the production and manufacturing of surgical implants and orthopedic devices. Titanium alloy medical equipment products such as artificial joints, dental implants and vascular stents are used for clinical diagnosis, treatment, repair, replacement of human tissues or organs, or to enhance the function of human tissues or organs, and their role cannot be replaced by drugs. Studies have shown that the biofilm formed by *S. aureus* is easy to adhere to the surface of metal implants such as titanium alloys, while *S. epidermidis* is easy to adhere to the surface of polymers.[26] Previous studies in the literature regarding the efficacy of LL-37 against preformed biofilms vary. Some studies suggest that LL-37 does not disrupt preformed biofilms, inhibit bacterial attachment, or prevent early biofilm formation.[27,28] In contrast, other studies have demonstrated that LL-37 can disrupt 24 and 48 hours mature *S. aureus* biofilms.[29] Therefore, in this study, a bacterial biofilm of *S. aureus* in vitro was constructed on the surface of titanium alloy to simulate the infection related to orthopedic implants.

In this study, the *in vitro* antibacterial activity of LL-37 on *S. aureus* was measured first, and the effect of LL-37 on the adhesion of *S. aureus* was determined. Then, an *in vitro* static model of *S. aureus* biofilm, a common pathogen of orthopedic implants, was established to further observe the damaging effect of LL-37 on the *S. aureus* biofilm and the *S. aureus* biofilm formed on the surface of titanium alloy. The purpose of this study was to clarify the role of LL-37 in the process of *S. aureus* infection, provide a theoretical basis for clinical treatment of *S. aureus* infection of orthopedic implants.

2. Materials and methods

2.1. Materials

Staphylococcus aureus subsp. *aureus* (ATCC 6538P) obtained from Guangdong Microbial Culture Collection center (GDMCC). The human antimicrobial peptide LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), with 98.67% purity, was purchased from China Peptides Co., Ltd. (Shanghai, China). Titanium plate (Ti6A14V, diameter 1.0 cm, thickness 3 mm) provided and processed by Tianjin Jinxingda Industrial Co., Ltd. (Tianjin, China). Tryptic soy broth (TSB), phosphate-buffered saline (PBS) and other reagents were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The equipment used in this study includes a constant temperature incubator (BJPX-400-1, Shandong Brocade Biological Industry Co., Ltd., Jinan, China), a Motic Digital BA310 Biological Light Microscope (Motic China Group Co., Ltd, Xiamen, China) an Olympus FluoView FV1000 laser scanning confocal microscope (Olympus Corp., Tokyo, Japan), an ultraviolet and visible spectrophotometer (UV-5100 manufactured by Shanghai Kunke Instrument Equipment Co., Ltd.) and a RT-6100 microplate reader (Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China). This study does not involve human or animal participants, hence ethical approval is not required.

2.2. Preparation of bacterial suspension

Firstly, 0.3 mL of normal saline was injected into the opened strain ampoule by pipetting to dissolve the freeze-dried strain in the ampoule into a bacterial suspension. Aspirated the above suspension and inoculated to a sheep blood agar plate was divided into 3 sectors, the plates were inverted and incubated at 37°C for 24 hours. Then picked a single colony and inoculated it in 5 mL TSB and placed it in a 37°C constant temperature incubator, shaked culture, and the oscillation frequency was set to 180 rpm. After shaking for 16 hours, an ultraviolet spectrophotometer used to adjust the standard bacterial solution with a concentration of about 1.5×10^9 CFU/mL (OD450 = 0.29).

2.3. Determine the minimum inhibitory concentration of LL-37

The MIC was determined according to the NCCLS standard. Briefly, in 96-well microtiter plates, added 180 μL TSB to the first well, each of 2 to 11 wells added 100 μL. Then added 20 μL of LL-37 supernatant (20 μM) to the first well, mixed and aspirated 100 μL into the second well, the same method was use to dilute to the tenth well, discard 100 μL. Then 100 μL of standard bacterial solution was added to each well. The concentration of LL-37 in each well is 10, 5, 2.5, 1.25, 0.62, 0.31, 0.16, 0.08, 0.04, and 0.02 μM, respectively. The negative control was 100 μL of normal saline and all experiments were repeated 3 times. After incubating at 37°C for 24 hours, the OD value at 450 nm of each well was measured with microplate reader.

2.4. Determination the effect of LL-37 on the adhesion of S. aureus

Inoculated 100 μL of standard bacterial solution in a 96-well plate and incubated at 37°C for 1, 2, and 4 hours respectively. Aspirated and discarded the medium, then gently rinsed with PBS 3 times to wash away non-adherent bacteria. Added LL-37 in 2-fold serial dilutions (10, 5, 2.5, 1.25, 0.62, 0.31, 0.16, 0.08, 0.04, and 0.02 μM) to wells 1 to 10. The negative control was added the same volume of normal saline and incubated continuously at 37°C for 24 hours. Then a microplate reader was used to detect the absorbance value (OD450) of each well. The experiment was repeated 3 times, 8 replicate holes were set for each drug concentration, and the average value was taken.

2.5. Establishment of an in vitro bacterial biofilm model

Pipetted 10 μL of standard bacterial solution into a 24-well plate, added 1 mL TSB to each well, cultured at 37°C, replaced the culture medium once every 24 hours, and cultured for 4 consecutive days. The culture medium supernatant was stained with crystal violet, and the 24-well plate was gently washed with sterile PBS 3 times to remove the floating *S. aureus*. After air-drying in the ultra-clean workbench, stained with 1% Fuchsin for 30 seconds, rinsed gently with PBS solution 3 times, let it air dry.

Finally, the images were recorded using the microphotography system (BA310 Digital, McAudi Industrial Group Co., LTD.).

2.6. Destructive effect of LL-37 on S. aureus biofilm

Pipetted 10 μL of standard bacterial solution into a 24-well plate, and 1 mL TSB was added to each well. After autoclaving the titanium alloy plate, placed it in a 24-well plate containing the above-mentioned bacterial solution, and incubated at 37°C for 1 week, and replaced the culture solution every day. One week later, they were randomly divided into experimental group and control group. The experimental group was added with 5 μM LL-37, and the control group was added with the same volume of broth medium. After incubating at 37°C for 24 hours, rinsed with PBS several times and fixed with 2.5% glutaraldehyde solution at 4°C overnight. 30%, 50%, 70%, 90%, 100% ethanol gradient dehydration was used for 10 minutes, and 100% ethanol was used twice. The sample is placed in a desiccator, and the dried specimen is placed in a high-vacuum evaporator, to be tested after gold spraying by an ion sprayer. Finally, an Olympus Fluo View FV1000 laser scanning confocal microscope was used to observe the ultrastructure of the bacterial biofilm on the titanium plate.

The above experiment was repeated without adding titanium alloy plate to observe the ultrastructure of the *S. aureus* biofilm in vitro. In addition, the quantitative detection of *S. aureus* biofilm by crystal violet staining method. The specific method is as follows: added 200 μL of standard bacterial solution to the 96-well plate, incubated at 37°C for 1 week, and replaced the culture solution every day. One week later, different concentrations of LL-37 (10, 5, 2.5, 1.25, 0.62, 0.31, 0.16, 0.08, 0.04, and 0.02 μM) were added to wells 1 to 10, and the control group was added with the same volume of TSB. Standing at 37°C for 24 hours, rinsed the 96-well plate with PBS. After air drying, added 200 μL of 1% crystal violet, dyed for 30 minutes at room temperature, then rinsed the dye solution with distilled water. After natural air drying, added 95% ethanol and stand for 10 minutes. 95% ethanol was used as a blank control, and the absorbance value at 450 nm (OD450) was measured with microplate reader.

2.7. Statistical analysis

The results were analyzed using SPSS17.0 statistical software, and all data were expressed as mean ± standard deviation. An analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test was used to determine significance between groups, and a P value of $<.05$ was considered statistically significant.

3. Results

3.1. Determine the minimum inhibitory concentration of LL-37

The antibacterial activity of LL-37 against *S. aureus* was obtained by two-fold micro dilution method at the concentration of 10, 5, 2.5, 1.25, 0.62, 0.31, 0.16, 0.08, 0.04, and 0.02 μM (Fig. 1). After incubation, the MIC was read as the lowest concentration of antimicrobial agent that visibly inhibited bacterial growth. The results showed that the MIC of LL-37 against *S. aureus* was about 0.62 μM.

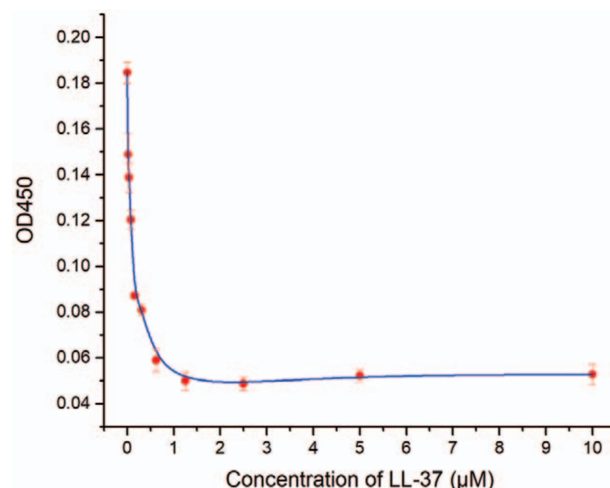

Figure 1. Inhibition curve of LL-37 against *S. aureus.* Three replicates were performed at each concentration, and the results were expressed as the mean ± standard deviation.

3.2. The effect of LL-37 on the adhesion of S. aureus

After culturing *S. aureus* for 1, 2, and 4 hours, different concentrations of LL-37 were added, and negative controls were set. The absorbance (OD450) was measured with a spectrophotometer. The results show (Fig. 2A, B, and C) that different LL-37 concentrations have a significant impact on the adhesion of *S. aureus*. Compared with the negative control group, when the LL-37 concentration at 0.16 μM, the amount of bacteria attached was significantly reduced ($P < .05$). That is, LL-37 can inhibit the initial attachment behavior of *S. aureus* at 1/4 MIC concentration, and the inhibitory effect shows a dose enhancement. However, the different culture time of *S. aureus* (after 1, 2, and 4 hours) did not have a significant effect.

3.3. The static bacterial biofilm model in vitro

To establish an in vitro static biofilm model, the standard bacterial solution was added to the 24-well plate and incubated at 37°C for 4 consecutive days. The results show that *S. aureus* could form mature biofilm after 4 days of continuous cultivation. After fuchsin staining, the bottom of 24 well plate was covered with red dense substance. Under the microscope, lots of bacteria can be seen to grow and form membranous colonies (Fig. 3A). The planktonic *S. aureus* in the culture medium was observed under high power microscope after Gram staining (Fig. 3B). The results showed that *S. aureus* formed stable biofilm *in vitro* under the current condition, this static biofilm model could be used for follow-up study.

3.4. Anti-staphylococcal biofilm effects of LL-37

In order to observe the anti-staphylococcal biofilm effects of LL-37, we prepared static biofilm models in vitro and titanium alloy carrier biofilm models, and 5 μM LL-37 was used to experimental group. Scanning electron microscope observation revealed that the control group was significantly different from the LL-37 group, as shown in Fig 4. The mature biofilm structure of the control group is still intact, and the bacteria are densely arranged (Fig. 4A, B, E, and F). In the LL-37 treatment group, the arrangement of the biofilm was loose, the total amount of mature plaque was significantly reduced, and only a few intact bacteria were seen (Fig. 4C, D, G, and H). From the above results, we can

Effect on adhesion (1h)

OD450

Concentration of LL-37 (μM)

A

Effect on adhesion (2h)

OD450

Concentration of LL-37 (μM)

B

Effect on adhesion (4h)

OD450

Concentration of LL-37 (μM)

C

Inhibition on biofilm

OD450

Concentration of LL-37 (μM)

D

Figure 2. The effect of LL-37 on the adhesion of *S. aureus*. After culturing *S. aureus* for 1 (A), 2 (B), 4 (C) hours, inhibitory activity of LL-37 on *S. aureus* biofilm (D). Statistical analysis was calculated by Tukey's honestly significant difference (HSD) test. N=3 independent experiments, 8 replicates in each ($^{*}P<.05$, $^{**}P<.01$; $^{***}P<.001$).

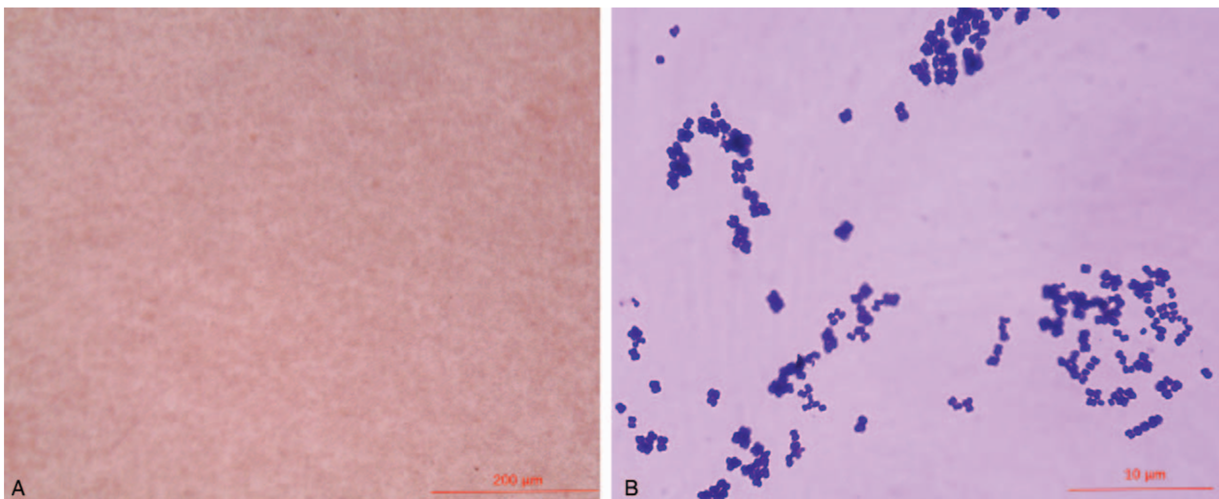

Figure 3. Micrographs of *S. aureus* and its biofilm. (A) *S. aureus* biofilm formed in vitro, stained with 1% acid fuchsin. Magnifications: 40×. (B) Planktonic *S. aureus* in the medium, stained with 1% crystal violet solution. Magnifications: 1000×.

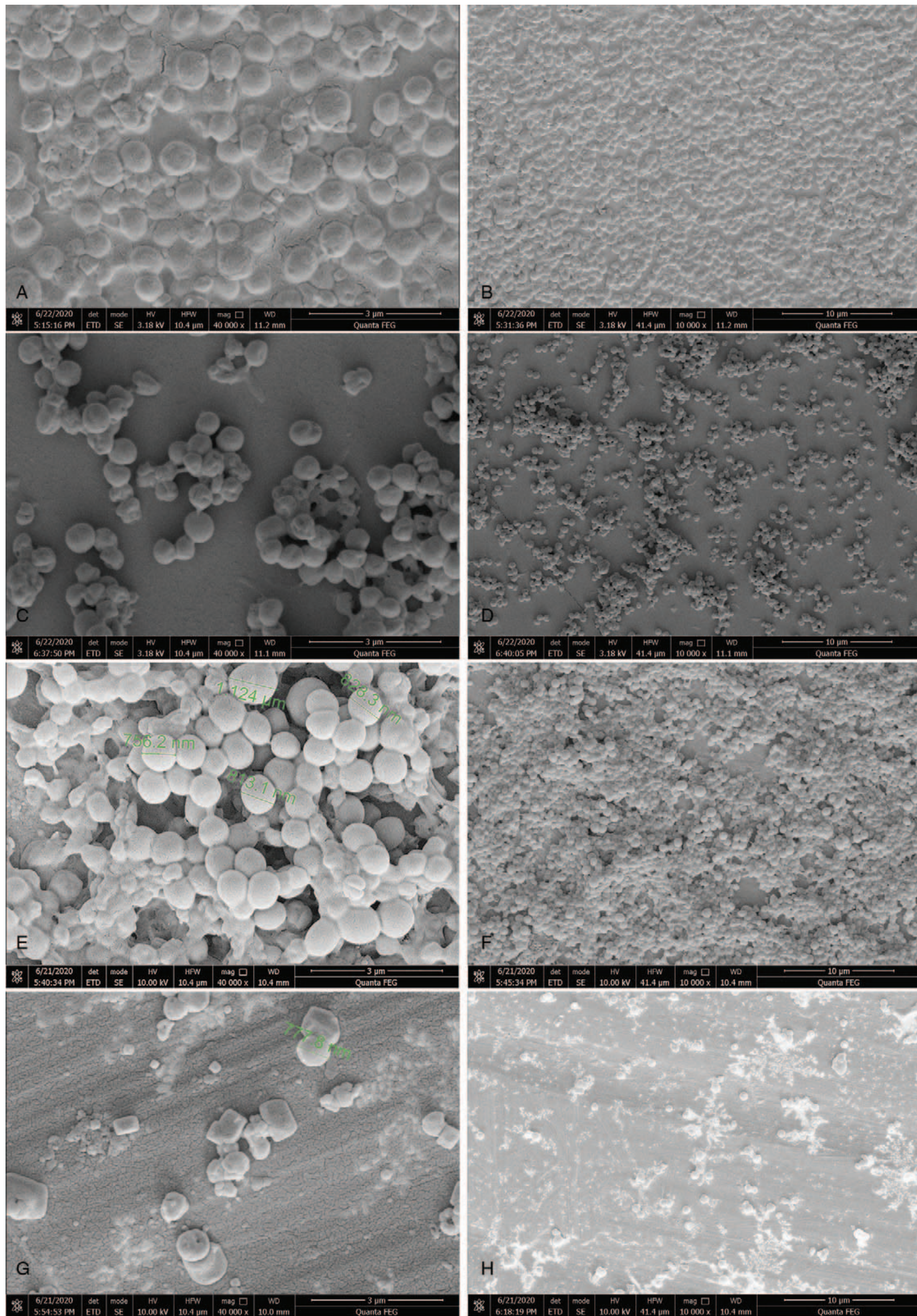

Figure 4. Scanning electron microscopy (SEM) micrographs of the *S. aureus* biofilm. (A) and (B) are the control group micrographs of preformed biofilm in vitro at 40000x and 10000x. (C) and (D) are LL-37 treatment group micrographs of preformed biofilm in vitro at 40000x and 10000x. (E) and (F) are the control group micrographs of preformed biofilm on titanium plate at 40000x and 10000x. (G) and (H) are LL-37 treatment group micrographs of preformed biofilm on titanium plate at 40000x and 10000x.

see that LL-37 at a concentration of 5 μM exert a strong inhibitory effect on the mature biofilm of *S. aureus* (Fig. 4C, D). Moreover, the scanning electron microscope observation results show that LL-37 has a significant anti-staphylococcal biofilm effects on the titanium alloy surface (Fig. 4G, H). As shown in Figure 4G, the structure of biofilm and mature plaque almost

disappeared, and only a small number of damaged bacteria were seen, while the complete biofilm structure can be observed under the control group (Fig. 4H).

The analysis results of crystal violet staining method showed that compared with the control group, the total amount of biofilm in the different concentrations of LL-37 treatment group decreased to varying degrees, and the 1/4 MIC concentration group had a statistically significant difference ($P < .05$) (Fig. 2D). This indicates that LL-37 can affect the formed biofilm at the MIC concentration and exhibit a dose-dependent enhancement.

4. Discussion

The results showed that LL-37 had strong antibacterial activity in a dose-dependent manner at nanomolar concentrations in vitro. This result is consistent with the research results reported previously. Noore et al found that LL-37 was effective in killing extracellular *S. aureus at* nanomolar concentrations, while lactoferricin B was effective at micromolar concentrations and doxycycline and cefazolin at millimolar concentrations. LL-37 was found to exhibit over 90% killing efficacy at as low as 250 nM, over 99% at 500 nM, and 100% at 3.0 μM.[30] In addition, related studies have shown that LL-37 has higher antibacterial activity than commonly used antibiotics, and it is not easy to develop drug resistance[22,30,31] These studies indicate that LL-37 is an ideal non-antibiotic bacteriostatic agent.

Surface attachment is regarded as the first step for biofilm formation. In previous studies, the experimental results of LL-37 effect on the adhesion of *S. aureus* were different. Mishra et al found that LL-37 in the concentration range from 3.1 to 25 μM was unable to inhibit the attachment of *S. aureus* USA300.[27] Luo et al used the crystal violet assay biofilm biomass showed that LL-37 had significant efficacy in preventing biofilm formation by *S. aureus* but was unable to inhibit early biofilms of *S. aureus*.[28] Our experimental results show that in the presence of LL-37, the initial attachment rate of *S. aureus* is significantly reduced, which directly reduces the number of initially attached bacteria on the surface of the carrier. LL-37 played an important intervening role in the first step of biofilm formation.

In recent years, the effect of LL-37 on the *S. aureus* biofilm has attracted lots of attention from researchers. Related research shows that LL-37 can effectively inhibit *S. aureus* biofilm.[14,22,32,33] Luo et al believe that LL-37 can prevent the formation of biofilms, but has no effect on the formed biofilms.[28] Mishra *et al* observed that LL-37 was unable to inhibit bacterial attachment or disrupt preformed biofilm.[27] In our research, the inhibitory effect of LL-37 on the formed biofilm was verified. In addition, we also found that LL-37 has obvious inhibitory activity on the formed biofilm on the titanium alloy surface.

5. Conclusion

This study further confirmed the role of antimicrobial peptide LL-37 in the process of *S. aureus* infection, including antimicrobial activities, inhibition of bacterial adhesion, and inhibition of mature biofilm. Briefly, LL-37 in vitro on *S. aureus* has a strong antibacterial effect, and the MIC is about 0.62 μM. When the concentration of LL-37 is 0.16 μM, it has a significant impact on the adhesion of *S. aureus* and prevents the formation of bacterial biofilm. In addition, LL-37 can significantly destroy the mature biofilm structure and the stable biofilm structure on the titanium alloy surface. The biofilm in this study is a model in vitro, which has a certain gap with the infection biofilm of clinical cases. In the future, a more mature biofilm model should be established to simulate clinical infection cases.

Author contributions

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