

Original Article

Insulin treatment enhances pseudomonas aeruginosa biofilm formation by increasing intracellular cyclic di-GMP levels, leading to chronic wound infection and delayed wound healing

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Abstract: Diabetes-related infections have become challenging and important public health problems in China and around the world. *P. aeruginosa* plays an important role in diabetic foot infections. As a gram-negative opportunistic pathogen, *P. aeruginosa* causes recurrent and refractory infections that are characterized by biofilm formation. Previous studies have demonstrated that biofilm-challenged wounds typically take longer to heal than non-biofilm-challenged normal wounds in diabetic mouse models. In the present study, we sought to explore the mechanism via which insulin treatment affects cyclic di-GMP signaling in *P. aeruginosa*-infected chronic wounds in db/db diabetic mice. We found that the wounds of diabetic mice healed more slowly than those of nondiabetic mice. Moreover, wound healing in diabetic mice treated with insulin exhibited a considerable delay. Peptide nucleic acid-fluorescence in situ hybridization (PNA-FISH) was used to detect biofilms on *P. aeruginosa*-infected wound tissues. Increased intracellular c-di-GMP levels promoted biofilm formation in wound tissues from nondiabetic mice. Greater biofilm formation was observed in the wounds of insulin-treated diabetic mice than in the wounds of untreated diabetic mice or nondiabetic mice, in both the PAO1/*p_{lac}-yhjH*- and PAO1-infected groups. Quantitative RT-PCR indicated that upon infection with PAO1/*P_{lac}-yhjH* (the low c-di-GMP expression strain), the expression of IL-4 RNA was significantly higher in diabetic mice treated with insulin than in untreated diabetic mice or nondiabetic mice at each observation time point. Peak expression of IFN- γ occurred earlier in diabetic mice treated with insulin than in untreated diabetic mice with each of the experimental strains. Finally, *P. aeruginosa* harboring the plasmid pCdrA:*gfp*^s was used as a reporter strain to monitor c-di-GMP levels. We found that insulin could promote biofilm formation by increasing intracellular c-di-GMP levels in vitro. Taken together, these data demonstrate that insulin treatment increases intracellular c-di-GMP levels, promotes biofilm formation and prolongs the inflammation period during the healing of infected wounds, resulting in delayed wound healing.

Keywords: Insulin, *pseudomonas aeruginosa*, biofilm, cyclic di-GMP, wound infection, inflammation

Introduction

In China, approximately 92.4 million adults (50.2 million men and 42.2 million women), or 9.7% of the adult population, have diabetes, and more than 148.2 million adults (76.1 million men and 72.1 million women) have prediabetes [1]. There are several complications associated with diabetes, ranging from heart disease to blindness. In addition, the most common complications experienced by patients with diabetes are infections, such as skin and lung infections. Foot infections are a common

and serious problem among patients with diabetes [2]. Therefore, diabetes-related infectious diseases have become a challenging and important public health problem in China and around the world.

Pseudomonas aeruginosa plays an important role in chronic wound infections and cystic fibrosis-associated pneumonia by forming biofilms. Although a wide range of bacterial species have been cultured from infected chronic wounds, including *Staphylococcus aureus*, *P. aeruginosa*, *Enterococcus faecalis*, coagulase-negative

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staphylococci, *Proteus* species, and anaerobic bacteria [3, 4], *P. aeruginosa* is the most common gram-negative rod-shaped bacterium isolated from the soft tissue or ulcerated skin of patients with diabetic foot infections (DFIs) [5, 6]. *P. aeruginosa* is also a major pathogen in patients with chronic lung infections and is the primary cause of morbidity and mortality in cystic fibrosis (CF) [7].

P. aeruginosa is a gram-negative opportunistic pathogen that causes recurrent and refractory infections characterized by biofilm formation. Biofilms are defined as structured communities of a large number of bacteria that adhere to inert or living surfaces; secrete a polysaccharide matrix, fibrinogen, lipoprotein, and DNA; and are enveloped in this self-produced polymeric matrix [8]. Biofilms are significantly different from planktonic bacteria, and biofilm bacteria are protected against antibacterial treatment and phagocytosis and are able to evade the innate and adaptive immune systems [9]. Therefore, *P. aeruginosa* biofilm infection leads to serious clinical problems.

Multiple mechanisms are involved in the regulation of *P. aeruginosa* biofilm formation, and these mechanisms mainly involve cyclic diguanosine-5'-monophosphate (c-di-GMP), small RNAs (sRNAs) and quorum sensing (QS) [10]. C-di-GMP was discovered by Benziman et al. [11] as an activator of cellulose synthase in *Gluconacetobacter xylinus* in 1987 [12] and was recently identified as a novel bacterial global secondary messenger that is considered to be a master regulator of the transition between the biofilm and planktonic lifestyles. Diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) regulate the formation and degradation of c-di-GMP, respectively. DGC activity is associated with the GGDEF domain, while PDE activity is associated with the EAL or HD-GYP domain. High intracellular levels of c-di-GMP promote biofilm formation by increasing the surface adherence of bacterial cells, cell aggregation and the secretion of extracellular polymeric substances. In contrast, low intracellular levels of c-di-GMP inhibit biofilm formation and stimulate bacterial motility [13]. Among the processes associated with biofilm formation, surface attachment, swimming motility, swarming motility, twitching motility, and the production of the extracellular matrix are regulated by c-di-GMP signaling [14].

A previous study demonstrated that biofilm-challenged wounds typically take longer to heal than non-biofilm-challenged normal wounds in a diabetic mouse model [15]. Currently, there is much controversy regarding whether insulin can be used to treat *P. aeruginosa* infections. One study reported that low-dose insulin treatment can attenuate the sepsis caused by *P. aeruginosa* infection after severe burn injuries [16]. However, Chase Watters' group found that insulin treatment could not decrease the *P. aeruginosa* bacterial load in diabetic wounds and instead enhanced biofilm formation by this bacterium [17, 18]. To date, there have been no studies on the role of the c-di-GMP signaling pathway in wound healing in diabetic mouse models, and no pathway studies have confirmed whether intervention with insulin clears bacterial loads or promotes the formation of *P. aeruginosa* biofilms.

Therefore, the main purpose of this study was to explore the effect of insulin on the healing of wounds infected with *P. aeruginosa* in diabetic mice. In addition, we examined whether these effects were associated with intracellular c-di-GMP signaling. Finally, in this study, the effect of insulin on *P. aeruginosa* biofilm formation in the diabetic mouse model was confirmed at the level of the c-di-GMP signaling pathway. Three *P. aeruginosa* strains with different levels of intracellular c-di-GMP and different biofilm formation abilities were chosen as experimental strains: the control strain PAO1, the low c-di-GMP expression strain PAO1/*P*_{lac}-*yhjH* and the high c-di-GMP expression strain PAO1 Δ *wspF*. We assessed the intracellular levels of c-di-GMP in *P. aeruginosa* by transcriptionally fusing the c-di-GMP-responsive *cdrA* promoter to the gene encoding green fluorescent protein (GFP), a method that was developed by Rybtke et al. [19]. We found that insulin treatment promoted biofilm formation by increasing intracellular c-di-GMP levels in vitro. In vivo, the wounds of diabetic mice healed more slowly than those of nondiabetic mice, and among these mice, the slowest wound healing was observed for diabetic mice treated with insulin. We observed increased inflammatory cell infiltration (via hematoxylin-eosin (HE) staining), increased levels of released inflammatory factors and enhanced biofilm formation (by peptide nucleic acid-fluorescence in situ hybridization (PNA-FISH)) in diabetic mice treated with insulin. Taken together, these data demonstrated that insulin treatment increased intracellular c-di-

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GMP levels, promoted biofilm formation, and prolonged the inflammatory period, leading to delayed diabetic wound healing.

Materials and methods

Bacterial strains and growth conditions

This study used the sequenced *P. aeruginosa* PAO1 wild-type strain, the PAO1 Δ wspF strain (a *wspF* mutant of PAO1 constructed by allelic exchange), the PAO1/P_{lac}-*yhjH* strain (PAO1 harboring the P_{lac}-*yhjH* vector), and a PAO1 strain harboring the P_{cdrA}-*gfp* vector (to provide a fluorescent readout of the intracellular level of c-di-GMP in *P. aeruginosa*), all of which were generously donated by Dr. Liang Yang (Nanyang Technological University, Singapore). The *P. aeruginosa* strains were grown at 37°C in Luria-Bertani (LB) medium or ABTGC [20] medium and on LB agar or ABTGC agar at 37°C. Antibiotics were supplied where necessary at the following concentrations: the PAO1/P_{lac}-*yhjH* strain was grown with 10 µg/ml tetracycline, and the *P. aeruginosa* strain harboring the P_{cdrA}-*gfp* vector was grown with 200 µg/ml carbenicillin. Before each experiment, frozen *P. aeruginosa* cells stored at -80°C were resuscitated and inoculated onto an LB agar plate with or without antibiotics by using a 1-µL inoculation loop. A single colony was picked and subcultured in LB broth in a temperature-regulated shaker set at 37°C, with shaking at 200 rpm for 14~16 hours. Then, the overnight culture was diluted with fresh ABTGC medium to the appropriate bacterial cell density for subsequent experiments.

Animals

Male, 8-week-old, db/db mice were purchased from the Biomedical Research Institute of Nanjing University. Homozygous BKS.Cg-Dock7m+/+Leprdb/Nju mice were deemed diabetic if their blood glucose levels were greater than 20 mmol/L, while wild-type BKS.Cg-Dock7m+/+Leprdb/Nju mice were deemed nondiabetic. All the mice were raised in specific pathogen-free (SPF)-level rooms in the Animal Center Laboratory of Guangxi University (Nanning, People's Republic of China), fed a standard chow diet, provided free access to tap water and acclimated for 1 week prior to surgery. This protocol was conducted according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes

of Health (NIH publication 85-23, revised 1996) and was approved by the Animal Care and Use Committee of Guangxi Medical University, People's Republic of China.

Chronically wounded diabetic mouse model

A diabetic mouse model with chronic wounds was established as described by Watters C and colleagues. Briefly, the mice were anesthetized and shaved, and a dorsal full-thickness surgical excision wound was created (size: 1.5 × 1.5 cm). Then, the wounds were covered with a transparent wound care accessory, i.e., a semi-permeable polyurethane dressing (OPSITE, Smith & Nephew, Hull, England). Approximately 10⁴ colony forming units (CFUs) of *P. aeruginosa* was injected into the gap between the polyurethane dressing and the wound. Some groups of diabetic mice were administered hypodermic injections with 2 units of Humulin® N insulin (Eli Lilly, Indianapolis, IN, USA) daily.

Pathological analysis of infected wound tissue

The infected wound tissue (size range: more than 2 mm of the visible wound margin and as deep as the surface of the mouse crest) was extracted completely from the back of the mouse at each observation time point. The tissue was then fixed in 10% (v/v) formalin solution, embedded in paraffin, sectioned, and stained with HE to visualize the inflammatory reactions of the cells. PNA-FISH was used to identify the bacteria in infected chronic wounds according to the protocol described by Fazli et al. [20]. In brief, paraffin-embedded tissue samples were deparaffinized by treatment with xylene (twice for 5 min), 99.9% ethanol (twice for 3 min), and 96% ethanol (twice for 3 min) and then washed three times in Milli-Q water for 3 min each. One drop of an approximately 300 nmol/L concentration of a PNA-FISH probe that was conjugated to Texas Red and specific for *P. aeruginosa* 16S rRNA was applied to the tissue section, which was then covered with a cover slip. The samples were incubated at 55°C for 90 min. The cover slip was removed, and the slides were washed in warm washing buffer at 55°C for 30 min and then air-dried in the dark.

Antibiotic resistance assay

The amikacin resistance of *P. aeruginosa* in the wounds was examined in accordance with Chase Watters' method. Chronically wounded

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diabetic mice infected with *P. aeruginosa* were obtained as described above. A sterile gauze pad was placed over the wound and under the transparent wound care accessory (OPSITE). On days 4, 8, 12, and 16 post surgery, the mice were euthanized, and the gauze pads were removed from the wounds. The gauze pads were sheared in half, weighed, and then immersed in sterile PBS or a 200 mg/mL amikacin solution for 5 hours. The amikacin-treated gauze pad segments were neutralized in PBS for 10 min and then immersed in PBS. Then, the gauze pads were thoroughly homogenized. The obtained suspension was serially diluted and plated to determine the CFU/g of the gauze pad. The amikacin tolerance of *P. aeruginosa* in the wounds was determined from the CFU/g value obtained after treatment, which was divided by the CFU/g value obtained for the PBS-treated gauze section and multiplied by 100.

Quantitative RT-PCR assay

Infected wound tissue samples were collected as described above. On days 4, 8, 12, and 16 post surgery, the tissue samples were weighed and placed in a precooled 1.5-ml EP tube with RNA Protector and then stored at -80°C . The total RNA was extracted from the above tissue samples by following the manufacturer's instructions for the MiniBEST Universal RNA Extraction Kit (TaKaRa, Japan). The first-strand complementary DNA (cDNA) was synthesized according to the manufacturer's recommendations for the PrimeScript™ RT Reagent Kit and then purified by using gDNA Eraser.

Specific primer sets for genes encoding murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5'-AAGGTCGGAGTCAACGGATT-3'; reverse: 5'-TTGATGACAAGCTTCCCGTT-3'), interferon- γ (IFN- γ) (forward: 5'-TATAGCTGCCATCGGCTGAC-3'; reverse: 5'-AAGCC-AAGATGCAGTGTGTAGC-3'), interleukin-4 (IL-4) (forward: 5'-TACCAGGAGCCATCCACGGATG-3'; reverse: 5'-TGTGGTGTCTTCGTTGCTGTGAG-3'), and interleukin-6 (IL-6) (forward: 5'-TCCATCCAGTTCCTTCTTG-3'; reverse: 5'-AAGCCTCCGACTTGTAAGT-3') were used to amplify DNA templates on a real-time PCR system (ABI 7500, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions for the SYBR Premix Ex Taq II PCR Kit. These samples were used to study the effect of insulin on *P. aeruginosa* in vitro.

Biofilm formation assay

A microtiter plate-based biofilm assay was used to quantitate the biofilm mass after treatment, as described by Merritt et al. [21]. In brief, an overnight culture of *P. aeruginosa* was diluted to approximately 10^6 CFU/ml with ABTGC medium supplemented with denatured insulin or Humulin® N insulin at a final concentration of 200 nU/ml, 200 $\mu\text{U}/\text{ml}$, or 200 mU/ml. The bacteria were inoculated directly into sterile microtiter plates at 200 μl per well, and five wells were used per concentration per strain. The plates were incubated in a temperature-regulated shaker at 37°C and 110 rpm for 12 hours. The planktonic bacteria were removed from each microtiter plate by pipetting gently, and the plates were then washed three times with PBS. The plates were air-dried for 15 min at room temperature, and 220 μl of 0.1% (w/v) crystal violet solution was then added to each well. The wells were stained for 15 min at room temperature. The crystal violet solution was removed by rinsing the wells three times with sterile distilled water. The plates were air-dried, and 33% (v/v) acetic acid was then added to each stained well. The plates were incubated for 15 min at room temperature. The crystal violet/acetic acid solution in each well was mixed by pipetting, and 200 μl of the contents of each well was then transferred to a separate well in a new 96-well plate. The optical density (OD) of the sample in each well was measured spectrophotometrically at a wavelength of 595 nm.

c-di-GMP reporter assay

The fluctuations in intracellular c-di-GMP levels were monitored indirectly by detecting changes in the output of the $P_{\text{cdrA}}\text{-gfp}$ reporter in *P. aeruginosa* according to a method previously described by Chua et al. [19] and Christensen et al. [22]. Briefly, an overnight culture of *P. aeruginosa* was diluted to approximately 10^6 CFU ml^{-1} with fresh ABTGC medium supplemented with Humulin® N insulin or denatured insulin (the final amount of insulin was 200 nU, 200 μU , or 200 mU). Next, 200 μl of each culture was transferred into individual wells of a 96-well microplate. Then, the microplate was sealed with Parafilm and incubated at 37°C , with shaking at 185 rpm for 12 hours. The optical density at 600 nm (OD_{600}) and the GFP expression (excitation 490 nm/emission 515 nm) from $P_{\text{cdrA}}\text{-gfp}$ in *P. aeruginosa* were measured using a Thermo Fisher spectrophotome-

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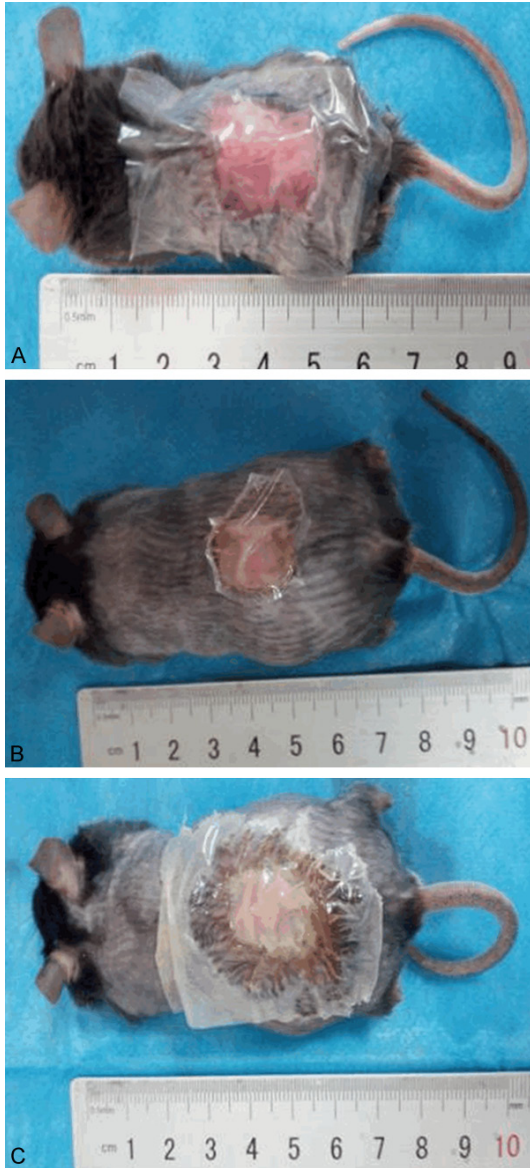


Figure 1. A mouse model of chronic wounds infected with *P. aeruginosa* was established for three groups: nondiabetic mice (A), diabetic mice (B) and diabetic mice treated with insulin (C).

ter (SN: 3020-128). The OD₆₀₀ and GFP fluorescence (in relative fluorescence units (RFUs)) were recorded for each well of the 96-well microplate. The RFU values are the fluorescence intensity units corrected for cell density.

Effects of Humulin® N insulin on P. aeruginosa growth in vitro

An overnight *P. aeruginosa* culture was diluted to approximately 10⁶ CFU ml⁻¹ with fresh ABTGC medium supplemented with Humulin® N insulin or denatured insulin. Humulin® N insulin or

denatured insulin was added to a final concentration of 200 nU/ml, 200 µU/ml, or 200 mU/ml. Next, 3 ml of diluted overnight culture was transferred to a sterile 10-ml BD Falcon tube and incubated at 37°C, with shaking at 185 rpm for 24 hours. Growth was monitored by determining the CFU count at 4, 8, 16, and 24 hours.

All the in vitro experiments described above were performed in triplicate and repeated at least three times, and the results are shown as the mean ± SD.

Statistical analysis

The results were analyzed by the SPSS18.0 software package. The mean values of the statistical results for the measured data were presented as the mean ± standard error. Significant differences between groups were determined by analysis of variance (ANOVA) based on the mean values for the groups, which corresponded to a normal distribution. *P*-values less than 0.05 were considered to be statistically significant.

Results

The db/db mouse model is the most appropriate model for studying diabetic wound-healing interventions, as it is more appropriate than Akita and streptozocin-induced C57BL/6J mice [23]. This model is reproducible and can provide more than 28 days to demonstrate delayed wound healing following biofilm challenge, with low mortality [24]. *P. aeruginosa* biofilm-infected diabetic wounds in db/db mice are suitable as a chronic wound infection model for studying the role of microbial biofilms in chronic wound infections and the effects of specific biofilm treatments on wound healing. However, there have been no published studies on the role of *P. aeruginosa* biofilms and the c-di-GMP signaling pathway in wound healing in diabetic mouse models with insulin intervention. Therefore, we compared the factors that affected the healing of wounds infected with the PAO1 wild-type strain, PAO1/*P_{lac}-yhjH* strain (with low intracellular c-di-GMP expression) and PAO1Δ*wspF* strain (with high intracellular c-di-GMP expression). Subsequently, for each strain, the mice were divided into three groups: nondiabetic mice (**Figure 1A**), diabetic mice (**Figure 1B**) and diabetic mice treated with insulin (**Figure 1C**). If purulence under the surgical semipermeable

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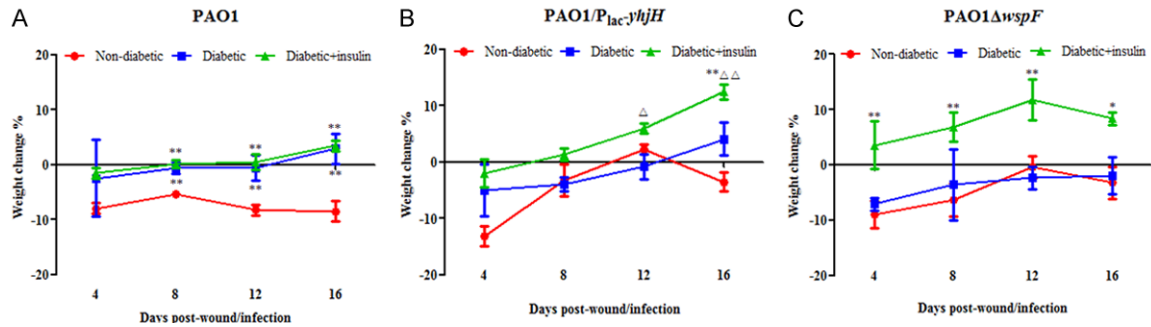


Figure 2. The nondiabetic and diabetic mice exhibited a loss of body weight, while the diabetic mice treated with insulin gained body weight, among mice infected with the (A) PAO1 wild-type strain, (B) PAO1/p_{lac}-yhjH strain and (C) PAO1ΔwspF strain over the 16 days of the experiment. The mice were weighed every 4 days, and the percent change in body weight was calculated by the following equation: $(W_t - W_0)/W_0 \times 100$, where W_t was the weight on the day of observation, and W_0 was the weight on the day of surgery. One-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test was used to determine the differences between groups. Note: * $P < 0.05$ compared with the nondiabetic group, ** $P < 0.01$ compared with the nondiabetic group, $\Delta P < 0.05$ compared with the diabetic group, $\Delta\Delta P < 0.01$ compared with the diabetic group.

polyurethane dressing was observed on the third day post infection, the model was considered to have been successfully generated (Figure 1B, 1C). Based on this criterion, a mouse model with wounds infected with *P. aeruginosa* strains exhibiting different levels of intracellular c-di-GMP expression was successfully established. The mortality rate of this model was low, with a value of 1.04%, and only 2 deaths occurred among the 196 diabetic mice, one in the group infected with the PAO1 control strain and one in the group infected with the PAO1/p_{lac}-yhjH strain (with low c-di-GMP expression).

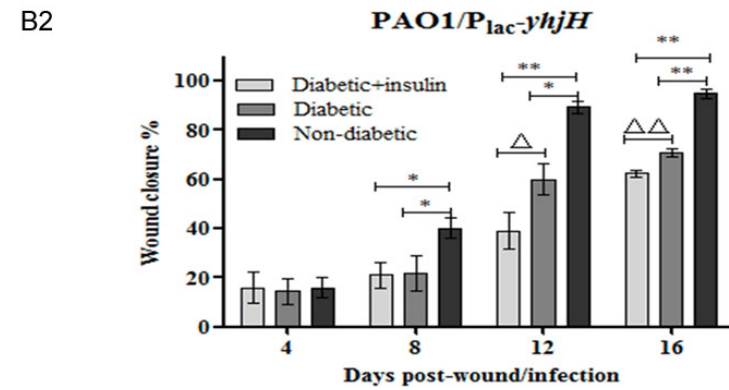
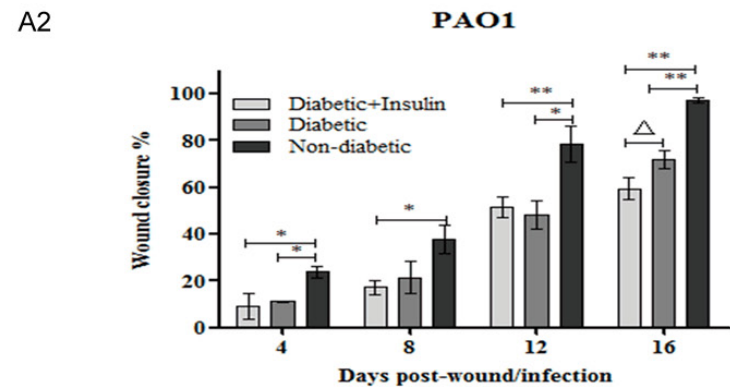
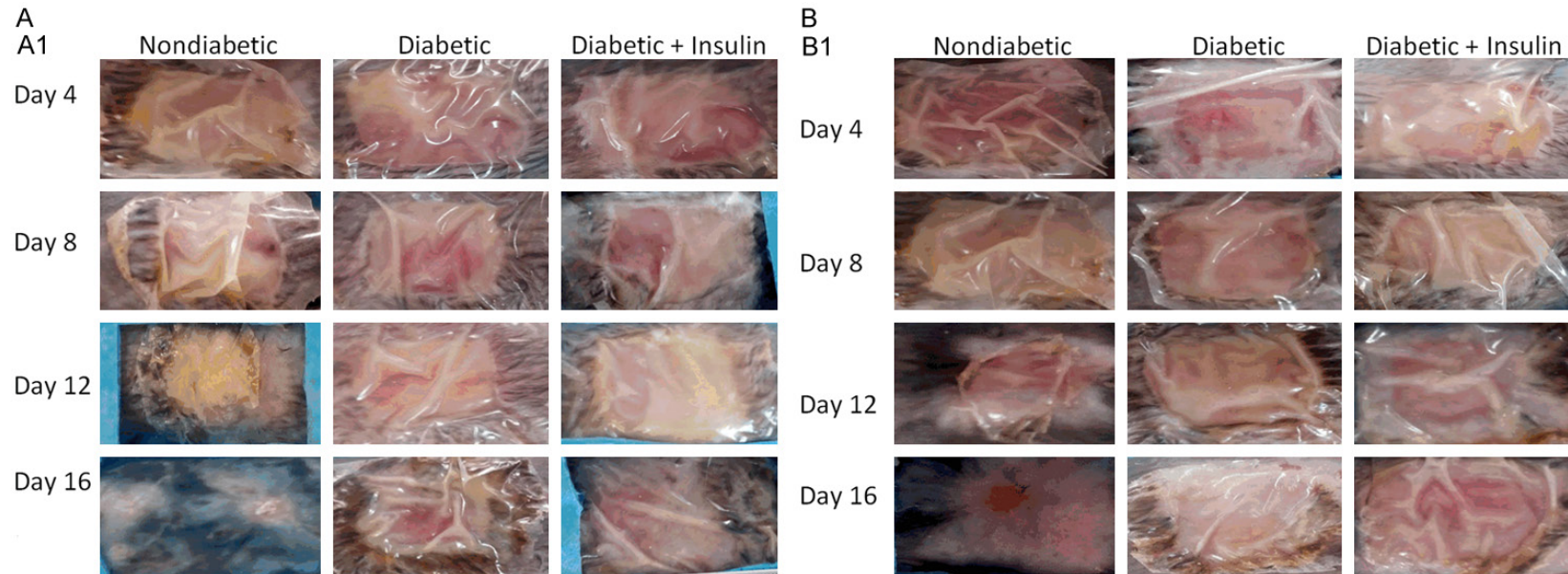
The effect of insulin on the body weights and wound healing rates of diabetic mice with chronic wounds infected with *P. aeruginosa*

Among the PAO1 wild-type-, PAO1/p_{lac}-yhjH- and PAO1ΔwspF-infected mice, the nondiabetic and diabetic mice lost an average of 3.22% to 8.53% of their body weight, while the diabetic mice treated with insulin gained an average of 3.38% to 12.4% of their body weight over the 16 days of the experiment. Among these mice, the percent gain in body weight observed for the diabetic mice treated with insulin was significantly higher than the loss in weight observed for the diabetic mice infected with PAO1/p_{lac}-yhjH or PAO1ΔwspF (Figure 2A-C). These results demonstrated that the diabetic mice treated with insulin were healthier than the nontreated diabetic mice.

The percent wound closure was calculated by the following equation: $(A_0 - A_t)/A_0 \times 100$, where

A_0 was the wound area on the day of surgery, and A_t was the wound area on the day of observation. The mice were anesthetized, and the area of wound closure was measured by a ruler on the day of surgery and at 4-day intervals post surgery. On day 4 post surgery, only the wounds of the nondiabetic mice infected with PAO1/p_{lac}-yhjH were similar to those of diabetic mice and diabetic mice treated with insulin, and the average percent wound closure values were 16.04, 14.5 and 16.01% respectively. The wound-healing rates of the nondiabetic mice infected with PAO1/p_{lac}-yhjH were higher than those of the other two groups. Subsequently, on each day of observation post surgery, the percent wound closure for nondiabetic mice was higher than that for diabetic mice or diabetic mice treated with insulin among the mice infected with the PAO1 wild-type, PAO1/p_{lac}-yhjH and PAO1ΔwspF strains (Figure 3A-C). Among the mice infected with the PAO1 wild-type, PAO1/p_{lac}-yhjH and PAO1ΔwspF strains, by day 16, the wounds of the nondiabetic mice were almost completely closed, with percent wound closure values of 97.11, 94.83 and 81.07%, respectively (Figure 3A-C). Interestingly, by day 16 in mice infected with the PAO1 wild-type strain and by day 12 and day 16 in mice infected with the PAO1/p_{lac}-yhjH strain, the percent wound closure in diabetic mice treated with insulin was significantly lower than that in untreated diabetic mice (Figure 3A and 3B). By day 16 in mice infected with the PAO1 wild-type strain, the percent wound closure values for diabetic mice and diabetic mice treated with insulin were 71.8% and 59.46%, respec-

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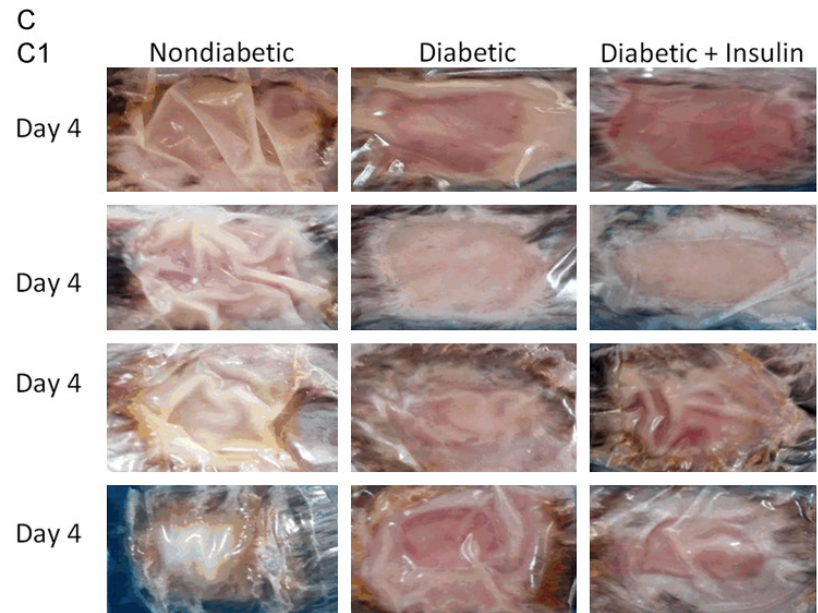
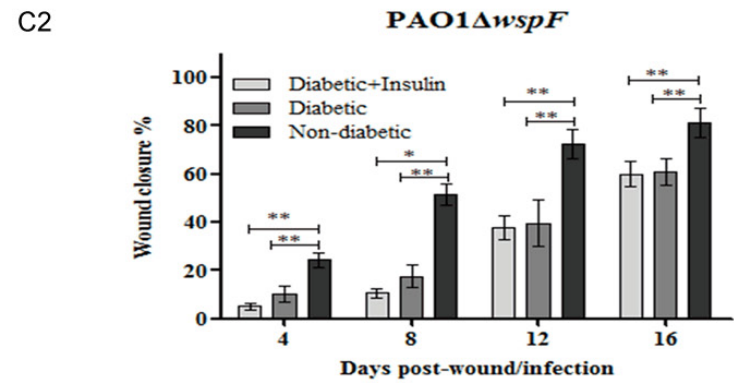


Figure 3. (A) Wound closure in the *P. aeruginosa* infection model was delayed in the PAO1 control group of diabetic mice. All wounds were measured at 4-day intervals to assess wound closure (representative images are shown in A1). The percent wound closure was determined using the following equation: $(A_0 - A_t) / A_0 \times 100$, where A_0 was the wound area on the day of surgery, and A_t was the wound area on the day of observation (A2). One-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test was used. Note: $*P < 0.05$ compared with the nondiabetic group, $**P < 0.01$ compared with the nondiabetic group, $\Delta P < 0.05$ compared with the diabetic group. (B) Wound closure in the *P. aeruginosa* infection model was delayed in the PAO1/*P_{lac}-yhjH*-infected group of diabetic mice. All wounds were measured at 4-day intervals to assess wound closure (representative images are shown in B1). The percent wound closure was determined using the following equation: $(A_0 - A_t) / A_0 \times 100$, where A_0 was the wound area on the day of surgery, and A_t was the area of the wound on the day of observation (B2). One-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test was used. Note: $*P < 0.05$ compared with the nondiabetic group, $**P < 0.01$ compared with the nondiabetic group, $\Delta P < 0.05$ compared with the diabetic group, $\Delta\Delta P < 0.01$ compared with the diabetic group. (C) Wound closure in the *P. aeruginosa* infection model was delayed in the PAO1 Δ *wspF*-infected group of diabetic mice. All wounds were measured at 4-day intervals to assess wound closure (representative images are shown in C1). The percent wound closure was determined using the following equation: $(A_0 - A_t) / A_0 \times 100$, where A_0 was the wound area on the day of surgery, and A_t was the area of the wound on the day of observation (C2). One-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test was used. Note: $*P < 0.05$ compared with the nondiabetic group, $**P < 0.01$ compared with the nondiabetic group.



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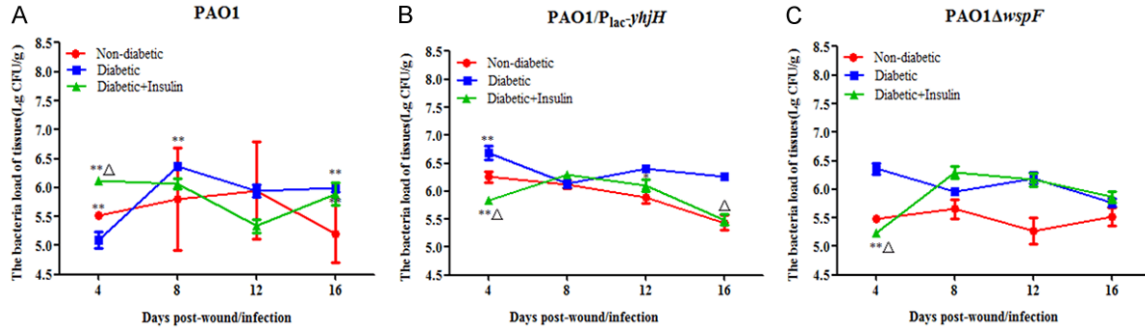


Figure 4. CFU/g values were measured to determine the bacterial loads in the wounds. Insulin treatment did not have any effect on the bacterial loads in chronic wounds of diabetic mice infected with the (A) PAO1 wild-type strain, (B) PAO1/P_{lac}-yhjH strain and (C) PAO1ΔwspF strain. One-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test was used. Note: **P* < 0.05 compared with the nondiabetic group, ***P* < 0.01 compared with the nondiabetic group, Δ*P* < 0.05 compared with the diabetic group, ΔΔ*P* < 0.01 compared with the diabetic group.

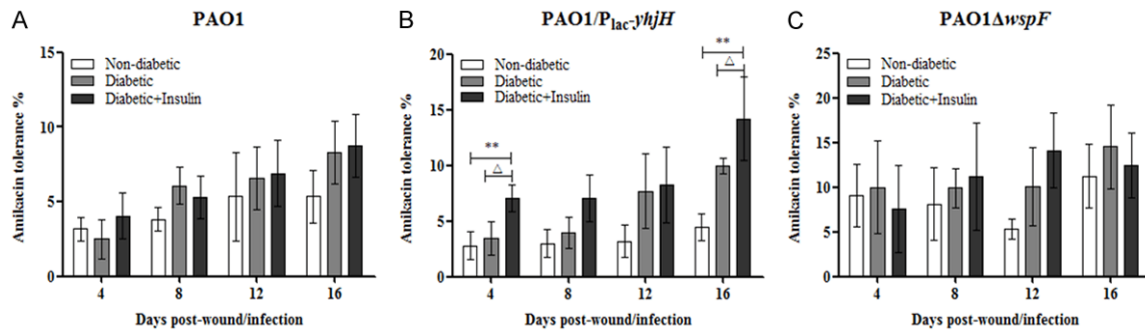


Figure 5. The resistance of *P. aeruginosa* to amikacin in diabetic mice treated with insulin was higher than that in nondiabetic mice and untreated diabetic mice. In particular, in the PAO1/P_{lac}-yhjH-infected group, the resistance of diabetic mice treated with insulin was significantly higher than that of nondiabetic mice or untreated diabetic mice at days 4 and 16. The mice were anesthetized, and the gauze pads were removed from the wounds and treated with amikacin or PBS on the day of surgery and at 4-day intervals post surgery. The amikacin resistance of *P. aeruginosa* in the wounds was calculated by the following equation: (Ra CFU/g)/(Rp CFU/g) × 100, where Ra CFU/g refers to gauze pads treated with amikacin, and Rp CFU/g refers to gauze pads treated with PBS. One-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test was used. Note: **P* < 0.05 compared with the nondiabetic group, ***P* < 0.01 compared with the nondiabetic group, Δ*P* < 0.05 compared with the diabetic group, ΔΔ*P* < 0.01 compared with the diabetic group.

tively. In addition, by day 12, the percent wound closure values for diabetic mice and diabetic mice treated with insulin were 60 and 38.98%, respectively, and by day 16, these values were 70.9 and 62.32% respectively, for mice infected with the PAO1/p_{lac}-yhjH strain (Figure 3B).

Insulin treatment did not have any effect on the bacterial loads of P. aeruginosa-infected chronic wounds in diabetic mice but increased resistance to amikacin

Every 4 days during observation, one group of mice was euthanized, and the tissue removed

from the backs of the mice was weighed. The CFU/g value was determined as a measure of the bacterial loads in the wounds (Figure 4). The CFU count in the wound tissue was determined by a standard colony counting method. The bacterial load increased from an infecting dose of 10⁴ CFU to approximately 10⁶⁻⁷ CFU/g of wound tissue by day 4 or day 8 in all the groups of mice. By day 16, the CFU/g value for the nondiabetic mice was lower than that for the diabetic mice infected with PAO1/P_{lac}-yhjH or PAO1. In general, the wounds of the nondiabetic mice closed by approximately 81.07 to 97.11% during the experiment, and it took an additional 12 to 16 days before the wounds of

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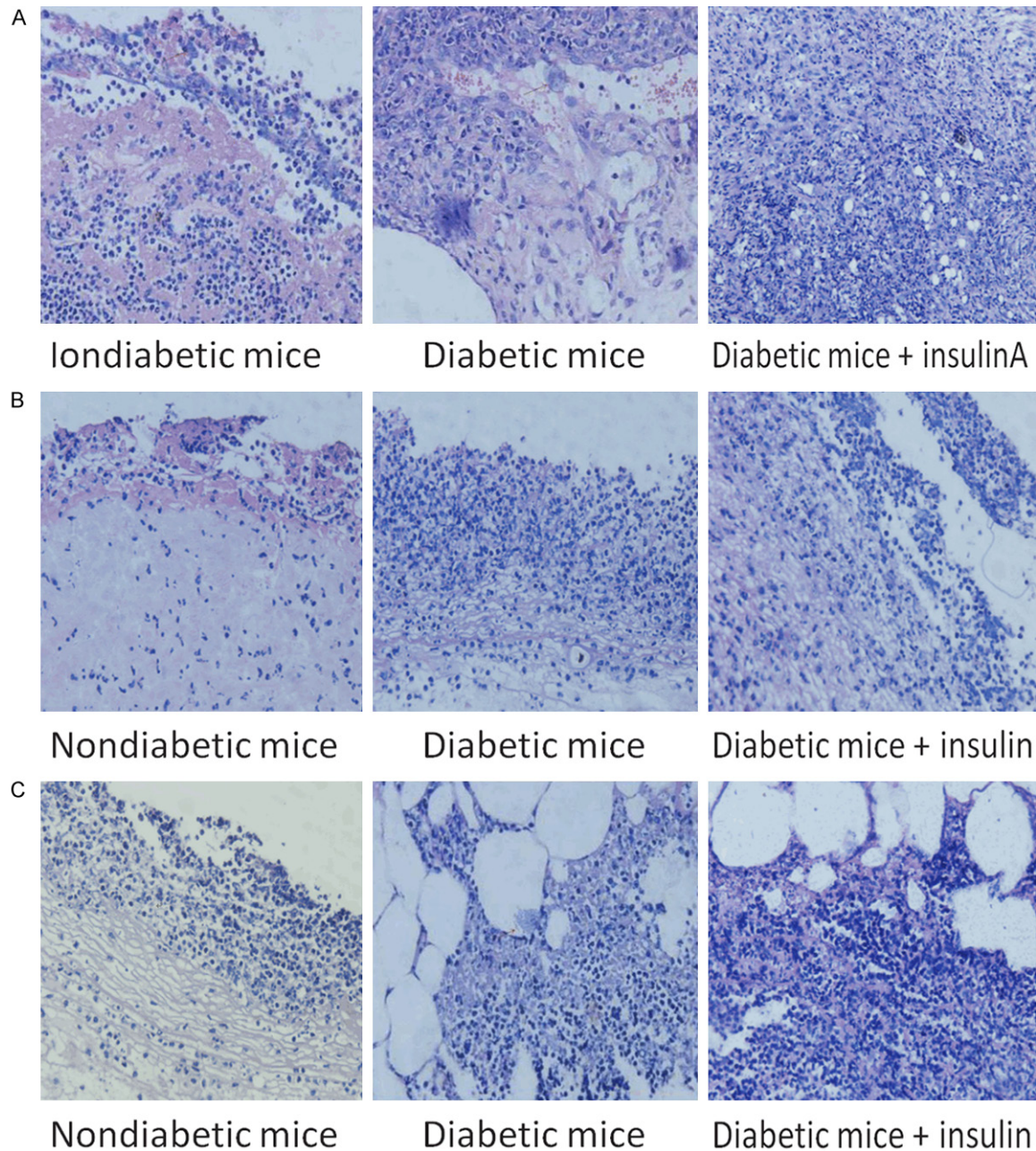


Figure 6. Deparaffinized pathological tissue samples stained with HE to observe the inflammatory reactions of the cells in the infected sections. Arrows indicate *P. aeruginosa* cells observed by microscopy. Tissues infected with the (A) PAO1 wild-type strain, (B) PAO1/ p_{lac} -*yhjH* strain and (C) PAO1 Δ *wspF* strain.

the diabetic mice closed to the same degree. These data indicate that the nondiabetic mice may have been more effective at clearing the infection than the diabetic mice, leading to faster wound healing. The blood, liver and spleen tissues that were extracted from the infected mice were cultured; however, no colonies were obtained in this experiment, suggesting that the *P. aeruginosa* infection did not spread systemically but was localized to only the wound bed.

Next, we determined the resistance of the bacteria in the chronic wounds to amikacin, which is commonly used as an antipseudomonal agent. We did not observe any changes among the nondiabetic mice, diabetic mice and diabetic mice treated with insulin in the groups infected with either the PAO1 wild-type strain or the PAO1 Δ *wspF* strain (Figure 5A and 5C). However, in the group infected with the PAO1/ p_{lac} -*yhjH* strain (Figure 5B), we observed that the resistance of *P. aeruginosa* to amikacin

Insulin treatment in *P. aeruginosa* biofilm infection

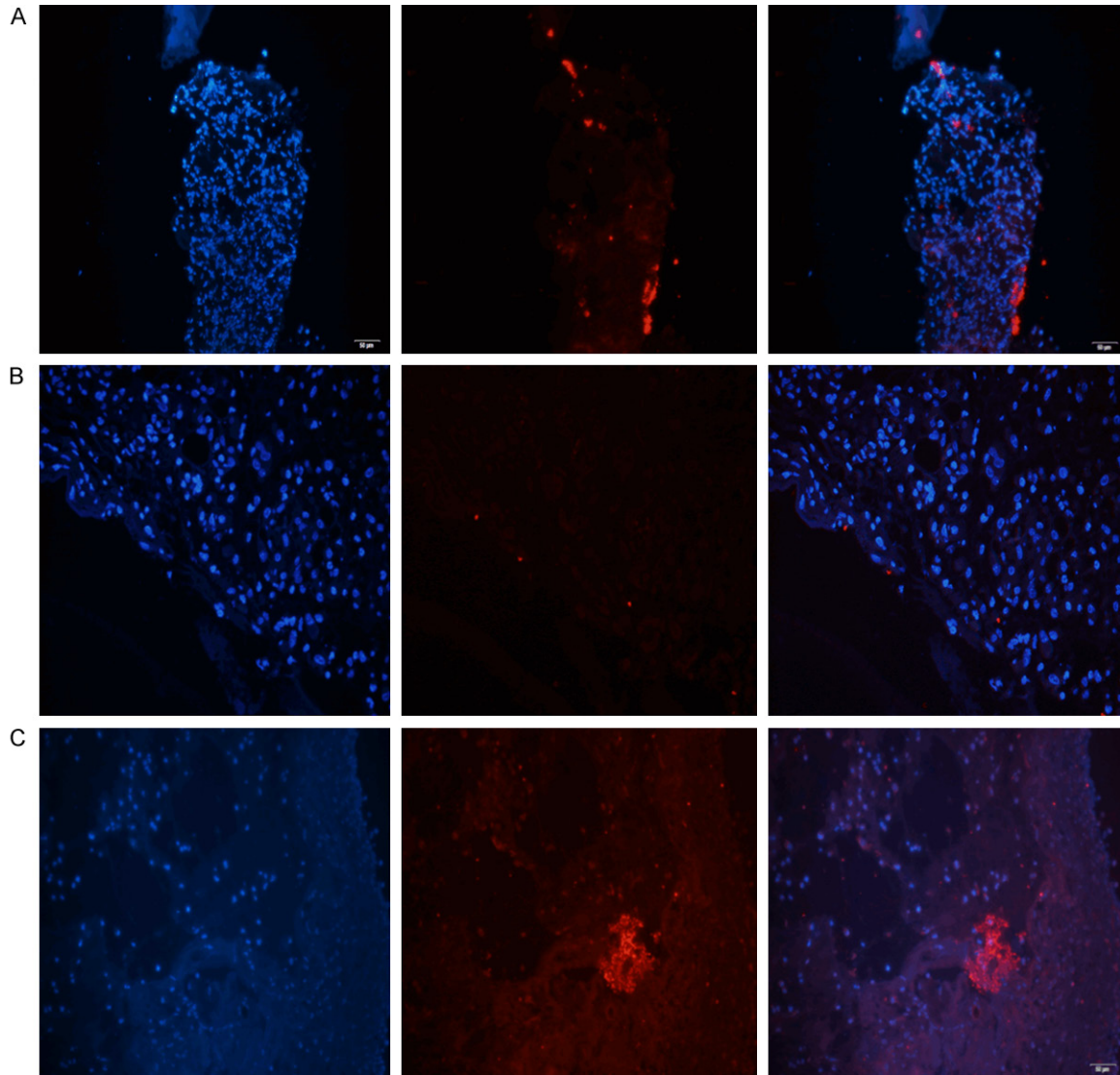


Figure 7. *P. aeruginosa* aggregates were observed by PNA-FISH with a Texas Red-labeled *P. aeruginosa*-specific probe (red). The (A) PAO1 wild-type strain, (B) PAO1/ P_{lac} -*yhjH* strain and (C) PAO1 Δ *wspF* strain observed with a fluorescence microscope. A high level of *P. aeruginosa* aggregation was observed with the PAO1 Δ *wspF* strain (with high intracellular levels of c-di-GMP). Some scattered *P. aeruginosa* aggregates were observed with the PAO1/ P_{lac} -*yhjH* strain (with low intracellular levels of c-di-GMP).

was significantly higher for the diabetic mice treated with insulin than for the nondiabetic mice or the untreated diabetic mice at days 4 and 16, with values of 2.79, 3.44, and 7.09%, respectively, on day 4 and 4.47, 9.97, and 14.2%, respectively, on day 16 (Figure 5B).

P. aeruginosa aggregates were most prevalent in the wounds of diabetic mice treated with insulin

HE staining was used to observe the inflammatory reactions of the cells in the infected sec-

tions (Figure 6). There were more polymorphonuclear leucocytes (PMNs) in the wounds of the diabetic mice treated with insulin than in those of untreated diabetic mice and nondiabetic mice. *P. aeruginosa* aggregates were observed by PNA-FISH with a fluorescence microscope. More *P. aeruginosa* aggregates were observed with the PAO1 Δ *wspF* strain (with high intracellular levels of c-di-GMP) than with the other strains. Some scattered *P. aeruginosa* aggregates were observed with the PAO1/ P_{lac} -*yhjH* strain (with low intracellular levels of c-di-GMP) (Figure 7). A high level of *P. aeruginosa* aggre-

Insulin treatment in *P. aeruginosa* biofilm infection

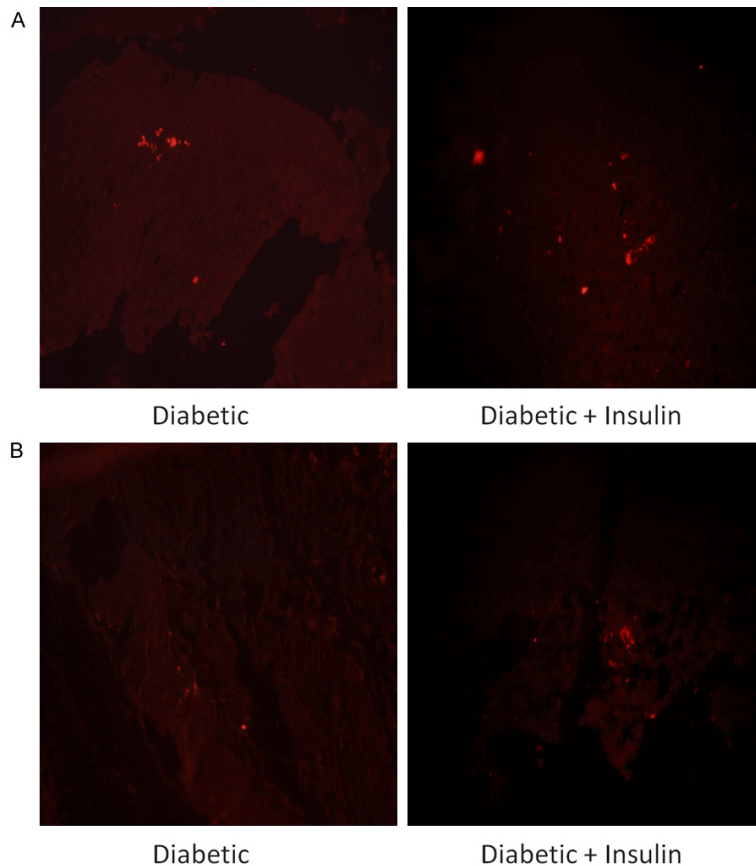


Figure 8. Effect of insulin treatment on the biofilms in wound tissue infected with the (A) PAO1 wild-type strain or (B) PAO1/P_{lac}-yhjH strain. A high level of *P. aeruginosa* aggregation was observed in diabetic mice treated with insulin and infected with either the PAO1 wild-type strain or the PAO1/P_{lac}-yhjH strain.

gation was observed in diabetic mice treated with insulin and infected with either the PAO1 wild-type strain or the PAO1/P_{lac}-yhjH strain (Figure 8).

*Insulin treatment promoted the expression of IL-4 and IFN- γ RNA in *P. aeruginosa*-infected wounds in diabetic mice*

To identify the mechanism by which insulin participates in the immune response, RT-qPCR was used to detect the expression levels of IL-4 and IFN- γ RNA in *P. aeruginosa*-infected wounds in diabetic mice. We found that the expression of IL-4 RNA in each group increased with time post infection (Figure 9). For the PAO1/P_{lac}-yhjH strain (with low c-di-GMP expression levels), at each observation time point, the expression of IL-4 in diabetic mice treated with insulin was significantly higher than that in the untreated diabetic group or the

nondiabetic group ($P < 0.05$). The peak expression of IFN- γ in diabetic mice treated with insulin occurred earlier than it did in the untreated diabetic group with each of the strains tested (Figure 10).

Insulin affected biofilm formation via the c-di-GMP signaling pathway in vitro

Chase Watters' group found that insulin treatment promoted biofilm formation activity in the *P. aeruginosa* wild-type PAO1 strain. Therefore, we tested whether insulin treatment enhanced biofilm formation by increasing the intracellular c-di-GMP levels of *P. aeruginosa* in a microtiter plate-based biofilm assay. First, we determined whether insulin or denatured insulin could affect the growth of *P. aeruginosa* strains with different intracellular c-di-GMP levels. Among the mice infected with the PAO1, PAO1 Δ wspF or PAO1/P_{lac}-yhjH strain, no changes were observed in the growth of the *P. aeruginosa* strains with different intracel-

lular c-di-GMP levels in response to Humulin[®] N insulin, at amounts ranging from 200 nU to 200 μ M, or denatured Humulin[®] N insulin (Figure 11). We next tested whether Humulin[®] N insulin affected the ability of *P. aeruginosa* to form biofilms by a microtiter plate-based biofilm assay. We observed that Humulin[®] N insulin but not denatured insulin could promote the formation of biofilms in the PAO1 and PAO1/P_{lac}-yhjH strains in a manner that was independent of insulin concentration (Figure 12A and 12B). However, we did not observe any effect on the biofilm formation of the PAO1 Δ wspF strain (Figure 12C). Finally, we examined whether insulin could increase intracellular c-di-GMP levels in *P. aeruginosa* under the conditions described above. We found that Humulin[®] N insulin significantly promoted the expression of the intracellular c-di-GMP biosensor P_{cdtA}-gfp in the PAO1 strain treated with 200 μ M of insulin

Insulin treatment in *P. aeruginosa* biofilm infection

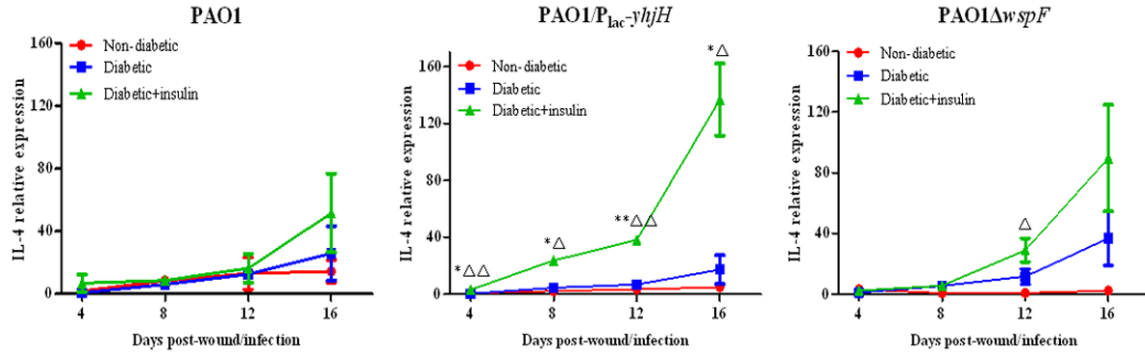


Figure 9. Effect of insulin treatment on the expression of IL-4 RNA in infected chronic wound tissue from diabetic mice. Note: * $P < 0.05$ compared with the nondiabetic group, ** $P < 0.01$ compared with the nondiabetic group, $\Delta P < 0.05$ compared with the diabetic group, $\Delta\Delta P < 0.01$ compared with the diabetic group.

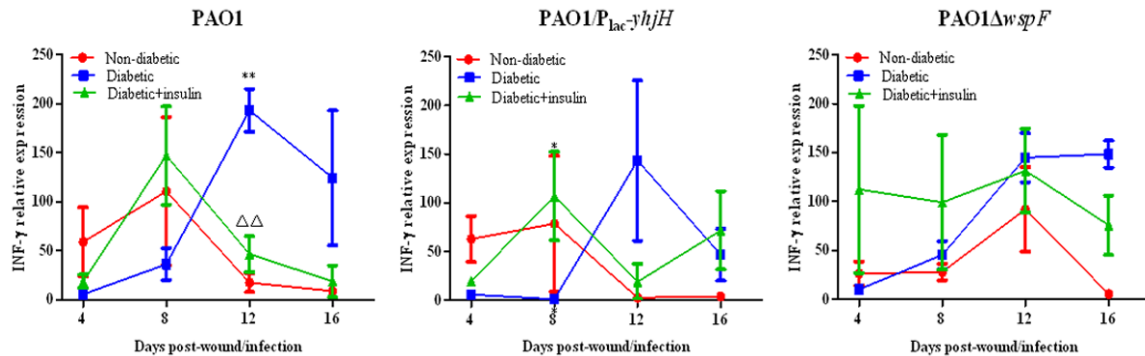


Figure 10. Effect of insulin treatment on the expression of INF- γ RNA in infected chronic wound tissue from diabetic mice. Note: * $P < 0.05$ compared with the nondiabetic group, ** $P < 0.01$ compared with the nondiabetic group, $\Delta P < 0.05$ compared with the diabetic group, $\Delta\Delta P < 0.01$ compared with the diabetic group.

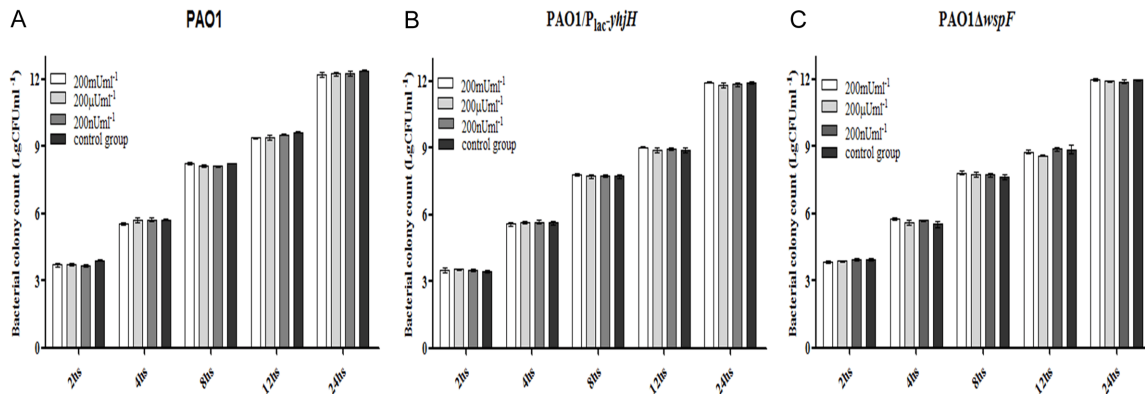


Figure 11. Effect of insulin on the growth of the (A) PAO1 strain, (B) PAO1/ P_{lac} -*yhjH* strain and (C) PAO1 Δ *wspF* strain. We found that there were no changes in the growth of *P. aeruginosa* strains with different intracellular c-di-GMP levels in response to any concentration of insulin or denatured insulin.

and in the PAO1/ P_{lac} -*yhjH* strains treated with 200 nU to 200 mU of insulin (Figure 13B). However, in contrast, Humulin® N insulin did not promote, and instead suppressed, the expression of the intracellular c-di-GMP biosensor

P_{cdrA} -*gfp* with 200 mU of insulin in the PAO1 Δ *wspF* group (Figure 13B). These data demonstrate that Humulin® N insulin promoted *P. aeruginosa* biofilm formation by increasing intracellular c-di-GMP levels in *P. aeruginosa*

Insulin treatment in *P. aeruginosa* biofilm infection

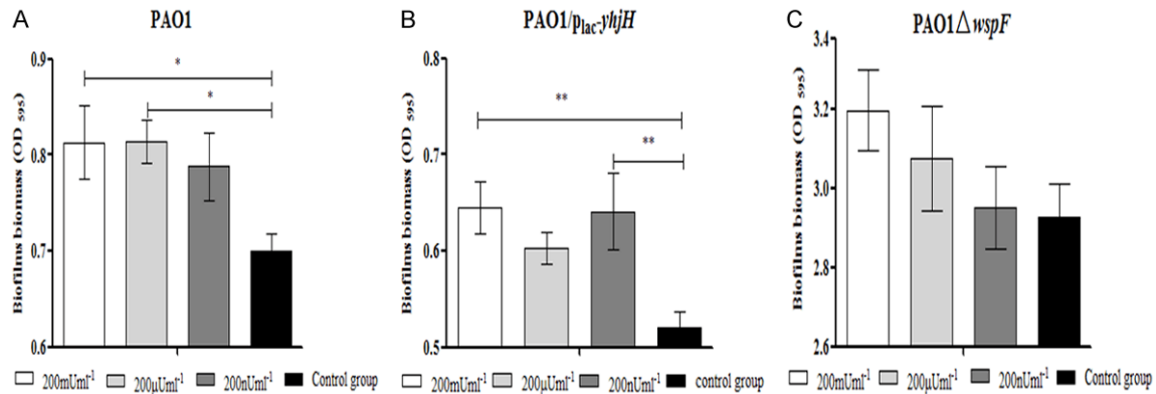


Figure 12. Effect of insulin on biofilm formation of the (A) PAO1 strain, (B) PAO1/P_{lac}-yhjH strain and (C) PAO1ΔwspF strain in microplates. An overnight culture was diluted with ABTGC medium supplemented with Humulin® N insulin or denatured insulin with a final amount of 200 nU, 200 μU, 200 mU or 200 mU. Biofilm formation was monitored after 12 hours of continuous cultivation. The mean and SD from at least triplicate experiments are shown. Note: **P* < 0.05 compared with the control group, ***P* < 0.01 compared with the control group.

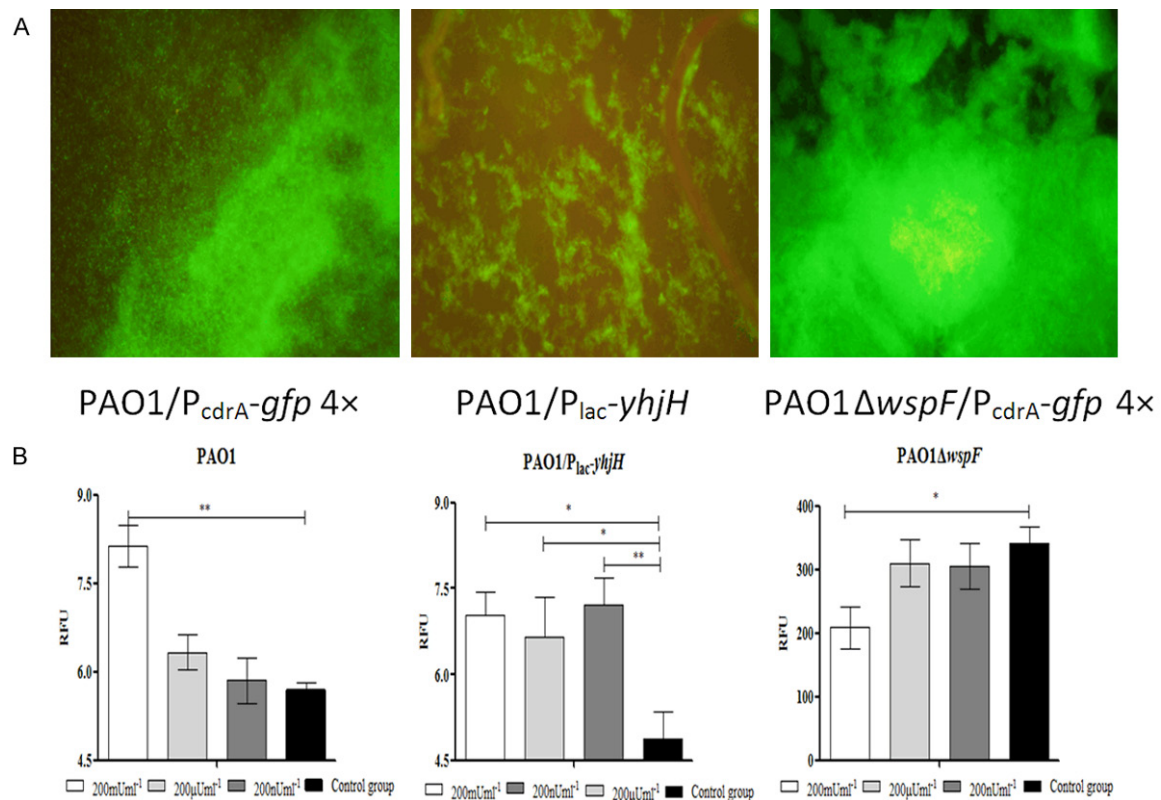


Figure 13. Effect of insulin on P_{cdrA}-gfp expression in the PAO1 strain, PAO1/P_{lac}-yhjH strain and PAO1ΔwspF strain in microplates. The PAO1 strain containing the P_{cdrA}-gfp vector, which can provide a fluorescent readout of the intracellular level of c-di-GMP in *P. aeruginosa*, was resuscitated and grown at 37 °C in Luria-Bertani (LB) medium. After 24 hours of cultivation, the microplates were observed by fluorescence microscopy (A). P_{cdrA}-gfp fluorescence and OD₆₀₀ values were measured and recorded as relative fluorescence unit (RFU) values after 12 hours of incubation. The mean and SD of RFUs from at least triplicate experiments are shown (B). Note: **P* < 0.05 compared with the control group, ***P* < 0.01 compared with the control group.

strains with intermediate or low intracellular c-di-GMP levels, while the opposite trend was

observed for the strain with high c-di-GMP levels.

Discussion

The presence of biofilms has been confirmed in infected chronic wounds via PNA-FISH, scanning electron microscopy (SEM), and detection of the extracellular polymeric substance (EPS) matrix by illumination of the alginate surrounding *P. aeruginosa*; these specimens originated from patients with DFIs or from chronic wound models of diabetes [2, 25-28]. However, whether biofilms in wounds hinder wound healing is a subject of debate. Increasing evidence has shown that the presence of biofilms in wounds leads to delayed healing [2, 28, 29]. It is well known that the presence of biofilms in wounds delays wound healing in a variety of ways, including by activating the host response to the bacteria, which is characterized by persistent inflammation, continuous oxidative damage, fibroblast senescence and depletion of beneficial growth factors needed for tissue resolution in a chronic wound [30]. However, Trøstrup H reported that *P. aeruginosa* biofilms hamper murine central wound healing by suppressing the vascular epithelial growth factor [31]. Whether insulin treatment can be used to treat *P. aeruginosa* infections is currently a controversial subject. Low-dose insulin treatment has been reported to decrease the mortality rate of sepsis caused by *P. aeruginosa* infection after severe burn injury. However, as reported by Watters et al. [16], in chronic wound models of diabetes, biofilms are highly prevalent in STZ-induced wounds in diabetic mice, and most importantly, high biofilm formation was observed in the wounds of insulin-treated diabetic mice [18]. Subsequently, studies have shown that high levels of lysed neutrophils in the wounds of diabetic mice treated with insulin, combined with decreased levels of macrophages that clear the cellular detritus, contribute to enhanced eDNA levels, thereby enhancing *P. aeruginosa* biofilm formation [17].

In our study, we observed that the increase in the body weights of diabetic mice treated with insulin was significantly greater than that observed for untreated diabetic mice between the PAO1/p_{lac}-*yhjH*- and PAO1Δ*wspF*-infected groups, which indicated that insulin treatment indeed improved the overall condition of diabetic mice. For example, in the PAO1-wild-type-infected group, only the diabetic mice treated with insulin gained body weight. As weight loss

is a sign of disease progression or infection, these data showed that insulin treatment can improve the overall health of diabetic mice with *P. aeruginosa*-infected chronic wounds [32]. Brown et al. showed that wound closure was impaired, with significantly greater severity, in diabetic mice infected with *P. aeruginosa* than in nondiabetic mice infected with *P. aeruginosa* [33]. Our result was similar to the results reported by Brown et al. [33]. However, we found dramatic differences in wound closure between nondiabetic and diabetic mice among the groups infected with the PAO1 wild-type, PAO1/p_{lac}-*yhjH* or PAO1Δ*wspF* strain. The slowest wound healing was observed with the PAO1Δ*wspF* strain (with high intracellular c-di-GMP levels) among the nondiabetic mice, diabetic mice and diabetic mice treated with insulin. Most importantly, we observed that the wound closure in diabetic mice treated with insulin was significantly slower than that in untreated diabetic mice in the PAO1 wild-type-infected group (day 16) and the PAO1/p_{lac}-*yhjH*-infected group (day 12 and day 16). *P. aeruginosa* produces biofilms that increase the resistance of this species to antibiotics [34, 35]. C-di-GMP is involved in the regulation of cellular functions, especially biofilm formation and dispersion; high intracellular levels of c-di-GMP promote biofilm formation, and low c-di-GMP levels cause biofilm dispersal. We also observed that the PAO1/p_{lac}-*yhjH* strain in the wounds of the diabetic mice was significantly more tolerant to amikacin (day 4 and day 16) than that same strain in the wounds of nondiabetic mice, and the highest antibiotic tolerance was observed in diabetic mice treated with insulin. However, the changes in wound healing and amikacin resistance were not related to the changes in the colony counts of the wound tissues. Therefore, these data suggest that one of the primary factors underlying the changes in wound healing and amikacin resistance was increased biofilm formation.

We used PNA-FISH to determine whether the wounds were infected with *P. aeruginosa*, and ConA-FITC was then used to stain the exopolysaccharide to confirm biofilm formation. Notably (and unexpectedly), more biofilm was observed in the wounds of insulin-treated diabetic mice infected with PAO1/p_{lac}-*yhjH* or PAO1 than in the wounds of untreated diabetic mice or nondiabetic mice. These in vivo data indicate

that the formation of biofilms in the wounds of diabetic mice treated with insulin was promoted at intermediate or low levels of intracellular c-di-GMP, which was associated with c-di-GMP signaling in *P. aeruginosa*. In other words, insulin treatment likely promoted *P. aeruginosa* biofilm formation by affecting the c-di-GMP signaling pathway, resulting in delayed wound healing and increased antibiotic resistance. In vitro studies showed that there were no significant changes in the growth of *P. aeruginosa* strains with different intracellular c-di-GMP levels in response to insulin or denatured insulin. We also found that insulin could significantly promote the expression of the intracellular c-di-GMP biosensor P_{cdtA} -*gfp* in the PAO1 wild-type strain with 200 mU of insulin and in the PAO1/ p_{lac} -*yjhH* strain with 200 nU to 200 mU of insulin. However, in the PAO1 Δ *wspF*-infected group, in vitro studies showed that insulin and denatured insulin did not affect biofilm formation. Similarly, we found that insulin treatment did not significantly prolong wound healing in diabetic mice or lead to increased rates of drug resistance in vivo. We speculate that there is already a very high concentration of intracellular c-di-GMP in the PAO1 Δ *wspF* strain, leaving no room to improve biofilm formation. The intracellular c-di-GMP levels in the PAO1 Δ *wspF* strain were not enhanced by drugs or other means. Rybtke et al. [19] showed that reporter constructs provided a fluorescent readout of the intracellular levels of c-di-GMP in *P. aeruginosa* strains with different levels of c-di-GMP and could detect the increased turnover of c-di-GMP mediated by the treatment of *P. aeruginosa* with the phosphodiesterase-inducer nitric oxide. These data proved that insulin did not promote the growth of *P. aeruginosa* but promoted the formation of biofilms by increasing intracellular c-di-GMP synthesis in *P. aeruginosa* strains with intermediate and low levels of c-di-GMP.

In the clinic, chronic wounds are also frequently infected by multispecies biofilms [4], but only one pathogen, *P. aeruginosa*, which is a ubiquitous, clinically important, opportunistic pathogen, was used in this study; therefore, the results obtained in this study did not accurately simulate an infected human chronic wound. In this study, we observed the effect of insulin treatment on only the amount of biofilm in the *P. aeruginosa*-infected chronic wounds, but determining whether insulin treatment can

influence the production of biofilm components such as rhamnolipids requires further research. In our in vivo study, we observed that insulin amounts from 200 nU to 200 mU promoted biofilm formation, but it remains unclear whether these insulin amounts correspond to the levels in the infected wounds of diabetic mice in vivo. Information regarding the levels of insulin that reach the peripheral tissue under the mouse skin is scarce; however, it has been reported that 40~70 μ U/mL insulin was detected in the periphery 90 s after injection of 400 ng of insulin into the vena cava in rats [36]. Assuming that this result was true in our mice, when the mice were treated with approximately 69 μ g of insulin (equal to 2 U), the peripheral insulin concentration would have been 7~12 mU/mL, which clearly promoted the biofilm formation of *P. aeruginosa* directly in vitro.

The presence and persistence of *P. aeruginosa* biofilms in chronic wounds can affect the functions of cells such as leukocytes, keratinocytes, endothelial cells, and fibroblasts, and the affected functions include the inflammatory cellular response, the cutaneous innate immune response, and the repair phase of wound healing [37]. In the course of normal wound healing, once the wound tissue is removed, the end of the inflammatory reaction period follows, which is followed by the beginning of the proliferative phase. A chronic wound is characterized by a prolonged duration of the inflammatory reaction, which interferes with wound healing. It was reported that insulin had anti-inflammatory and antioxidant effects in both clinical trials and animal experiments [38, 39]. Insulin promotes the polarization of T cells to the Th2 cell type by promoting the phosphorylation of the Th2 extracellular signal-regulated kinase, which plays an anti-inflammatory role not only in septicemia but also in chronic inflammation associated with obesity or type 2 diabetes [40]. Animal experiments and clinical studies have indicated that the immune response to *P. aeruginosa* infection is a Th2-type response, as observed mainly in patients with pulmonary cystic fibrosis infected with *P. aeruginosa* [41-43]. According to the latest *European Society of Clinical Microbiology and Infectious Diseases (ESCMID)* guidelines [44, 45], the inflammatory cells in *P. aeruginosa*-infected wound tissues are mainly PMNs or macrophages, which will determine whether the predominant immune response to the

microbes present in *P. aeruginosa* biofilms consist of either Th2 (antibody) or Th1 (cell-mediated) polarization. Our study found that the RNA expression of IL-4, a characteristic inflammatory factor that is secreted by Th2 cells, increased with time. In particular, in the PAO1/*P_{lac}-yhjH* strain (with low c-di-GMP expression), the expression of IL-4 RNA in diabetic mice treated with insulin was significantly higher than that in untreated diabetic and nondiabetic mice at each observation time point. In terms of the expression of IFN- γ RNA, a characteristic inflammatory factor secreted by Th1 cells, the peak local expression of this inflammatory factor observed in the *P. aeruginosa*-infected wounds of diabetic mice treated with insulin was lower and occurred earlier than that in diabetic mice, with expression levels gradually decreasing thereafter. These data demonstrate the dual effects of insulin; on one hand, the clearance of *P. aeruginosa* decreased via inhibition of the Th1-type immune response, but on the other hand, biofilm formation was promoted by enhanced Th2-type polarization, leading to promotion of the Th2-type immune response, which resulted in a prolonged period of inflammation and the healing of infected wounds.

In conclusion, the delayed healing of infected chronic wounds is affected by a variety of factors, which are complex and variable. In our study, for the first time, insulin was shown to promote *P. aeruginosa* biofilm formation by increasing intracellular c-di-GMP levels in vitro and in vivo, resulting in delayed wound healing in diabetic mice. In addition, for the first time, insulin was shown to decrease the clearance of *P. aeruginosa* by inhibiting the Th1-type immune response and promoting biofilm formation by enhancing Th2-type polarization, thereby promoting the Th2-type immune response, which resulted in a prolonged period of inflammation and the healing of infected wounds. However, there have been no clinical studies linking the chronic DFIs caused by *P. aeruginosa* with c-di-GMP signaling. It has been reported that [46] the naturally induced dispersal agent nitric oxide can reduce intracellular c-di-GMP levels and decrease cell permeability. Compared with other c-di-GMP synthase inhibitors, nitric oxide can induce *P. aeruginosa* biofilm dispersal [47, 48] at relatively low concentrations. Further clinical studies are required to determine whether a nitric oxide donor, such as

sodium nitroprusside solution, can be used locally to induce the dispersal of *P. aeruginosa* biofilms in strains with high intracellular c-di-GMP levels.

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Disclosure of conflict of interest

None.

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