

Platelet-rich plasma plays an antibacterial, anti-inflammatory and cell proliferation-promoting role in an in vitro model for diabetic infected wounds

Tao Li,¹ Yu Ma,² Min Wang,¹ Tao Wang,³ Jing Wei,⁴ Rui Ren,¹ Min He,¹ Guixue Wang,² Johnson Boey,⁵ David G Armstrong,⁶ Wuquan Deng,² Bing Chen¹

¹Department of Endocrinology, Southwest Hospital, Army Medical University, Chongqing, People's Republic of China; ²Department of Endocrinology and Nephrology, Key Laboratory for Biorheological Science and Technology of Ministry of Education, Chongqing University, Affiliated Central Hospital of Chongqing University, Chongqing, People's Republic of China; ³Institute of Combined Injury, State Key Laboratory of Trauma, Burn and Combined Injury, Army Medical University, Chongqing, People's Republic of China; ⁴Department of Endocrinology, General Hospital of Xinjiang Military Region, The Chinese People's Liberation Army, Urumqi, People's Republic of China; ⁵Department of Podiatry, Singapore General Hospital, Singapore; ⁶Southwestern Academic Limb Salvage Alliance (SALSA), Department of Surgery, Keck School of Medicine of the University of Southern California, Los Angeles, CA, USA

Correspondence: Wuquan Deng
Department of Endocrinology and Nephrology, Key Laboratory for Biorheological Science and Technology of Ministry of Education, Chongqing University, Affiliated Central Hospital of Chongqing University, I Health Road, Yuzhong District, Chongqing 400014, People's Republic of China
Tel +86 236 369 2185
Email wuquandeng@gmail.com

Bing Chen
Department of Endocrinology, Southwest Hospital, Army Medical University, 30 Gaotanyan Street, Shapingba District, Chongqing 400038, People's Republic of China
Tel +86 236 875 4138
Email chenbing3@medmail.com.cn

Aim: This study was designed to examine the potential mechanism underlying these roles of platelet-rich plasma in treating diabetic foot ulcers (DFUs).

Methods: *Staphylococcus aureus* and HaCaT were co-cultured under high glucose conditions to serve as an in vitro model for infected cells in DFUs. Platelet-rich gel (PRG) or extract liquid of platelet-rich gel (EPG) were used to interfere with the model to observe the growth of HaCaT cells and *S. aureus*, and the effect of miR-21 changes in HaCaT cells on PDCD4, NF-κB activity and related inflammatory factors.

Results: Incubation of HaCaT cells with *S. aureus* promoted the decline of cell proliferation. Under this condition, the level of PDCD4 and the activity of NF-κB were increased in HaCaT cells with concomitant increased of IL-6, TNF-α and decreased IL-10, TGF-β1 in cultured supernatant. Both of PRG and EPG exhibited specific anti-*S. aureus* activity where they protect HaCaT cells from bacterial damage and promote cell proliferation. Meanwhile, EPG was observed to increase intracellular miRNA-21 while reduce PDCD4 expression and inhibit NF-κB activity to suppress the inflammation in HaCaT cells.

Conclusion: This in vitro model provides a valuable tool for study of wound healing in the treatment of DFUs. Our results suggest that miRNA-21 may regulate the expression of NF-κB through PDCD4 where it plays an anti-inflammatory role and promote proliferation in infected DFUs treated by PRP. These findings could provide novel therapeutic targets for refractory wounds.

Keywords: platelet-rich plasma, antibacterial, anti-inflammatory, cell proliferation-promoting, diabetic infected wound

Introduction

The global incidence of diabetes mellitus is increasing rapidly. The International Diabetes Federation estimated that there will be ~630 million of people diagnosed with diabetes by 2045.¹ Among the complications associated with diabetes, diabetic foot ulcers (DFUs) severely debilitate the quality of life, and it was found that one in four individuals diagnosed with diabetes will develop DFU in their lifetime.² Many pathophysiological mechanisms have been proposed to explain delayed wound healing in DFUs, one of which, wound infection plays an important role.³ Gram-positive bacterial infections are relatively common in DFUs, predominately caused by *Staphylococcus aureus*.⁴ Bacterial colonization in wounds causes direct cellular damage to the host cell, and an imbalance in the microenvironment. Once the bacterial bioburden reaches a critical level, the host body mounts an immune response, which is often deranged and

yet insufficient to eradicate the offending bacteria. Persistent inflammation ensues, which further impedes diabetic wound healing.^{5,6} Antibiotics are often used to treat wound infections in DFUs. However, the frequent recurrence of DFUs for patients in DFU remission and emergence of multiple-drug resistance bacteria rendered antibiotics ineffective or poor efficacy, when used alone.^{2,7}

In recent years, studies has revealed the wound healing potential of platelet-rich plasma (PRP) in the treatment of DFUs from their antimicrobial and regenerative properties.⁸⁻¹⁰ Platelet-rich gel (PRG) is produced from PRP which is activated by thrombin and/or calcium. These platelet derivatives have been shown to possess several important functions such as antibacterial and promotion of wound healing. For example, PRG has been proven to inhibit *S. aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Klebsiella pneumoniae*, as well as methicillin-resistant staphylococcus aureus in a previous study.¹¹ Moreover, PRG does not cause drug resistance and has a synergistic effect with antibiotics. The combination of these characteristics makes it more promising over traditional antibiotic prescriptions.¹²

Within activated platelets, platelet-derived antimicrobial peptides, chemokines, growth factors, and miRNAs have been observed to exert antibacterial effects and promote wound healing.¹³ There are more than 200 miRNAs found in platelets, in particularly, miRNA-21 and miRNA-223 are highly abundant.¹⁴ The level of miRNAs in DFU is directly correlated to wound healing.¹⁵ The expression of miR-21 is up-regulated during wound healing while the suppression of its expression is found in wounds of diabetic mice.¹⁶ Taken the above literature together, it has been speculated that miRNA-21 may play an important role in PRG treatment of DFUs. Furthermore, it has been reported that miRNA-21 can also play a role in inflammation by regulating nuclear factor-kappa (NF- κ B),¹⁷ but the exact mechanism is not yet clear.

Programmed cell death factor 4 (PDCD4) is one of the currently validated miRNA-21 regulatory targets in which miRNA-21 inhibit PDCD4 expression at the post-transcriptional level.^{18,19} It has been reported that inhibition of miRNA-21 expression in mice may up-regulate PDCD4, induce the activation of NF- κ B which eventually leads to increased synthesis of pro-inflammatory cytokines, and decreased synthesis of anti-inflammatory cytokines.²⁰ Based on the above findings, we hypothesize that PRG may affect the activity of NF- κ B, regulate wound inflammation, and promote wound healing through regulating PDCD4 mediated by miRNA-21.

To confirm this assumption, we firstly devised a diabetic wound infected in-vitro study model. The PRG was

subsequently added into this model to observe the growth of HaCaT cells and *S. aureus*. In addition, to evaluate the potential ability of PRP in antibacterial and wound healing, the changes in miRNA-21, PDCD4 and NF- κ B in HaCaT cells and related inflammatory factors were assessed.

Materials and methods

Preparation of platelet-poor plasma (PPP) and PRP

Whole blood samples were drawn from 21 patients (age 69 \pm 12) with DFUs but free of active infection and existing systemic coagulation disorders. PPP was isolated from the blood specimens with the first centrifugation in the department of blood transfusion. Platelets were sequestered by full automatic blood separator (CS-3000 plus; Baxter International, Inc., Deerfield, IL, USA), and condensed by the second centrifugation to produce PRP. The concentrations of platelets in PRP and PPP were determined by the automatic hematology analyzer (KX-21N; Sysmex Corporation, Kobe, Japan). The average platelet count was (1024 \pm 111) \times 10⁹/L in PRP, while it was <10 \times 10⁹/L in PPP. The majority of the extracted PRP was used to treat the blood donor's DFUs while the remaining PRP will be used in our study. The above processes were approved by the institutional review board of the first hospital affiliated to the Army Medical University. Written informed consent was obtained from all patients. The study was approved by the medical ethics committee of the first hospital affiliated to the Army Medical University (Chongqing, People's Republic of China) and conducted in compliance with the Declaration of Helsinki.

Preparation of PRG

The PRG was harvested by platelet activation. The 10 mL PRP was activated by 1 mL calcium gluconate solution (100 g/L) and 1,000 IU thrombin.

Preparation of the extract liquid from PRG

The PRG was incubated at 37°C for 1 hour with constant thorough stirring with a sterile glass rod. Then, the mixture was centrifuged for 15 minutes with a centrifugation force of 3,000 \times g at room temperature. The supernatant was collected as extract liquid of platelet-rich gel (EPG).

Establishment of an in vitro diabetic infected wound model

HaCaT cells and *S. aureus* (ATCC 25923) were bestowed gifts from the Institute of Combined Injury of the Army

Medical University (Chongqing, People's Republic of China) for research purposes. *S. aureus* was cultured in Tryptic Soy Broth (TSB) medium in a shaking 37°C incubator overnight. Then, the cultured bacteria were collected by centrifugation and re-suspended in high glucose DMEM culture medium (Hyclone). Bacterial density was adjusted to 10⁸ CFU/mL and serial dilutions were prepared. Simultaneously, HaCaT cells were cultured in high glucose DMEM supplemented with 10% FBS at 37°C with 5% CO₂ for 4 days. Then cells were harvested and seeded into 96-well plates at a density of 30,000 cells/cm². After 48 hours, the culture medium was replaced by either fresh high glucose DMEM (HaCaT control) or *S. aureus* dilutions. To examine potential damage ability to HaCaT cells, four serial bacterial concentrations, from 10 to 10⁴ CFU/mL, were chosen to set up the co-culture system. After pre-incubation of HaCaT cells with *S. aureus* for 1 hour, PRG or EPG accounted for different volume ratio was added to the co-culture system. In addition, in order to observe the direct effect on HaCaT cells, EPG was also added to the cell culture medium without bacteria. After 12, 24, 36 and 48 hours of intervention, cell proliferation was determined by Cell Counting Kit-8 (CCK-8) assay. Western blotting, quantitative reverse transcription (qRT-PCR), immunofluorescence and ELISA were used to determine the expression of miRNA, protein and cytokines, and the co-culture system was established in 6-well plates and divided into four groups: the H group, culture of HaCaT cells alone; the HP group, uninfected HaCaT cells interfered with EPG; the HS group, co-culture of HaCaT cells with *S. aureus*; and the HSP group, *S. aureus*-infected HaCaT cells interfered with EPG. The components of the co-culture system are shown in Table 1.

Evaluation of antibacterial activity

To compare the differential antibacterial ability of PRG and EPG, the bacterial concentration of 10⁴ CFU/mL was chosen. Either PRG or EPG was prepared from equivalent PRP. After 12, 24, 48 and 72 hours of intervention, the bacteria were quantified by plate colony counting method. Furthermore, the antibacterial performance of EPG accounting for 10%, 20% and 30% volume were tested after 24 hours of intervention in the co-culture system with the initial bacterial concentration as 10⁶ CFU/mL.

CCK-8 assay

Cell proliferation was measured according to the instructions of CCK-8. Briefly, 100 µL of DMED containing 10

Table 1 Components of the four co-culture groups

Component \ Group	H	HP	HS	HSP
DMEM	1.6 mL	1.6 mL	1.4 mL	1.4 mL
<i>S. aureus</i> ^a	–	–	0.2 mL	0.2 mL
EPG	–	0.4 mL	–	0.4 mL
PPP	0.4 mL	–	0.4 mL	–

Notes: ^a*Staphylococcus aureus* suspension was diluted with high glucose DMEM to 10,000/ mL. The H group, culture of HaCaT cells alone; the HP group, uninfected HaCaT cells interfered with EPG; the HS group, co-culture of HaCaT cells with *S. aureus*; and the HSP group, *S. aureus*-infected HaCaT cells interfered with EPG.

Abbreviations: EPG, extract liquid of platelet-rich gel; PPP, platelet-poor plasma; *S. aureus*, *Staphylococcus aureus*.

µL of CCK-8 solution was added to each well and the plate was incubated for 2 hours at 37°C. Then the OD of wells was measured at 450 nm using a microtiter plate reader. Cell proliferation of experimental groups was expressed as a percentage of the HaCaT control group. There is a linear relationship between cell number and OD.

Plate colony counting

The bacterial suspension or cell lysate were collected and underwent serial dilution of up to seven times. Then 100 µL from each dilution was plated onto TSB agar plates. After incubation overnight at 37°C, the number of bacterial colonies was counted manually. The dilution times were adjusted to ensure the number of colonies in each plate was between 30 and 300.

Quantitative reverse transcription

Total RNA from HaCaT cells was extracted by Trizol (Thermo Fisher Scientific, Waltham, MA, USA). The purity of isolated RNA was determined by Nanodrop ND-1000 (Thermo Fisher Scientific). Reverse transcription was performed with PrimeScript RT reagent Kit (Takara Bio Inc., Kusatsu, Shiga, Japan). Quantitative PCR of miRNA-21, IL-6, IL-10, tumor-necrosis factor alpha (TNF-α) and TGF-β1 were performed using Mir-X™ miRNA SYBR® Kit, TB Green™ Premix Ex Taq™ II (Takara Bio Inc.) and iQ5 (Bio-Rad Laboratories Inc., Hercules, CA, USA) detection system supplied with analytical software. Primers were synthesized by RiboBio Co. Ltd (Guangzhou, People's Republic of China). Forward (F) and reverse (R) primers are described in Table 2. The PCR results were normalized with U6 snRNAs for miR-21, and β-actin mRNAs for IL-6, IL-10, TNF-α and TGF-β1 as internal controls respectively. Results were expressed as relative expression compared with control samples.

Western blotting

Total proteins from HaCaT cells were extracted by cell lysis buffer, containing supplementary protease and phosphatase inhibitor. Then, the proteins were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membrane. Subsequently, the membrane was blocked with 5% non-fat dry milk in tris buffered saline with Tween 20 buffer and incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies at 37°C for 1 hour. Protein blots were visualized with super-efficient chemiluminescence kit and detected by ChemiDoc XRS device (Bio-Rad Laboratories Inc.) supplied with Image Lab software. Antibodies purchased from Cell Signaling Technology (Danvers, MA, USA) were as follows: rabbit anti-PDCD4 antibody, rabbit anti- β -actin antibody, rabbit anti-phospho-NF- κ B p65 antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG.

Immunofluorescence analysis

Sterile coverslips were pre-placed into a 6-well plate, then HaCaT cells were seeded on the coverslips and the co-culture system of each group was performed according to Table 1. After EPG intervention for 12, 24 and 48 hours, the cells were washed three times by PBS and fixed in 4% paraformaldehyde for 15 minutes. Subsequently the cells were treated with 0.5% Triton X-100 for 20 minutes, blocked with 10% goat serum at 37°C for 30 minutes and incubated with NF- κ B p65

antibodies (1:200; Cell Signaling Technology) at 4°C overnight, followed by incubation with Cy3-conjugated secondary antibodies at 37°C for 30 minutes. Then the cell nucleus was stained with a DAPI for 5 minutes at room temperature. After rinsing with PBS, the coverslips were mounted on glass slides and observed under a fluorescence microscope.

ELISA

Cell culture supernatants in the four groups were collected for cytokine evaluation. The level of IL-6, IL-10, TNF- α , TGF- β 1 were measured by enzyme immunoassay kit (Bosterbio, Wuhan, People's Republic of China). The content of cytokines was calculated through a standard curve obtained by measuring the standards.

Statistical analysis

Data were expressed as mean \pm SD. One-way ANOVA and LSD tests were used to analyze all experimental data. Analysis was carried out by SPSS 20.0 (IBM Corporation, Armonk, NY, USA). We used an alpha of 5% (* P <0.05; ** P <0.01).

Results

Effect of *S. aureus* on the proliferation of HaCaT cells

Using the proposed co-culture system in-vitro model for infected cells in DFUs, we found that increasing initial concentrations of *S. aureus* results in a dose-dependent decline

Table 2 Primers used in the study

Primers	Sequences (5'→3')	
Reverse transcription PCR		
U6	AAAATATGGAACGCTTCACG	
miR-21	CTCAACTGGTGTCTCGTGGAGTCGGCAATTCAGTTGAGTCAACATC	
Primers used for RT-qPCR		
U6	Forward	CGCTTCGGCAGCACATATACTAAAATTGGAAC
	Reverse	GCTTCACGAATTTGCGTGTCATCCTTGC
miR-21	Forward	ACACTCCAGCTGGGTAGCTTATCAGACTGA
	Reverse	TGGTGTCTGGAGTCG
IL-6	Forward	ACTCACCTCTTCAGAACGAATTG
	Reverse	CCATCTTTGGAAGGTTCCAGTTG
IL-10	Forward	TCAAGGCGCATGTGAACTCC
	Reverse	GATGTCAAACCTCACTCATGGCT
TNF- α	Forward	CCTCTCTAATCAGCCCTCTG
	Reverse	GAGGACCTGGGAGTAGATGAG
TGF- β 1	Forward	GGCCAGATCCTGTCCAAGC
	Reverse	GTGGGTTTCCACCATTAGCAC
β -actin	Forward	CTTAGTTGCGTTACACCCTTTCTT
	Reverse	CACCTTCACCGTTCCAGTTTT

Abbreviation: TNF- α , tumor-necrosis factor alpha.

of HaCaT cells proliferation (Figure 1). For instance, there was significant reduction in cell proliferation after co-culture for 12 hours at a baseline bacterial concentration of 10^3 CFU/mL ($P<0.05$). Furthermore, independent to the initial concentration of bacteria, cell proliferation was severely impaired after 36 hours of co-culture ($P<0.01$) and almost complete destroyed by bacteria after 48 hours ($P<0.01$).

The antibacterial effect of PRG and EPG

Initially, the baseline bacterial concentration was set at 10^4 CFU/mL to evaluate the antibacterial activity of PRG and EPG (Figure 2A). Compared to the PPP group, both PRG and EPG had a significant reduction in bacterial count within 12 hours ($P<0.01$). In the next 36 hours, there were gradual increases in the bacterial counts but there were no statistical differences between the two groups ($P<0.05$). Moreover, in the previous 24 hours, the EPG group exhibited a higher bacterial growth rate than the PRG group ($P<0.05$). Secondly, the higher initial bacterial concentration (10^6 CFU/mL) was used to further assess the antibacterial activity of EPG. It was found that the bacterial count decreased considerably after EPG intervention for 24 hours ($P<0.05$) and this antibacterial effect was concentration dependent. For instance, 30% EPG suppressed the extracellular (Figure 2B) and intracellular (Figure 2C) bacteria concentration significantly ($P<0.01$).

EPG protects cells from bacterial damage and promotes cell proliferation

In Figure 3, EPG demonstrate that it could protect HaCaT cells from *S. aureus* with an initial bacterial concentration of 10^3 CFU/mL. Cell proliferation was restored in the first

24 hours with 20% EPG and promotion of cell proliferation occurred during the next 24 hours, while 10% EPG was unable to achieve this effect. Compared to the control group, cell proliferation was stimulated after 36 hours in a concentration-dependent manner when EPG was added to HaCaT cells in the absence of *S. aureus* infection.

EPG regulate miRNA-21, PDCD4 and NF- κ B signaling pathway activity in HaCaT cells

Before extraction of RNA from HaCaT cells, the four groups were photographed with an inverted microscope (Figure 4). In the HS group, there were hardly any viable cells while EPG displayed remarkable antibacterial effect in the HSP group. Increased cell proliferation was observed more in HaCaT cells in the HP group than in the H group at 48 hours after treatment with EPG. As Figure 5 illustrates, in the early stage at the 12th hour, application of 20% EPG did not cause significant changes to miRNA-21 in the experimental group. But at the 24th hour, increased miRNA-21 level was observed in the HSP group. Meanwhile, miRNA-21 level in the HP group was significantly increased after 24 and 48 hours of EPG intervention. At the 48th hour, due to the extensive damage caused by bacteria in the co-culture system, insufficient RNA was extracted from residual HaCaT cells in the HS group. Thus, no data were available for comparison of HS and HSP groups. Moreover, miRNA-21 level was found to be elevated in the HP group, and this elevation was more pronounced at the 48th than the 24th hour when compared to the H group. In the setting of bacterial infection, the expression of PDCD4 and p-NF- κ B p65 protein was greatly

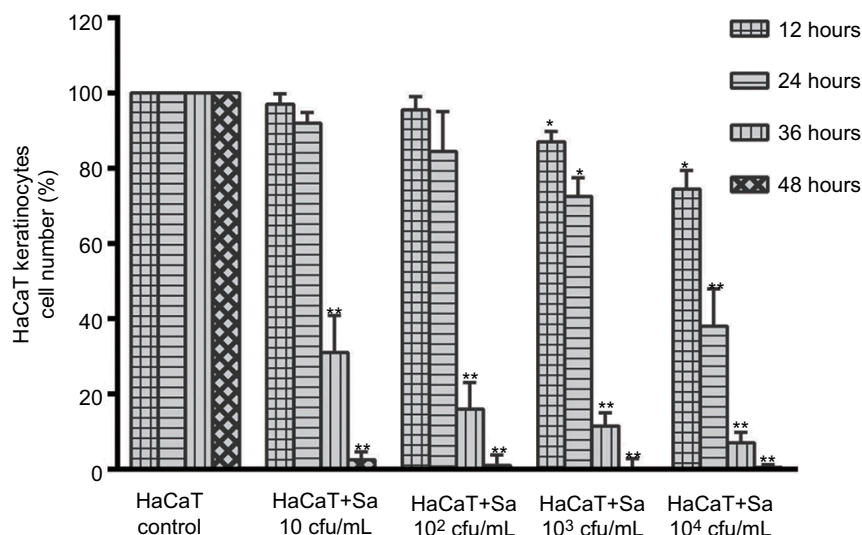


Figure 1 Incubation of HaCaT cells with increasing starting concentrations of *Staphylococcus aureus* led to a dose-dependent decline of the cell proliferation. **Notes:** Significant deviations from the HaCaT control at the respective incubation time (* $P<0.05$; ** $P<0.01$).

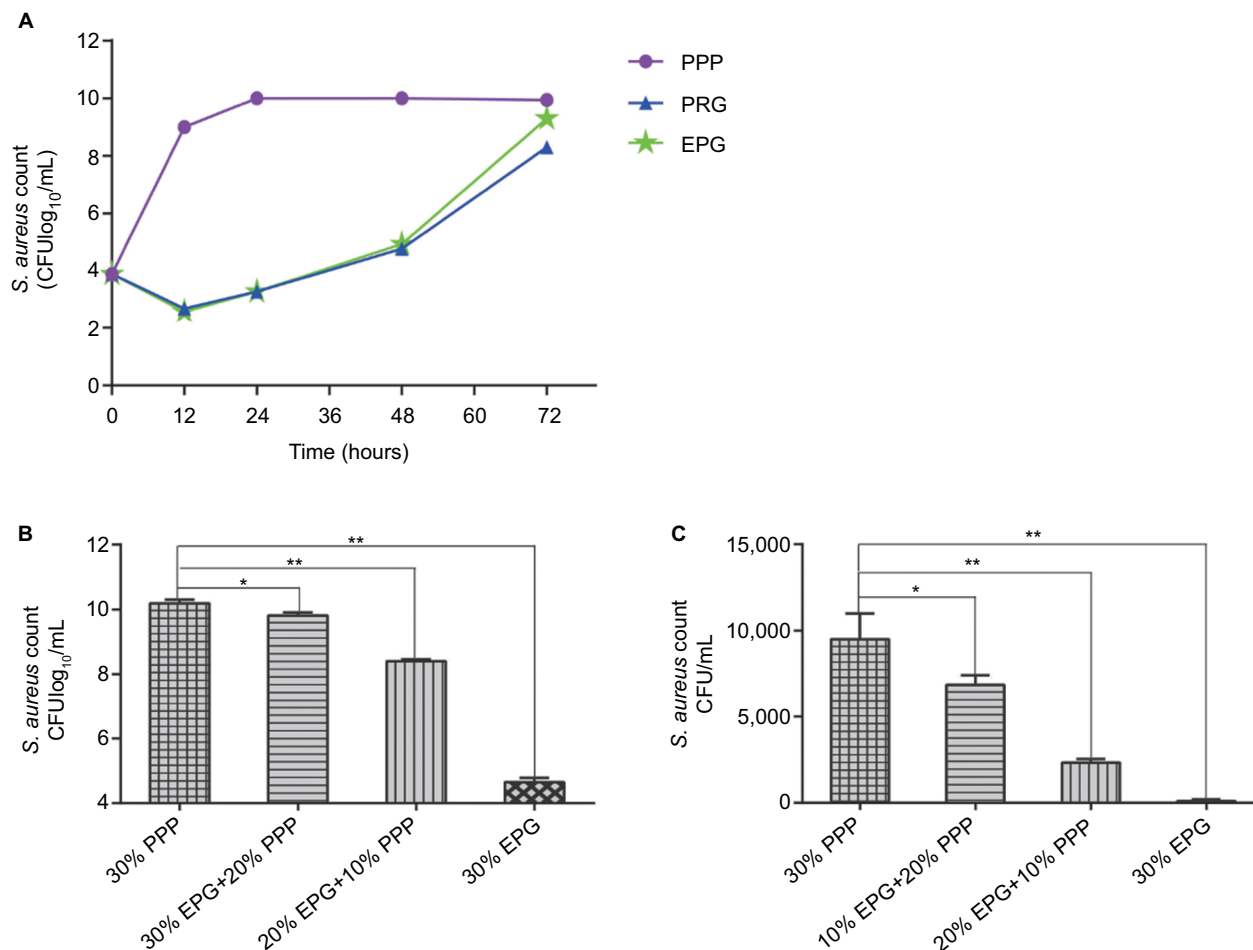


Figure 2 Comparison of antibacterial activity between PRG and EPG and antibacterial performance of EPG at different concentrations. **Notes:** (A) Comparison of the effects of PRG and EPG on the growth of *S. aureus*. (B) Effects of different concentrations of EPG on the growth of extracellular *S. aureus*. (C) Effects of different concentrations of EPG on the growth of intracellular *S. aureus*. Viable bacteria were quantified by the colony plate counting method. Significant deviations from the 30% PPP group (* $P < 0.05$; ** $P < 0.01$). **Abbreviations:** EPG, extract liquid of platelet-rich gel; PPP, Platelet-poor plasma; PRG, platelet-rich gel; *S. aureus*, *Staphylococcus aureus*.

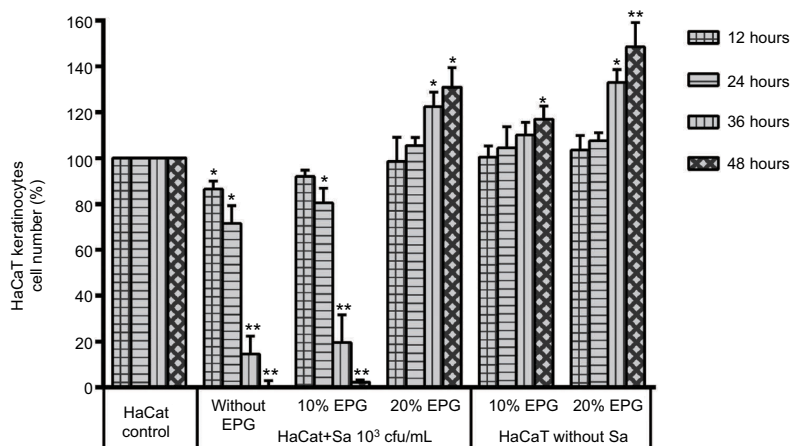


Figure 3 Amounts of 10% and 20% EPG were tested for their capacity to protect HaCaT cells from bacterial damage and promote HaCaT cells proliferation. **Notes:** Significant deviations from the HaCaT control at the respective incubation time (* $P < 0.05$; ** $P < 0.01$). **Abbreviation:** EPG, extract liquid of platelet-rich gel.

stimulated in the HS group at the 12th and 24th hour. After intervention with EPG, PDCD4 and p-NF-κB p65 proteins were significantly reduced when the HS was compared to

the HSP group. Results associated with bacterial infection at the 48th hour were not reported because of insufficient protein extracted from residual HaCaT cells. In comparison

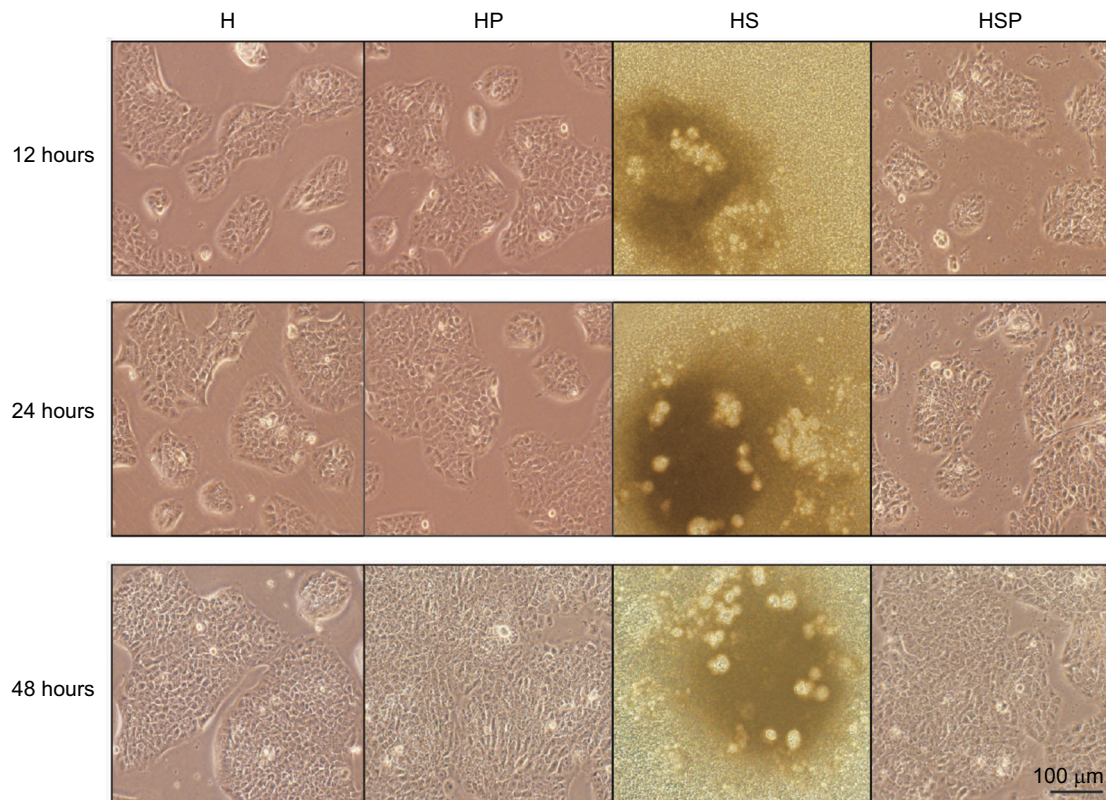


Figure 4 Images of the four groups under light microscope.

Notes: Photos were taken before extraction of RNA from HaCaT cells at various time points. Scale bar: 100 µm.

to the H group, PDCD4 and p-NF-κB p65 protein in the HP group were down-regulated by EPG at the 24th and 48th hour.

On the basis of detection in p-p65 protein expression, the translocations of p65 unit of NF-κB from cytosol to nucleus were determined in the four groups. As Figure 6 shows, antibody against NF-κB p65 produced red signal under fluorescence microscope, whereas the blue signal was produced by the DAPI staining of the cell nuclei. Areas where the red and the blue fluorescence overlapped appeared pink. In the H and HP groups, HaCaT cells exhibited red signal predominantly in the cytoplasm. Bacterial infection induced the pink staining in the HS group, indicating an increase in the nuclear translocation of NF-κB p65 protein. While after intervention with EPG, significantly reduced intensity of pink fluorescence was observed in the HSP group, suggesting EPG blocked the nuclear translocation of the NF-κB p65 protein.

EPG promotes anti-inflammatory cytokines and inhibits pro-inflammatory cytokines

To examine the effect of EPG intervention on inflammation during bacterial infection, the mRNA expressions and concentrations of anti-inflammatory (IL-10 and TGF-β1) and

pro-inflammatory cytokines (IL-6 and TNF-α) were measured in HaCaT cells and cultured supernatant respectively (Figures 7 and 8). Bacterial infection promoted the expression and secretion of IL-6 and TNF-α in the HS group. Whereas the intervention of EPG reduced the expression and release of these two pro-inflammatory cytokines in the HSP group, indicating an inhibition of inflammatory response. The same anti-inflammatory effect was also observed in the absence of bacterial infection in the HP group. In addition, a decrease in IL-10 expression and secretion were observed in the HS group but these were increased by EPG intervention in the HSP group. The expression and secretion of TGF-β1 were also increased by EPG intervention in the HSP group.

Discussion

Metabolic disorders and immune abnormalities in patients with diabetes increase the susceptibility of wounds to bacterial infections. Infection further aggravates these ulcers and impedes the healing process. *S. aureus* is a resident bacterium of human skin and colonizes in wounds after skin damage. It is one of the dominant sources of bacterial infection in DFUs.³ Bacteria causes cell necrosis and apoptosis by invading cells and releasing toxins. Bacteria also activates the body's

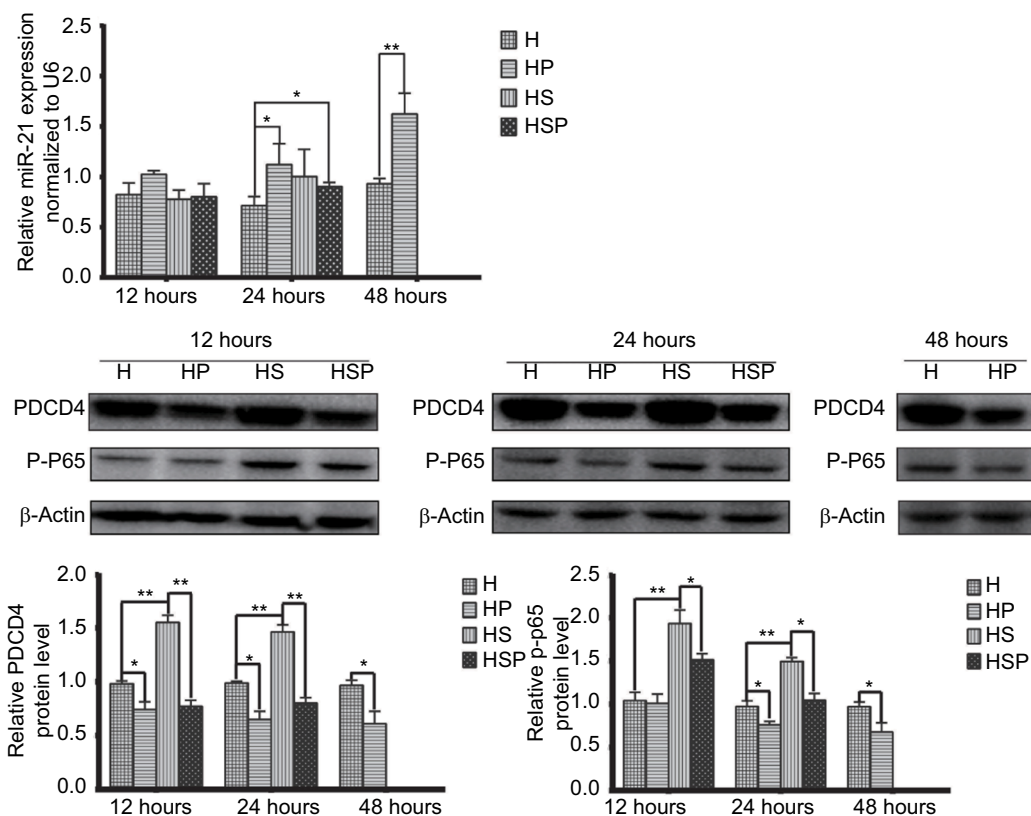


Figure 5 miR-21, PDCD4 and phospho-NF- κ B p65 (P-P65) level in HaCaT cells of the four groups.

Notes: PDCD4 and p-p65 increased because of bacterial infection but decreased by EPG at the 12th and 24th hour. Direct application of EPG to HaCaT cells down-regulated PDCD4 and p-p65 protein at the 24th and 48th hour. MiR-21 elevated with reduced PDCD4 and p-p65 at the 24th and 48th hour. In the setting of bacterial infection, results at the 48th hour are not shown because of insufficient RNA and protein extracted from the residual cells. Significant deviations from the HaCaT control at the respective incubation time (* $P < 0.05$; ** $P < 0.01$).

Abbreviations: EPG, extract liquid of platelet-rich gel; PDCD4, programmed cell death factor 4.

immune system, stimulating release of tissue-lysing enzymes and ROS from immune cells to cause tissue damage; causing sustained or excessive inflammatory reactions, stagnation of the wound healing process in the inflammatory response period and failure to enter the proliferative phase.²¹ Keratinocytes are the most important cell types in the epidermis and normally participate in the formation of barriers and protect the body from invading foreign bodies and pathogens. In the process of wound healing, keratinocytes proliferate, migrate and differentiate, and finally complete the re-epithelialization process.²² In addition, keratinocytes play various roles in the immune response of the skin, such as antigen presentation, release of immune mediators, and secretion of various cytokines.²³ Thus, with reference to a previous study,²⁴ we co-cultured human HaCaT keratinocytes with *S. aureus* under high-glucose conditions to establish an in-vitro high-glucose wound infection model. This model allows users to accurately set various conditions and avoid confounding factors. Compared to simple in-vitro antibacterial experiments, it is also possible to simultaneously observe the effects of bacteria and intervention on donor cells. The devised wound model is a

good study tool for simulating the infected cells in DFUs and observing wound repair mechanism in vitro.

This study illustrates that HaCaT cells damaged by *S. aureus* appears to be concentration-dependent. The low concentration of bacteria causes cell proliferation to be severely impaired after 24 hours of co-culture, suggesting that HaCaT cells have immunomodulatory effects on their own, and have a certain resistance to low concentrations of *S. aureus* virulence in the early stages of cellular damage. High concentrations of bacteria exacerbate the rate of damage. PRG exhibits an antibacterial activity to *S. aureus* at various time-intervals within 72 hours, while PPP cannot exert this antibacterial effect, suggesting that activated platelets are the cornerstone of PRG function. These observations are consistent with previous reports.^{25,26} Platelets share many structural and functional properties with other native immune cells and thus play an important role in host immune defense.²⁷ Platelets release alpha particles, antimicrobial peptides, chemokines, peroxides and other substances, all of which have antibacterial effect. These substances can directly inhibit or kill pathogens, and also exert indirect antibacterial

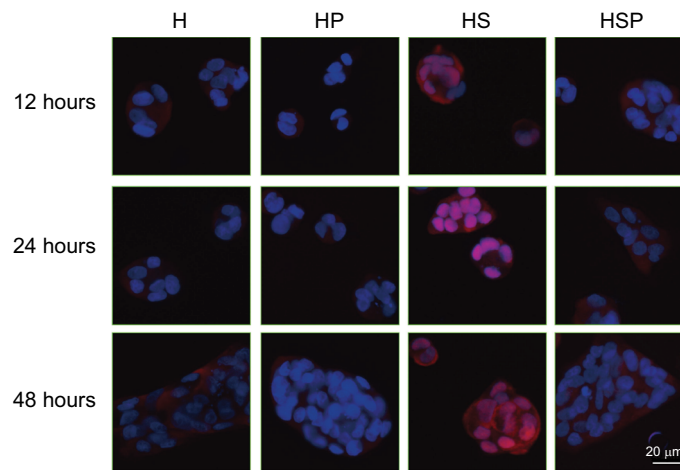


Figure 6 Immunofluorescence microscopy analysis of the spatial localization of the NF- κ B p65 protein.

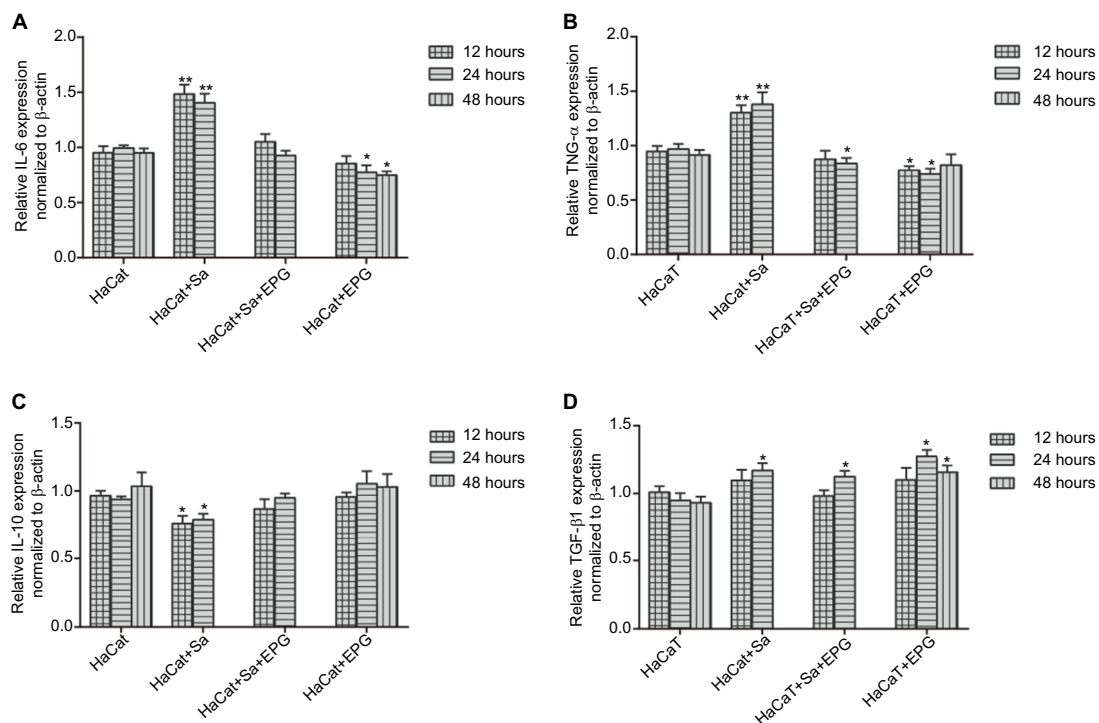


Figure 7 (A) IL-6, **(B)** IL-10, **(C)** TNF- α , **(D)** TGF- β 1 mRNA expression in HaCaT cells as measured by qRT-PCR.

Notes: In the setting of bacterial infection, results at the 48th hour are not shown because of insufficient RNA extracted from residual cells. Significant deviations from the HaCaT control at the respective incubation time (* P <0.05; ** P <0.01).

Abbreviations: EPG, extract liquid of platelet-rich gel; qRT-PCR, quantitative reverse transcription; TNF- α , tumor-necrosis factor alpha.

effects through chemotaxis and activation of immune cells.²⁸ Platelet concentration in PRG is three to five times that of normal human blood, and large amounts of active substances are released after platelet activation, which forms the basis of PRG to exhibit modest antibacterial effect.

In view of the fact that the jelly-like nature of PRG was difficult to pipette accurately, we extracted EPG from

PRG. Our study showed that both of them had equivalent antibacterial capacity within 48 hours, but PRG had a more lasting antibacterial effect than EPG. This phenomenon, on the one hand, may suggest that our extraction method could not completely obtain the platelets and their released substances in PRG. On the other hand, it may also be related to the three-dimensional scaffold structure formed by fibrin

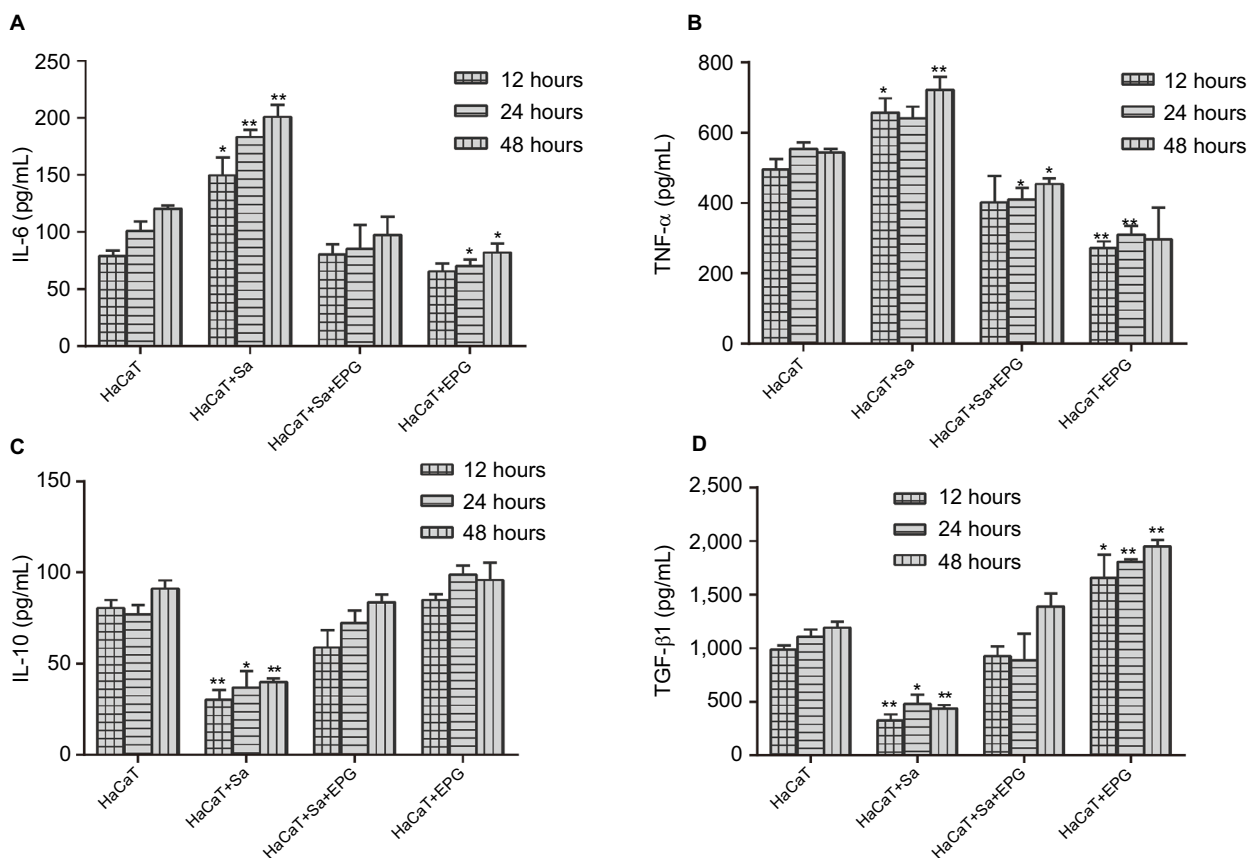


Figure 8 (A) IL-6, (B) IL-10, (C) TNF- α , (D) TGF- β 1 content in the supernatant as determined by ELISA.

Notes: Significant deviations from the HaCaT control at the respective incubation time (* P <0.05; ** P <0.01).

Abbreviations: EPG, extract liquid of platelet-rich gel; TNF- α , tumor-necrosis factor alpha.

polymerization in PRG, adhering to active substances released by platelets and slowly releasing these active substances.²⁹ The sustained-release effect that leads to a more long-lasting therapeutic effect also necessitates the need for the replacement of PRG after covering the wound for 5 days in clinical treatment. However, EPG is considered a more suitable candidate for micro-manipulation, owing to its convenience and accuracy in a liquid form. Combined with the comparative results of antibacterial activity, we believe that EPG was an effective substitute for PRG in experiments within 48 hours, and this finding has not yet been reported before. EPG is found to play a role in our concentration-dependent model. An amount of 20% of EPG can significantly inhibit intracellular and extracellular *S. aureus*, protect HaCaT cells against *S. aureus* infection, and promote cell proliferation. In the absence of *S. aureus* infection, EPG promotes cell proliferation more strongly.

miRNAs are a class of small, non-coding, single-stranded RNAs that specifically recognize and bind to a

target messenger RNA (mRNA), causing degradation of the mRNA or hindering its translation, thereby allowing post-transcriptional regulation of the gene.³⁰ Among the miRNA family, miRNA-21 is considered an important member which is involved in the proliferation, differentiation, and apoptosis of regulated cells and associated with the occurrence and prognosis of a variety of tumors.³¹ Studies have also suggested that miRNA-21 may stimulate re-epithelization of the wound surface.^{16,32} PDCD4 is a tumor suppressor regulated by miRNA-21 and inhibits cell proliferation by blocking protein translation in eukaryotic cells. The absence or low expression of PDCD4 is associated with tumorigenesis.³³ It has been reported that up-regulation of miRNA-21 in lipopolysaccharide-stimulated peripheral blood mononuclear cells can reduce PDCD4, inhibit NF- κ B activity and increase IL-10 expression.³⁴ It has also been reported that increased PDCD4 expression in coronary microembolized porcine cardiomyocytes will increase NF- κ B activity which result in an increase of TNF- α .³⁵ In either way, PDCD4 can activate NF- κ B signaling pathway to influence the regulation

of inflammation. NF- κ B is an important transcriptional regulator that specifically binds to promoters and enhancers of various genes and plays an important role in the body's immune response through its regulation of the expression of cytokines, adhesion molecules, chemokines, and immune receptors.³⁶ The most common form of NF- κ B is a dimeric form consisting of two family members of the p50 and p65 and is inactivated via the binding with I κ B. Upon activation, I κ B is degraded, p65 is phosphorylated, and NF- κ B is transported from the nucleus into the cytoplasm and functions as a transcription regulator.³⁷

This study found that *S. aureus* infection caused a simultaneous increase in the levels of PDCD4 and phosphorylated p65 (p-p65) in HaCaT cells. The increase in PDCD4 partially explained the impaired cell proliferation induced by bacterial infection, while the increase in p-p65 and its translocation from cytosol to nucleus reflected the activation of the NF- κ B signaling pathway. After 12 hours of EPG intervention, we found that both PDCD4 and p-p65 were down-regulated and the translocation of p65 was significantly reduced, but up-regulation of miRNA-21 was not yet statistically significant at this time point. We speculated that EPG-mediated up-regulation of miRNA-21 was a relatively slow process. The down-regulation of PDCD4 and p-p65 in the early stage was mainly attributed to the bacteriostatic effect of EPG. After 24 hours of EPG intervention, we found that up-regulation of miRNA-21 in HaCaT cells were coordinated by the down-regulation of PDCD4 and p-p65, as well as the reduced p65 translocation to the nucleus. We speculated that this phenomenon was partially related to the inhibition on bacteria by EPG. In addition, it may also be attributed to the up-regulation of miRNA-21 by EPG and subsequent down-regulation of PDCD4 and inhibition of NF- κ B activity. In the absence of bacterial infection, we used EPG to directly intervene in the cellular activity, in which the up-regulation of miRNA-21 appeared at the 24th hour but the degree of up-regulation was more pronounced at the 48th hour. This phenomenon also further supported our hypothesis. Synchronous reduction of PDCD4 and p-p65 observed with increased miRNA-21 in sterile conditions confirmed that EPG could regulate PDCD4 through miRNA-21 and thus affected the NF- κ B signaling pathway, regardless of its antibacterial effect.

The pro-inflammatory cytokines, IL-6 and TNF- α accumulate in large amounts at the DFUs' wound surface, making it difficult for inflammation to subside and aggravating tissue damage.^{5,38} Anti-inflammatory cytokine IL-10 can promote tissue repair by inhibiting these inflammatory responses.⁵

TGF- β 1 can promote the proliferation, migration and differentiation of fibroblasts, accelerate epithelial-mesenchymal transition, coordinate the action of other growth factors, and facilitate wound healing.³⁹ In the present study, it was found that the up-regulation of IL-6 and TNF- α caused by *S. aureus* infection was consistent with the activation of the NF- κ B signaling pathway, and the down-regulation of IL-10 and TGF- β 1 also reflects the perverse healing effect caused by the antibacterial infection. After intervention with EPG, the changes of the above-mentioned cytokines can be reversed; the anti-inflammatory cytokines are increased whereas the pro-inflammatory factors are reduced. In the absence of bacterial infection, direct action of EPG on cells validates this anti-inflammatory effect. Combined with the detection of relevant signal molecules, we consider miRNA-21/PDCD4/NF- κ B signaling pathway as a direct mechanism for EPG to exert its anti-inflammatory effects, and that EPG also indirectly produces an anti-inflammatory effect by inhibiting bacterial growth and reducing harmful stimuli.

miRNA-21 may play an important role in PRG mediating anti-inflammation and promoting wound repair, and provide further theoretical support for PRG to effectively treat DFUs in clinical practice. Yet our studies have presented some deficiencies. At present, we have not been able to elucidate the mechanism of miRNA-21 changes in HaCaT cells. It has been reported that platelet-released exosomes are rich in miRNAs, and these exosomes can be taken up by other cells,⁴⁰ suggesting that this change may be due to the direct release of miRNA-21 from platelets. It has also been reported that platelet-released growth factors, such as TGF- β can up-regulate miRNA-21,⁴¹ suggesting that this change may also be the result of the action of other platelet-derived substances. Further study is required to elaborate these changes. In addition, the elevated mRNA expression of TGF- β is contradicted by its reduced content in supernatants. This phenomenon may be involved in mRNA translation, protein modification, and protein secretion. The uncertain regulatory mechanism in these processes deserves our further study.

Conclusion

In conclusion, the high-glucose in vitro infection wound model established may be a valuable tool for future similar research. miRNA-21 appears to regulate NF- κ B through PDCD4 and play a triple role of antibacterial, anti-inflammatory while stimulating cell proliferation in the diabetic wound model. Therefore, we speculate that miRNA-21 is a

promising intervention target and may provide new strategies for the treatment of refractory wounds such as DFUs.

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Disclosure

The authors report no conflicts of interest in this work.

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