

Platelet-rich fibrin combined with naringin accelerates diabetic wound healing by enhancing re-epithelialization and reducing inflammation

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Abstract. Delayed wound healing, one of the fatal complications that affect about 15% of diabetic patients, is associated with impaired keratinocyte proliferation and migration and enhanced inflammation. In this research, we investigated the combined application of platelet-rich fibrin (PRF) and the natural flavonoid naringin for wound healing in diabetic conditions. High glucose (HG)-stimulated human keratinocytes HaCaT and high fat diet + streptozotocin-induced diabetic rats were treated with PRF and naringin alone or in combination. HaCaT cell proliferation and migration were detected by EdU staining and wound healing assay. On days 0, 3, 7, and 14 after wound creation, the wound healing rates in diabetic rats were calculated. The combined treatment of PRF and naringin was more effective than PRF or naringin alone in enhancing cell proliferation and migration and attenuating MMP2/9 and pro-inflammatory cytokine expression in HG-exposed HaCaT cells. PRF and naringin combination exhibited higher efficacy in accelerating wound closure, elevating cell proliferation, and reducing inflammation and NF- κ B phosphorylation in rat wound tissues vs. PRF or naringin treatment alone. In conclusion, the combined treatment of PRF and naringin is more effective than either treatment alone in promoting the healing of diabetic wounds through enhancing wound re-epithelialization and alleviating inflammation.

Key words: Diabetic wounds — Platelet-rich fibrin — Naringin — Keratinocytes — Inflammation

Introduction

Diabetes mellitus (DM) has become one of the most metabolic disorders globally in recent decades, which seriously endangers human health and creates a huge socioeconomic burden (Cloete 2022). According to the International Diabetes Federation, DM affects nearly 537 million adults in 2021 and is predicted to affect about 783 million adults in 2045 (Sun et al. 2022). Decreased physical activity, dysregulated lifestyles, highly supplemented diets, an aging population, urbanization, and rising obesity are all risk factors that might contribute to the occurrence of DM (Wu et al. 2014). DM is characterized by persistent hyperglycemia, which frequently

causes severe complications, including cardiovascular disease, retinopathy, kidney disease, neuropathy, hearing impairment, and delayed wound healing (Zheng et al. 2018). The injured tissue will initiate a complex and ordered process of acute wound healing under normal physiological conditions, which includes four classical stages: hemostasis, inflammation, proliferation, and remodeling (Morton and Phillips 2016). However, diabetes can disturb and delay the normal progression of wound healing, leading to long-term wound nonunion (Bai et al. 2020). A high-glycemic environment can promote the proliferation of bacteria and other microorganisms, increasing the risk of wound infections, and significantly slowing down the wound healing process (Sousa and Batista Kde 2016). Besides, diabetes is often accompanied by vascular lesions, which can lead to poor blood circulation and the lack of an adequate supply of oxygen and nutrients in the wound area, further impeding wound

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repair and healing (Yang et al. 2023). Currently available treatments such as strict blood glucose control, therapeutic biomaterial gels, anti-infection approaches, and wound dressing are only somewhat effective in treating diabetic wounds, while the persistence and deterioration of a large number of diabetic wounds will eventually cause amputation (Patel et al. 2019). Hence, it is urgent to develop more efficient therapeutic strategies to improve the management of chronic diabetic wounds.

The skin wound healing process involves the interaction of platelets, inflammatory cells, epithelial cells, keratinocytes, fibroblasts, various cytokines, growth factors, and other bioactive molecules (Sorg et al. 2017). Platelet-rich plasma (PRP), the first generation of blood concentrates, was initially extracted from human plasma by Assoian et al. in 1984 (Merchán et al. 2019). PRP is rich in platelets, and platelet degranulation releases a large amount of growth factors such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β), vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF-1), which promote wound healing as well as repair and regeneration of soft and hard tissues (Kobayashi et al. 2016). Since the 1990s, PRP treatments have been extensively applied in the fields of sports medicine, orthopaedics, cosmetic dermatology, and dentistry, showing potent therapeutic potential (Hesseler and Shyam 2019). However, the application of PRP is heavily controversial since the addition of heterologous thrombin and anticoagulants during the preparation process can result in the risk of immune rejection and transmission of infectious diseases (Wu et al. 2022). By contrast, platelet-rich fibrin (PRF), the second generation of blood concentrates prepared and extracted by Choukroun et al. in 2000 (Diab et al. 2023). PRF is composed of fibrin and the platelets, leukocytes, cytokines and circulating stem cells embedded in it (Strauss et al. 2018). PRF retains the advantages of PRP while offering a simpler preparation process as it requires no additional anticoagulants or thrombin, avoiding the risk of immune rejection and cross-infection (Miron et al. 2024). Accordingly, PRF has gradually replaced PRP in clinical practice. Previously, use of PRF was revealed to markedly accelerate skin wound healing on the back of diabetic mice (Ding et al. 2017). In addition, Wang et al. (2024) reported that treatment with autologous leukocyte-PRF brought significantly higher wound healing rate and overall cure rate than the traditional treatment method in patients with diabetic foot ulcers after debridement, suggesting that leukocyte-PRF effectively promotes wound healing in diabetic foot ulcers.

Natural compounds with vast diversities and few side effects have long been a good source for the development of new drugs (Thomford et al. 2018). Phytogetic compounds or derivations account for about half of the pharmaceuticals approved by the Food and Drug Administration (FDA)

(Jung et al. 2014). Till now, multiple plant-derived natural compounds have been identified to serve as therapeutics against diabetes and its complications (Bao et al. 2023). Naringin (4',5,7-trihydroxyflavanone-7-neohesperidoside) is an active flavanone glycoside that is abundant in various citrus fruits such as pomelo, lemon, lime, orange, and grapefruit (Alam et al. 2014). It is well-documented that naringin possesses anticancer, antiosteoporotic, antiulcer, antiapoptotic, anti-inflammatory, neuroprotective, antioxidant, and hypoglycemic properties (Shilpa et al. 2023). A great body of studies using cellular and animal models has demonstrated the benefits of naringin in preventing and treating various diseases, including diabetes (Ravetti et al. 2023). Naringin has been shown to improve cellular uptake of glucose and enhance overall blood sugar control (Den Hartogh and Tsiani 2019). Moreover, naringin is effective in attenuating insulin resistance, a major factor contributing to the development of type 2 diabetes (Den Hartogh and Tsiani 2019). Importantly, treatment with naringin was confirmed to improve wound healing of diabetic foot ulcers in rat models by inhibiting hyperglycemia, mitigating apoptosis, oxidative stress, and inflammatory response, and stimulating angiogenesis in wound tissues (Kandhare et al. 2014). Naringin activates HaCaT keratinocyte migration *in vitro* and accelerates the closure of full-thickness excisional wounds on the shaved dorsal skin of mice, showing its beneficial effects in wound healing (Yen et al. 2022). Kandhare et al. (2016) suggested that naringin ointment formulation exhibited wound-healing activities on experimental wound models through downregulating inflammatory and apoptotic factor expression and upregulating growth factor expression. Nevertheless, the pro-wound healing efficacy of the combination of PRF and naringin in the diabetic setting is yet to be investigated. Therefore, our study was designed to determine the effects of PRF combined with naringin on the proliferation, migration, and inflammation of high glucose (HG)-stimulated keratinocytes *in vitro* and wound healing and inflammation in diabetic animal models.

Materials and Methods

Cell culture

Human keratinocytes (HaCaT, #SNL-163), purchased from SUNNCELL (Wuhan, China), were cultivated in Dulbecco's modified Eagle's medium (DMEM; #90013; Solarbio, Beijing, China). The medium was supplemented with 10% fetal bovine serum (FBS; #LM87074C; LMAI Bio, Shanghai, China) and 1% Penicillin/Streptomycin (#LM1200; LMAI Bio). Cells were placed at 37°C in an incubator containing 5% CO₂. The purchased cells were tested and confirmed to

be free of mycoplasma contamination and authenticated by short tandem repeat (STR) profiling.

Preparation of PRF gel, film and leachate

Fresh venous blood (10 ml) from three healthy volunteers (aged 24–32 years) was collected into sterile negative pressure collection vessel and centrifuged for 10 min at 3000 rpm. After centrifugation, the fibrin clot in the middle layer consisted of PRF rich in growth factors. The PRF gel was removed and left to stand for 10 min to be slowly dehydrated to obtain PRF, which was extruded into a film (7×7 mm) with a thickness of 1 mm by two glass sheets and cut into small pieces (1×1×1 mm). The small pieces of PRF film were added into 5 ml of serum-free DMEM and soaked at a ratio of 1 ml medium to 100 mg PRF film. This mixture was maintained in a cell incubator (37°C, 5% CO₂) for 7 days, after which the resulting supernatant was collected and sterilized by filtration through a 0.22-μm syringe filter to obtain the PRF leachate. The PRF leachate was stored in a refrigerator at –80°C for standby. All volunteers had provided informed signed consents, and the experimental protocols were performed according to regulations and approval of the institutional ethical committee of the Hospital.

Cell treatment

Naringin (purity: ≥98%; #SS0171) was acquired from NJDULY (Nanjing, China) and dissolved in dimethyl sulfoxide (DMSO; #D8370; Solarbio). HaCaT cells were assigned into five groups: NG (normal glucose; treated with 5.5 mM glucose), HG (high glucose; treated with 50 mM glucose), HG+Naringin (treated with 50 mM glucose for 24 h and then with 200 μM naringin for 24 h), HG+PRF (treated with 50 mM glucose for 24 h and then with PRF leachate for 24 h), and HG+Naringin+PRF (treated with 50 mM glucose for 24 h and then with 200 μM naringin combined with PRF leachate for 24 h). The concentration of naringin was selected according to the previous study (Yen et al. 2022).

Cell proliferation assay

A 5-ethynyl-2'-deoxyuridine (EdU) assay kit (#C10310, RIBOBIO, Guangzhou, China) was used to analyze cell proliferation. HaCaT cells were inoculated (1×10⁵ cells/well) into 6-well plates and incubated overnight to allow adherence. After different treatment for 24 h, cells were incubated for 2 h with 100 μl EdU solution, followed by 15 min fixation with 4% paraformaldehyde, 10 min incubation with 0.2% glycine, and 10 min permeabilization with 0.5% Triton X-100. Thereafter, Apollo staining solution was added and cells were cultured for 30 min protected from light. Cell

nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; #CSD4080; Chemstan, Wuhan, China) for 10 min. Finally, a fluorescence microscope (Olympus) was utilized to image the stained cells.

Cell migration assay

Wound healing assay was conducted to assess the migratory capability of HaCaT cells. Cells after 24 h of treatment were harvested and seeded (2×10⁵ cells/well) in 6-well plates. When the cell density reached 80%, a sterile 200 μl pipette tip was used to gently make an incision in the monolayer to generate a scratch wound. The wells were washed thrice with phosphate buffer saline (PBS) to clear the floating cells, and cells were then cultivated for 24 h in serum-free medium. The wound area was photographed using an inverted microscope (Olympus) at 0 h and 24 h after scratching and measured using ImageJ software.

Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α (#E-EL-H0109), IL-1β (#E-EL-H0149), IL-6 (#E-EL-H6156), and IL-8 (#E-EL-H6008) in cell supernatant of HaCaT cells were examined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Elabscience, Wuhan, China) in line with the protocols.

Preparation of naringin ointment

The naringin ointment was composed of 30% PEG4000 (#AWC0188a; Abiowell, Changsha, China) and 70% PEG400 (#C107938; ChemeGen, Shanghai, China). The naringin, PEG400, and PEG4000 components were stirred and mixed at 80°C until uniformly distributed, then stirred at room temperature to a semi-solid state. The naringin cream (5 mg) was applied to the wound of each rat once *per day*.

Animal modeling and drug treatment

Male SPF-grade Sprague-Dawley rats (8-week-old, 200–220 g) were provided by Hunan SLRC Laboratory Animal Center (Changsha, China). Procedures involving animals and their care were approved by the Animal Care Committee of the institute (approval number: HLK-202404124; approval date: April 16, 2024) and performed in agreement with ARRIVE guidelines. Rats were housed 2/3 *per cage* under stable environmental conditions of temperature (21 ± 2°C) and humidity (60 ± 5%) with a 12/12 h light/dark cycle. Type 2 diabetes rat models were induced after one week of feeding with regular chow. The rats were fed with high-fat diet (HFD: 59% fat, 20% carbohydrates, and 15% protein) for 50 days and *intraperitoneally* injected with streptozo-

tocin (STZ; #S17049; Yuanye, Shanghai, China) dissolved in citrate buffer solution (0.1 M, pH 4.5) at a dose of 35 mg/kg at day 21 and 42 from the beginning of HFD. An *intra-peritoneal* glucose tolerance test (IPGTT) was performed 8 days following the second injection of STZ to verify diabetes induction. In brief, rats were fasted for 6 h prior to IPGTT and blood samples from tail vein were collected at the following time points: T0 (before glucose injection) and 30, 60, 90, and 120 min after glucose injection. An Accu-Chek Aviva glucometer (Hoffman-La Roche, Basel, Switzerland) was used to measure blood glucose levels. Rats with blood glucose exceeding 250 mg/dl at the end of the IPGTT were deemed diabetic and encompassed in our study (Cassano et al. 2020). Subsequently, diabetic rats were left to relax for one week and then intraperitoneally injected with ketamine (80 mg/kg) and xylazine (10 mg/kg) for anesthesia, after which the rats were depilated on the backs and a 10 mm diameter wound was created on the shaved dorsum using the full-thickness skin defect method. Thereafter, the wounded diabetic rats were randomly assigned into 4 groups ($n = 6/\text{group}$): Saline, Naringin, PRF, and Naringin+PRF. The wounded areas were treated daily for 14 consecutive days from the day of wound creation with normal saline, naringin ointment, PRF, and naringin ointment and PRF combination. The PRF was applied to the entire wound surface in a thin layer and the dressing was secured by using elastic gauze (Ding et al. 2017). In the Naringin+PRF group, the wound surface was first applied with naringin cream and then covered with PRF dressing. Naringin cream was applied and PRF dressing was replaced every day. In the Saline group, the wound surface was applied with PEG ointment (5 mg/rat) as a naringin vehicle. Only one rat was placed in each cage, which avoided mutual interference, reduced the risk of infection, and facilitated observation and care. On days 0, 3, 7, and 14 after wound creation, the wound area was photographed and the wound healing rate was calculated using ImageJ software. On day 14, the rats were sacrificed with an overdose of isoflurane, and the wound tissues were harvested for follow-up analysis.

Hematoxylin and eosin (H&E) staining

Rat wound skin tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned into five-micron thick sections using a cryostat (Thermo Fisher Scientific). The sections were dewaxed in xylene, dehydrated with gradient ethanol (75–100%), and stained with hematoxylin (#AWI0443a; Abiowell) for 5 min. Next, the slices were washed in 0.5% hydrochloric acid alcohol for 10 s and further stained in eosin (#AWI0119a; Abiowell) for 40 s. After dehydration with graded alcohol, transparency with xylene, and sealing with neutral gum, the sections were finally photographed using a fluorescence microscope.

Immunohistochemical staining

Paraffin wax-embedded sections of rat wound skin tissues were dewaxed in xylene and dehydrated with gradient ethanol, followed by the thermal repair of antigen in 0.01 M citrate buffer (pH 6.0) for 20 min. Subsequently, the slices were cleaned with PBS, added with 1% periodate acid for inactivation of the endogenous enzymes, and dropped with TNF- α (#AF7014; 1:100; Affinity Bioscience) and IL-1 β (#AF5103; 1:100; Affinity Bioscience) primary antibodies overnight at 4°C. The next morning, the slides were subjected to three washes (3 min/time) in PBS, and further 30 min incubation at 37°C with IgG secondary antibody (#S0001; 1:200; Affinity Bioscience). 3,3'-diaminobenzidine (DAB) working solution (#AR1027; Boster, Wuhan, China) was added for color development for 5 min at room temperature to visualize the antibody-antigen complexes. The sections were finally counterstained with hematoxylin, dehydrated in alcohol (from 60–100%), and ready for microscopic analysis under an optical microscope. The immunoexpression of the positive area was quantified in five randomly selected fields from each slice using ImageJ software.

RT-qPCR

Total RNA was isolated from HaCaT cells and rat wound skin tissues using TRIzol reagent (#mlsw-2615; mlbio, Shanghai, China). After detecting RNA concentration and integrity, 1 μg total RNA was prepared to perform the reverse transcription procedure using a reverse transcriptase kit (#R223-01; Vazyme, Nanjing, China) to synthesize the cDNA first chain. Afterwards, the qPCR SYBR Green Mix (#11200ES50; Yeason, Shanghai, China) was used to perform real-time PCR on the ABI 7500 Real-time PCR system (Applied Biosystems) with the following conditions: 95°C for 60 s, 40 cycles of 95°C for 5 s, and 60°C for 15 s. All primer sequences are displayed in Table 1. The relative expression levels (normalized to GAPDH) were calculated by employing the $2^{-\Delta\Delta C_t}$ method.

Western blotting

HaCaT cells and rat wound skin tissues were lysed in RIPA lysis buffer (#BL504A; Reanta, Beijing, China) to extract total protein. The protein concentration was determined by BCA protein quantitative kit (#abs9232; Absin, Shanghai, China). Equal amounts (40 μg) of protein samples were loaded onto gels, separated by 12% SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were incubated for 2 h with 5% non-fat dry milk in TBS/Tween20 (TBST) to block non-specific binding sites on membranes. MMP2 (#AF5330; 1:1000), MMP9 (#AF5228; 1:1000), p-NF- κB (#AF2006; 1:1000), NF- κB (#AF5006; 1:1000), and GAPDH

(#AF7021; 1:3000) primary antibodies (Affinity Bioscience, Jiangsu, China) were added to the membrane overnight at 4°C. After washing with TBST, the HRP-conjugated secondary antibody (#S0001; 1:3000; Affinity Bioscience) were used to develop the immunoblots at 37°C for 2 h. Visualization of the signals was performed using the ECL enhanced chemiluminescence substrate (#10144; Proandy, Shanxi, China). Images were captured using an Image Quant RT ECLTM imager (GE Healthcare, Chicago, IL, USA). Densitometric analysis was conducted using ImageJ software. GAPDH served as internal control.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, CA, USA), and the results collected are represented by mean $\pm$ standard deviation. As confirmed by the Shapiro-Wilk test, all data follow a normal distribution. The measurement data were compared using Student's *t*-test between two groups, and one-way ANOVA followed by Tukey's *post-hoc* test was used to compare multiple groups. Significance was established as $p < 0.05$.

Results

PRF combined with naringin enhances the proliferation and migration of HG-stimulated HaCaT cells

First, we evaluated the effects of PRF and naringin combined treatment on the proliferation and migration of HG-stimulated HaCaT cells. As shown by EdU staining, the number of EdU-positive cells was significantly reduced after HG induction ($p < 0.001$, HG vs. NG), which however, was largely recovered after PRF single treatment ($p < 0.01$, HG+PRF vs. HG) or PRF and naringin combined treatment ($p < 0.001$, HG+Naringin+PRF vs. HG). The promoting effect of combined treatment on the proliferation of HG-induced HaCaT cells was better than that of single treatment ($p < 0.05$, HG+Naringin+PRF vs. HG+PRF; $p < 0.01$, HG+Naringin+PRF vs. HG+Naringin) (Fig. 1A). Next, wound healing assay illustrated that HG-induced increase in the scratch width was reversed after PRF treatment alone ($p < 0.01$, HG+PRF vs. HG) or PRF and naringin treatment in combination ($p < 0.001$, HG+Naringin+PRF vs. HG), and the combined treatment showed superior efficacy in attenuating the inhibitory effects of HG on keratinocyte migration ($p < 0.05$, HG+Naringin+PRF vs. HG+PRF; $p < 0.001$, HG+Naringin+PRF vs. HG+Naringin) (Fig. 1B). Furthermore, the expression of matrix metalloproteinases (MMPs), extracellular matrix degradation enzymes, in HaCaT cells was measured by Western blotting. We found that MMP-2 and MMP-9 levels were markedly upregulated under

Table 1. Primer sequences used for RT-qPCR

Gene	Primer sequences
<i>Human</i>	
TNF- α	F: 5'-GAACCCCGAGTGACAAGCCT-3' R: 5'-TATCTCTCAGCTCCACGCCAT-3'
IL-1 β	F: 5'-CCCTCTGTCATTCGCTCCC-3' R: 5'-TAAAGAGAGCACACCAGTCCA-3'
IL-6	F: 5'-AGCACTGGCAGCACAAGGCAAAC-3' R: 5'-CAAGCCTGGG-ATTATGAAGAAGG-3'
IL-8	F: 5'-GGGCCATCAGTTGCAAATC-3' R: 5'-TTCCTTCCGGTGGTTTCTTC-3'
GAPDH	F: 5'-TCATTTCCCTGGTATGACAACGA-3' R: 5'-GTCTTACTCCTTGGAGGCC-3'
<i>Rat</i>	
TNF- α	F: 5'-TCCAGAACTCCAGGCGGT-3' R: 5'-TTGGTGGTTTGCTACGAC-3'
IL-1 β	F: 5'-TCAAGCAGAGCACAGACCTG-3' R: 5'-GAAGACACGGGTTCATGGT-3'
IL-6	F: 5'-AAATCTGCTCTGGTCTTC-3' R: 5'-AGGGTTTCAGTATTGCTC-3'
IL-8	F: 5'-GGTAAAGTCCGTAAGTCGTAGTAC-3' R: 5'-AGTTAACGTGGAGGTACCGTA-3'
GAPDH	F: 5'-TGTGTCCGTCGTGGATCTGA-3' R: 5'-CCTGCTTCACCACCTTCTTGAT-3'

F, forward; R, R.reverse.

HG conditions compared with NG conditions ($p < 0.001$, HG vs. NG), while a decline was observed in MMP-2 and MMP-9 levels after PRF and/or naringin treatment ($p < 0.001$, HG+Naringin/HG+PRF/HG+Naringin+PRF vs. HG), with even lower levels in HG-stimulated HaCaT cells after combined treatment than after single treatment ($p < 0.05$, HG+Naringin+PRF vs. HG+PRF; $p < 0.001$, HG+Naringin+PRF vs. HG+Naringin) (Fig. 1C).

PRF combined with naringin reduces inflammatory cytokine expression in HG-stimulated HaCaT cells

Studies have shown that chronic long-term inflammation is one of the hallmarks of diabetic wounds. Therefore, the anti-inflammatory activity of PRF and naringin in a single or combined treatment in HG-exposed HaCaT cells was determined by detecting the expression of inflammatory cytokines. As depicted in Figure 2A, HG treatment strikingly enhanced the mRNA levels of TNF- α , IL-1 β , IL-6, and IL-8 in HaCaT cells ($p < 0.001$, HG vs. NG). Of note, naringin and/or PRF treatment remarkably attenuated HG-induced upregulation in these inflammatory cytokine levels ($p < 0.001$, HG+Naringin/HG+PRF/HG+Naringin+PRF vs. HG), with anti-inflammatory activity further enhanced

by combination treatment ($p < 0.001$, HG+Naringin+PRF vs. HG+Naringin/HG+PRF). The results of ELISA were consistent with those of RT-qPCR. HG-induced increments in TNF- α , IL-1 β , IL-6, and IL-8 levels in keratinocyte culture supernatants were antagonized by single or combined treatment of naringin and PRF ($p < 0.001$, HG+Naringin/HG+PRF/HG+Naringin+PRF vs. HG). The combined treatment led to a further significant reduction in the above

inflammatory cytokine levels ($p < 0.001$, HG+Naringin+PRF vs. HG+Naringin/HG+PRF) (Fig. 2B).

PRF combined with naringin promotes skin wound healing in diabetic rats

After establishing a wound model in type II diabetic rats, pictures of rat dorsal wounds were captured on days 0, 3, 7,

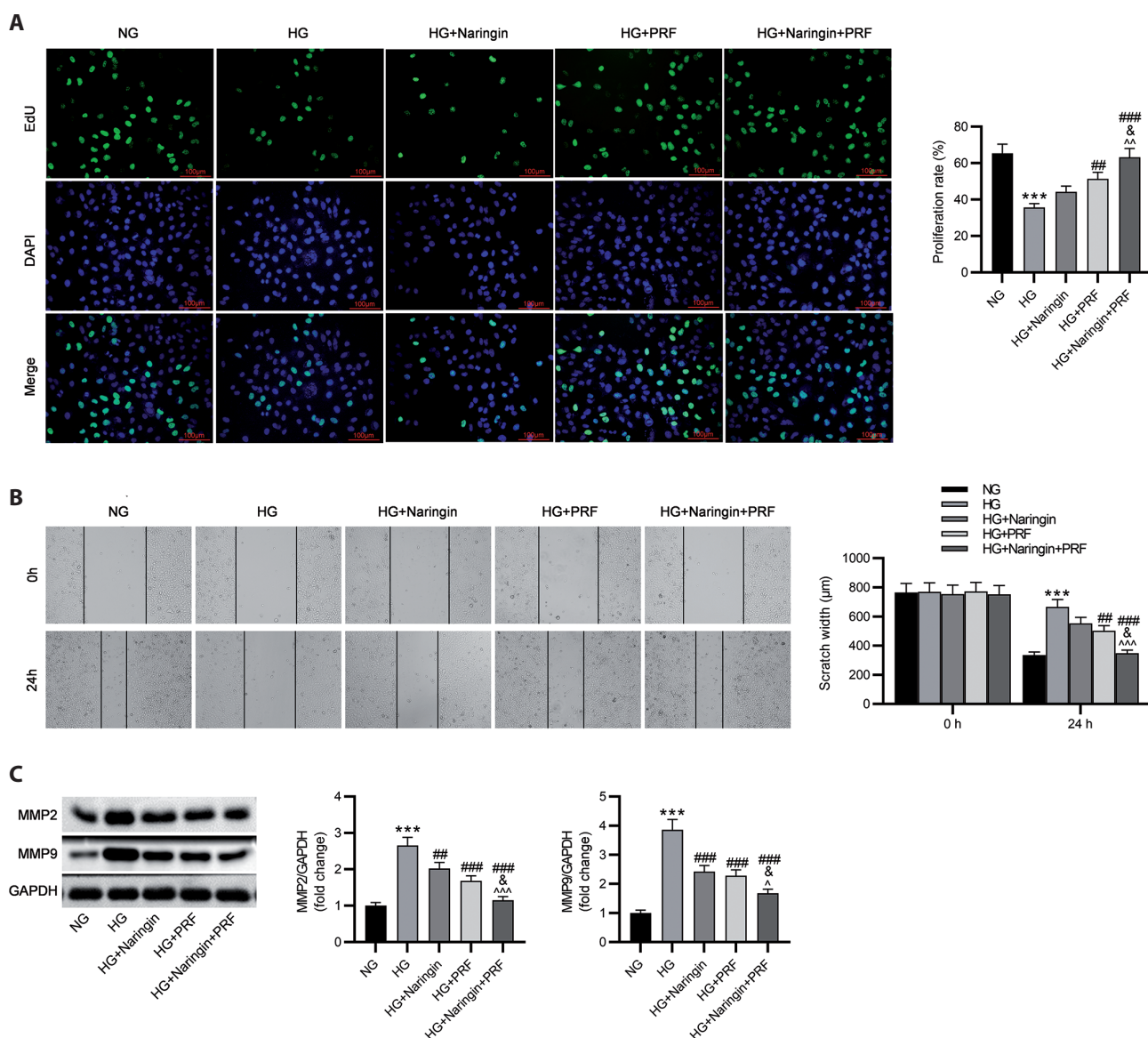


Figure 1. PRF combined with naringin enhances the proliferation and migration of HG-stimulated HaCaT cells. **A.** The proliferation of treated HaCaT cells was detected through EdU staining. **B.** The migratory ability of treated HaCaT cells was assessed by wound healing assay. **C.** The protein levels of MMP-2 and MMP-9 in treated HaCaT cells were measured by Western blotting. *** $p < 0.001$ vs. NG; ## $p < 0.01$, ### $p < 0.001$ vs. HG; & $p < 0.05$ vs. HG+PRF; ^ $p < 0.05$, ^^ $p < 0.01$, ^^ ^ $p < 0.001$ vs. HG+Naringin. NG, normal glucose (treated with 5.5 mM glucose); HG, high glucose (treated with 50 mM glucose); HG+Naringin, treated with 50 mM glucose for 24 h and then with 200 μ M naringin for 24 h; HG+PRF, treated with 50 mM glucose for 24 h and then with PRF leachate for 24 h; HG+Naringin+PRF, treated with 50 mM glucose for 24 h and then with 200 μ M naringin combined with PRF leachate for 24 h.

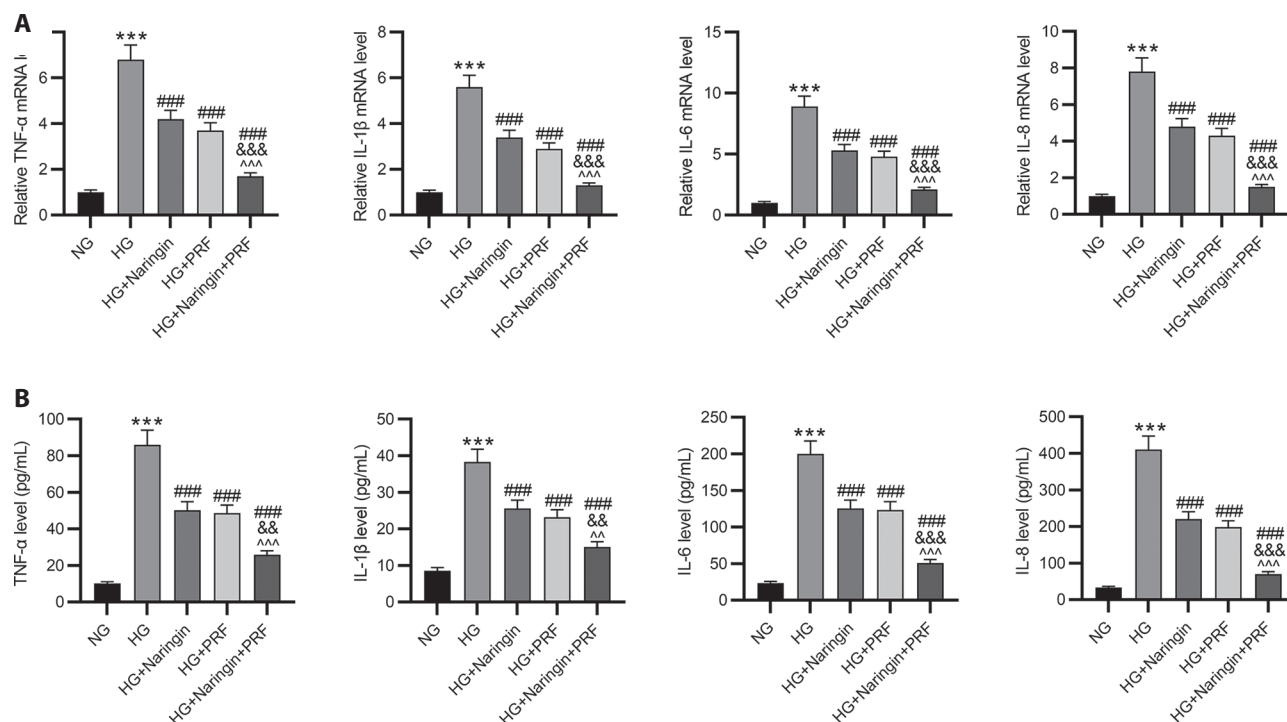


Figure 2. PRF combined with naringin reduces inflammatory cytokine expression in HG-stimulated HaCaT cells. **A.** The mRNA levels of TNF- α , IL-1 β , IL-6, and IL-8 in treated HaCaT cells were determined through RT-qPCR. **B.** The levels of TNF- α , IL-1 β , IL-6, and IL-8 in cell culture supernatants of treated HaCaT cells were evaluated by ELISA. *** $p < 0.001$ vs. NG; ### $p < 0.001$ vs. HG; && $p < 0.01$, &&& $p < 0.001$ vs. HG+PRF; ^ ^ $p < 0.01$, ^ ^ ^ $p < 0.001$ vs. HG+Naringin. For abbreviations, see Figure 1.

and 14 to record the wound size and calculate the wound healing rate. As observed in Figure 3A, the wound area was gradually reduced from days 3 to 14 after wound creation. The wound area at days 3 and 7 showed no significant differences in the four groups. The wounds were almost healed on day 14 in the Naringin+PRF group, while the wounds of the other treated groups were still in progress. The wound closure rate is shown in Figure 3B. We discovered that model rats administrated with naringin ($p < 0.05$, Naringin vs. Saline), PRF ($p < 0.001$, PRF vs. Saline), or naringin together with PRF ($p < 0.001$, Naringin+PRF vs. Saline) exhibited a faster wound healing rate than the saline-administrated model rats. Importantly, the Naringin+PRF group had a prominently higher wound closure rate than the Naringin ($p < 0.001$, Naringin+PRF vs. Naringin) and the PRF (Naringin+PRF vs. PRF) groups, which even reached nearly 100% on the 14th day. Besides, the histological evaluation of the healing progress in the four groups was performed on day 14 post-operation. The H&E-stained histological sections of the wound area showed that the migrating epidermal cells from both sides of the wound met under the wound scab to form a complete epidermis. Naringin or PRF administration notably accelerated the wound healing. Compared with the other three groups, the healing rate displayed high progress

in the naringin and PRF combination group, with wound scabs almost disappeared and hair follicles and sebaceous glands clearly visible (Fig. 3C). The expression of cell proliferation marker (Ki-67) in wound skin tissues was further monitored through immunohistochemical staining. The data manifested that naringin alone ($p < 0.05$, Naringin vs. Saline), PRF alone ($p < 0.01$, PRF vs. Saline), or naringin and PRF combined ($p < 0.001$, Naringin+PRF vs. Saline) administration considerably increased Ki-67 expression compared with the saline-administrated control group. In comparison, the naringin and PRF combination group exhibited a markedly higher Ki-67 expression than the Naringin ($p < 0.001$, Naringin+PRF vs. Naringin) and the PRF (Naringin+PRF vs. PRF) groups (Fig. 3D).

PRF combined with naringin alleviates skin wound inflammation in diabetic rats

Finally, the anti-inflammatory action of naringin and PRF combination treatment in diabetic wound healing was confirmed in rat models. As shown by RT-qPCR, the mRNA expression of TNF- α , IL-1 β , IL-6, and IL-8 in skin wounds of diabetic rats was attenuated after naringin and/or PRF treatment ($p < 0.001$, Naringin/PRF/Naringin+PRF vs. Saline),

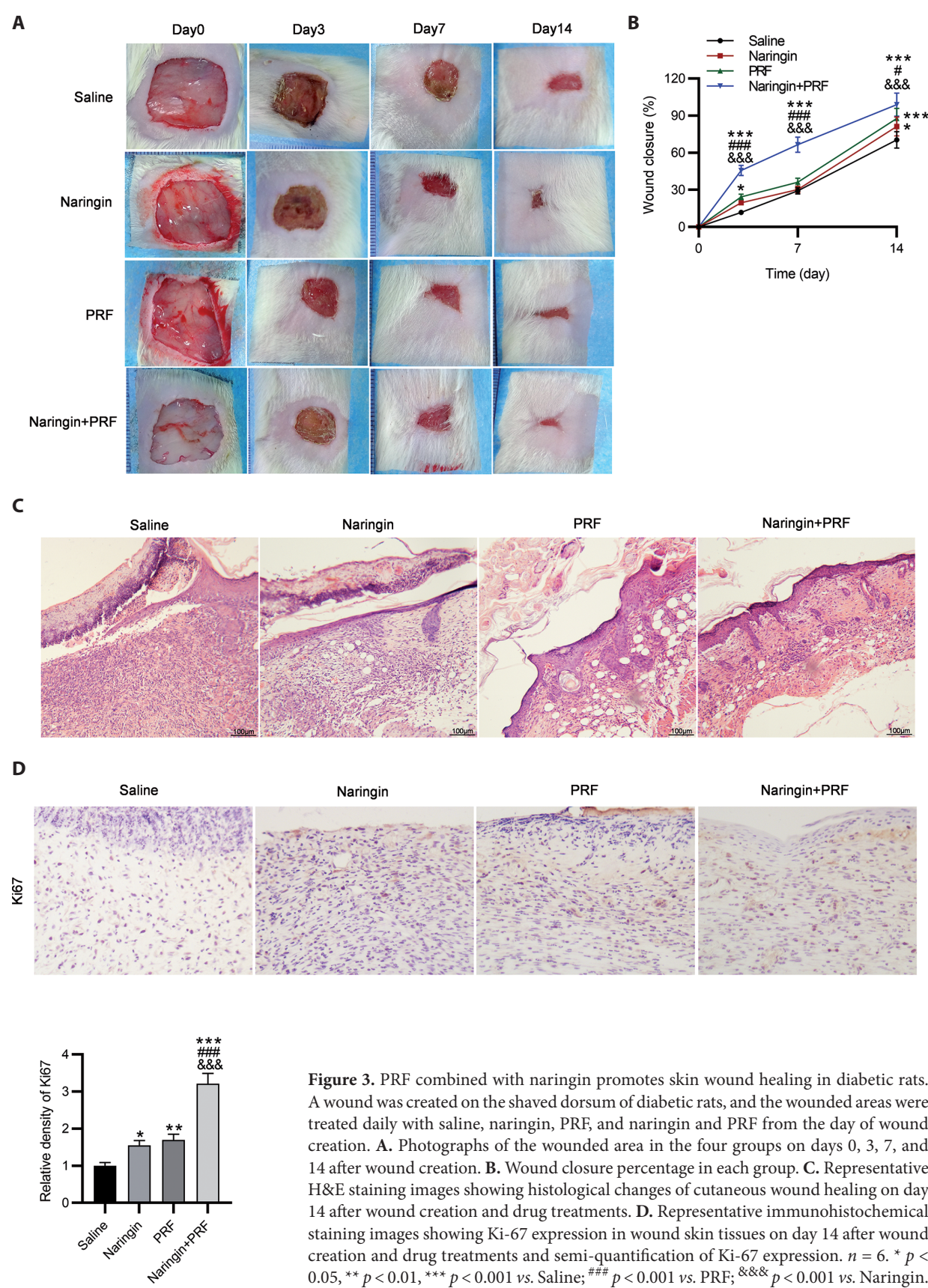


Figure 3. PRF combined with naringin promotes skin wound healing in diabetic rats. A wound was created on the shaved dorsum of diabetic rats, and the wounded areas were treated daily with saline, naringin, PRF, and naringin and PRF from the day of wound creation. **A.** Photographs of the wounded area in the four groups on days 0, 3, 7, and 14 after wound creation. **B.** Wound closure percentage in each group. **C.** Representative H&E staining images showing histological changes of cutaneous wound healing on day 14 after wound creation and drug treatments. **D.** Representative immunohistochemical staining images showing Ki-67 expression in wound skin tissues on day 14 after wound creation and drug treatments and semi-quantification of Ki-67 expression. $n = 6$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Saline; ### $p < 0.001$ vs. PRF; &&& $p < 0.001$ vs. Naringin.

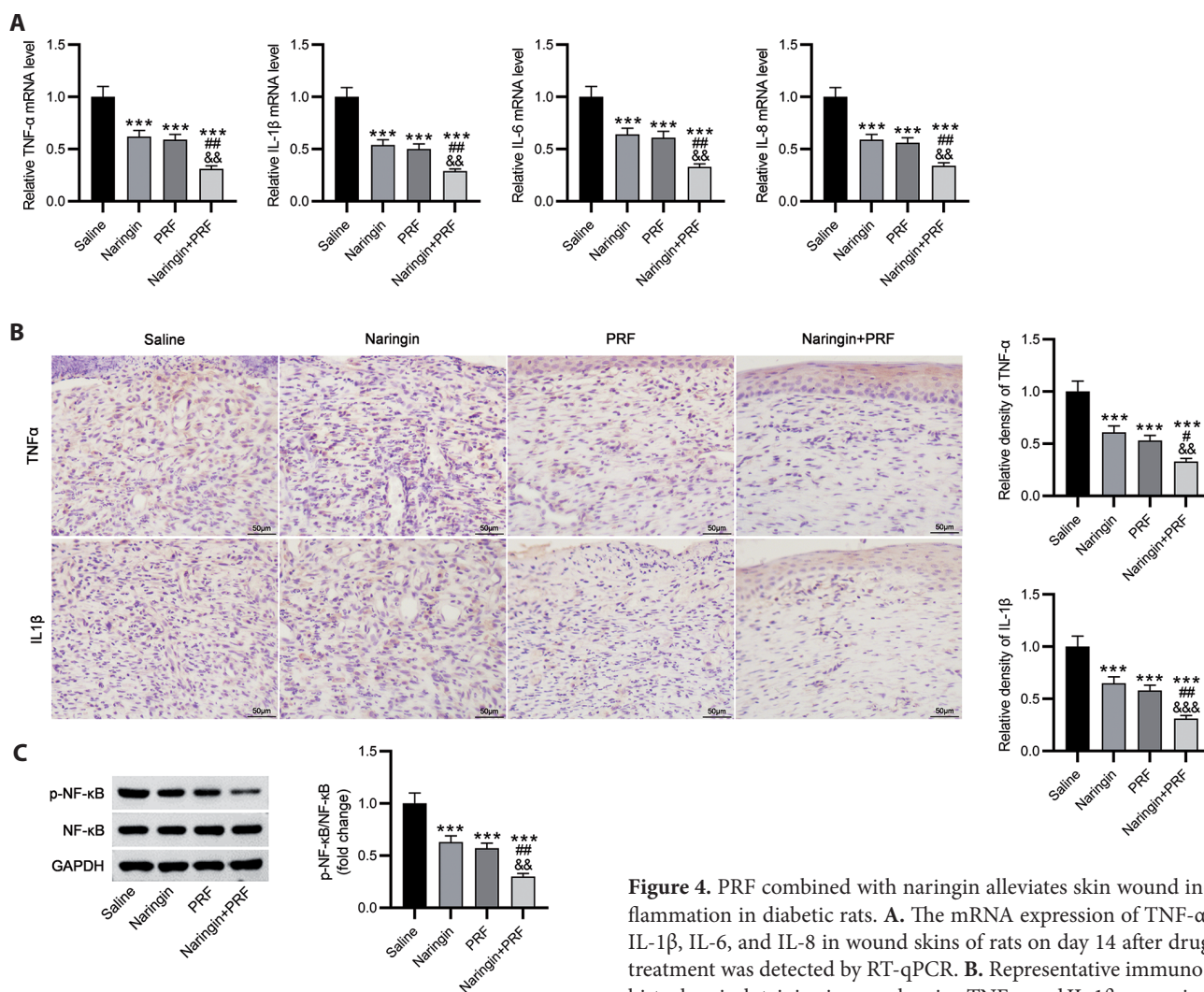


Figure 4. PRF combined with naringin alleviates skin wound inflammation in diabetic rats. **A.** The mRNA expression of TNF- α , IL-1 β , IL-6, and IL-8 in wound skins of rats on day 14 after drug treatment was detected by RT-qPCR. **B.** Representative immunohistochemical staining images showing TNF- α and IL-1 β expression in wound skin tissues of rats on day 14 after drug treatments and semi-quantification of TNF- α and IL-1 β expression. **C.** The protein levels of phosphorylated and total NF- κ B in wound skins of rats on day 14 after drug treatment were estimated through Western blotting. $n = 6$. *** $p < 0.001$ vs. Saline; # $p < 0.05$, ## $p < 0.01$ vs. PRF; && $p < 0.01$, &&& $p < 0.001$ vs. Naringin.

with drastically lower levels of pro-inflammatory cytokines in the combined treatment group than in the single treatment group ($p < 0.01$, Naringin+PRF vs. Naringin/PRF) (Fig. 4A). Consistently, immunohistochemical staining demonstrated that TNF- α and IL-1 β expression was pronouncedly reduced in wound tissues of rats administrated with naringin and/or PRF compared with the saline-administrated rats ($p < 0.001$, Naringin/PRF/Naringin+PRF vs. Saline). The reduction in TNF- α and IL-1 β expression was more apparent in the combined treatment group than in the single treatment group ($p < 0.01$, Naringin+PRF vs. PRF; $p < 0.001$, Naringin+PRF vs. Naringin) (Fig. 4B). In addition, the expression of NF- κ B, a transcription factor crucial for the secretion of pro-inflammatory cytokines and the activation of inflammatory responses, in wound tissues of rats was determined

by Western blotting. We observed that either single or combined treatment with naringin and PRF substantially downregulated the phosphorylated levels of NF- κ B in rat wound skins ($p < 0.001$, Naringin/PRF/Naringin+PRF vs. Saline), with combined treatment showing better suppressive effects on NF- κ B phosphorylation ($p < 0.01$, Naringin+PRF vs. Naringin/PRF) (Fig. 4C).

Discussion

DM is a common metabolic disease with several organ damages, whose prevalence is continuing to rise (Khan et al. 2020). Impaired wound healing is a major complication of DM, which affects approximately 15% of diabetic individuals

and can result in limb amputation and disability in severe cases (Baltzis et al. 2014). Despite great advances in therapeutic strategies, the clinical management of diabetic wounds has always faced a series of challenges. In recent years, PRF therapy, as a novel regenerative treatment technique, has been gradually applied in the fields of wound repair, orthopaedics, plastic surgery, and dentistry (Karimi and Rockwell 2019). Besides, natural products have emerged as an essential resource of pharmaceutical research and development for the treatment of non-healing or delayed healing of diabetic wounds (Oprita et al. 2023). In the present study, we assessed the functions of PRF combined with naringin in diabetic wound healing. We discovered that combined treatment with PRF and naringin better promoted keratinocyte proliferation and migration and reduced inflammatory cytokine expression *in vitro* as well as accelerated wound closure and mitigated inflammation in diabetic rat models than single treatment with either PRF or naringin.

Researches to date have elucidated that various factors including decreased immune function, lack of nutrition, chronic inflammation, poor circulation, peripheral vascular damage, and impaired neuropeptide signalling are related to wound healing defects in patients with diabetes (Kamal et al. 2024). Recently, abnormal keratinocyte function has been reported as a crucial underlying factor contributing to the poor healing capacity of diabetic wounds (Gao et al. 2020). As the predominant cells in the epidermis, keratinocytes play an indispensable part in wound repair and regeneration (Pastar et al. 2014). Keratinocytes migrate to the wound site, proliferate, and differentiate to re-form the epidermal barrier, thereby achieving re-epithelialization, which is a key event during the proliferation phase of the wound healing process (Hosseini Mansoub 2021). Studies have demonstrated that the proliferation status of keratinocytes is disturbed under *in vitro* HG conditions and in diabetic animal models (Hu and Lan 2016, Sakai et al. 2003). During the wound healing process in diabetic patients, hyperglycemic conditions remarkably suppress the proliferation and migration of keratinocytes (Lan et al. 2008). In addition to growth factors, cytokines, and chemokines, keratinocytes also regulate biological wound healing by releasing matrix metalloproteinase (MMPs), a class of endopeptidase enzymes (Rousselle et al. 2019). MMPs are mainly involved in degrading various extracellular matrix (ECM) proteins, including laminins, fibronectins, and collagens, thereby playing a vital role in the normal wound healing process, including removal of necrotic tissue and modulation of the inflammatory response (Dari et al. 2023). Moreover, the occurrence of several migratory and remodelling events requires the function of MMPs during the wound healing process (Martins et al. 2013). In the diabetic state, the activity of MMPs is enhanced, disrupting ECM components and leading to impaired wound healing (Zhang et al. 2023). In our study, keratinocyte proliferation

and migration were attenuated while MMP2 and MMP9 levels were elevated after HG stimulation, which was consistent with the previous studies (Gao et al. 2022, Lang et al. 2021). Previously, naringin was confirmed to promote wound healing *in vitro* by increasing HaCaT keratinocyte migration (Yen et al. 2022). Herein, we discovered that either single or combined treatment with PRF and naringin prominently facilitated proliferation and migration and reduced MMP2 and MMP9 protein levels in HG-exposed HaCaT keratinocyte. Importantly, the combined treatment exhibited better promoting effects on the above re-epithelialization process in wound healing than the single treatment.

Chronic and persistent inflammation is a central pathogenic characteristic of delayed wound healing in diabetes (Yang et al. 2021). Recent studies have shown that hyperglycemia is related to multiple immune disorders including impairment in leukocyte adhesion, movement, chemotaxis and phagocytosis (Shofler et al. 2021). Besides, increased infiltration of neutrophils and macrophages at the diabetic wound site leads to excessive ROS production, which interferes with the oxidant/antioxidant balance, not only activating the signalling pathways regulating the secretion of pro-inflammatory cytokines (IL-1 β , IL-6, IL-8, and TNF- α), and MMPs, but also contributing to fibroblast senescence (Jiang et al. 2023). Senescent fibroblasts produce high levels of proteases (MMP-2, MMP-3, and MMP-9) as well as fewer protease inhibitors, which results in the hydrolysis of excess ECM proteins and curbs the wound healing process (Serban et al. 2016). Elevated pro-inflammatory cytokine levels and reduced anti-inflammatory cytokine levels have been observed in the wound tissues of diabetic animal models (Chen et al. 2022, Zhao et al. 2022). Till now, multiple natural flavonoids with anti-inflammatory activities such as luteolin, apigenin, kaempferol, curcumin, hesperidin, and quercetin have been demonstrated to exert beneficial effects in promote diabetic wound healing (Chanu et al. 2023). Interestingly, naringin was reported to hinder the development of diabetes and its complications by mitigating inflammatory response. For example, Liu et al. (2017) clarified that naringin treatment downregulated pro-inflammatory cytokine TNF- α , IL-1 β , and IL-6 levels as well as inhibited NF- κ B p65 expression and nuclear translocation in diabetic rat retina and HG-induced rat Muller cells and diabetic rat retina, showing its anti-inflammatory effects in diabetic retinopathy. Kandhare et al. (2014) found that rats with diabetic foot ulcer exhibited reduced wound area, histopathological injury, and inflammatory mediator (TNF- α , IL-1 β and IL-6) levels after naringin administration. In addition, injectable PRF has been widely used for diabetic wound repair and regeneration, in part because of its ability to inhibit inflammation (Bai et al. 2023). Sericin/injectable-PRF hydrogel, a novel hydrogel, can effectively reduce inflammation levels, promote angiogenesis, and repair diabetic wounds of nude

mice (Bai et al. 2023). One previous open-label randomized controlled trial revealed that autologous PRF worked synergistically with hyaluronic acid to accelerate the diabetic foot ulcer healing process in patients by triggering angiogenesis and decreasing inflammation (Kartika et al. 2021). Herein, our results showed that PRF and naringin combination better attenuated TNF- α , IL-1 β , IL-6, and IL-8 levels in HG-stimulated HaCaT cells and wound tissues of diabetic rats than PRF or naringin treatment alone. The phosphorylation level of NF- κ B, a critical transcription factor that modulates a wide range of genes involved in immune and inflammatory response, in the skin wounds of diabetic rats was significantly downregulated after combined treatment with PRF and naringin.

Despite the encouraging results of this research, there are still some limitations to consider. First, our work focused on comparing the function of PRF and naringin single or combined treatment in inflammatory response and keratinocyte proliferation and migration, while their impacts on other pathways involved in wound healing, such as angiogenesis, apoptosis, autophagy oxidative stress, have not been investigated. Second, *in vitro* and *in vivo* models of diabetic wound healing used in our experiments can not necessarily reflect the disease complexity in humans. Further clinical trials are needed to validate the therapeutic potential of PRF and naringin combined treatment in diabetic wound healing. Third, the underlying molecular mechanisms through which PRF combined with naringin promotes re-epithelialization and inhibits inflammation in diabetic wound healing remain unknown.

In summary, our study proved that the topical application of PRF and naringin combination exerted a synergetic action in promoting excisional wound healing in diabetic rats. This beneficial effect was attributed to the fact that the combination therapy restored keratinocyte proliferation and migration and alleviated inflammation. Therefore, our results offered experimental evidence of PRF and naringin combination in treating diabetic wounds, which might afford opportunities for the development of novel therapeutic interventions for impaired wound healing in diabetes.

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Conflict of interests. The authors declare that they have no competing interests.

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Ethical approval. Procedures involving animals and their care were approved by the Animal Care Committee of Wuhan Myhalic Biotechnology Co., Ltd (approval number: HLK-202404124; approval date: April 16, 2024) and performed in agreement with

ARRIVE guidelines. All volunteers had provided informed signed consents, and the experimental protocols were performed according to regulations and approval of the institutional ethical committee of the Wuhan Third Hospital.

Authors' contributions. HT conceived and designed the experiments. HT, HG, JZ, RL and XZ carried out the experiments. HT, HG, JZ, RL and XZ analyzed the data. HT, HG, JZ, RL and XZ drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

Data availability statement. The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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