Ginsenoside (Rg-1) promoted the wound closure of diabetic foot ulcer through iNOS elevation via miR-23a/IRF-1 axis

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**ABSTRACT**

**Background:** Impaired wound healing in diabetes foot ulcers (DFUs) brings a great burden to diabetic patients. Pro-angiogenesis through elevating nitric oxide (NO) is beneficial to the wound healing process. Ginsenoside Rg1, the main active in Notoginseng, is reported to regulate the angiogenesis in endothelial cells through modulating miR-23a. However, the effect of Rg1 in diabetes remains elusive.

**Methods:** High fat diet combined with streptozotocin-induced diabetic rats were treated with Rg1. Then incision area and tissue NO level were measured to evaluate the wound closure efficacy of Rg1. Then high glucose cultured HUVECs were employed to mimic diabetic environment in vitro. Overexpression and knockdown plasmids of miR-23a or IRF-1 were constructed and transfected in HUVECs. qPCR and western blot were used to determine the mRNA and protein level, respectively. Dual-luciferase reporter assay was utilized to determine the interaction of IRF-1/miR-23a.

**Results:** Rg1 accelerated the wound closure speed in diabetic rats and increased NO level through elevating iNOS expression. Knockdown of iNOS reversed Rg1-induced VEGF expression, cell proliferation, anti-apoptotic efficacy and cell migration ability in high glucose cultured HUVECs. Further investigation revealed that Rg1 mediated iNOS through miR-23a. miR-23a inhibited the expression of IRF-1, a protein which could directly bind to the iNOS mRNA 3'UTR.

**Conclusion:** Rg1 promoted angiogenesis in diabetic wound healing process through NO signaling via miR-23a, providing a novel candidate for DFUs treatment.

1. Introduction

Diabetic foot ulcers (DFUs) are a major cause of amputation in diabetic patients, affecting over 170 million people worldwide, and it could increase the 5-year mortality risk to 80% [1]. Impaired wound healing process results from chronic inflammation, dysfunctional micro- and macro-circulatory et al., is the common trigger of DFUs. Current principles for DFUs treatment are wound debridement, pressure off-loading, revascularization and infection management [2]. Especially, it is reported that in non-healing diabetic wound site, angiogenesis is impaired and number of endothelial progenitor cells as well as the cells capable of de novo formation of new blood vessels, are reduced dramatically [3]. On the other hand, therapies that pro-angiogenic is shown to improve wound closure in diabetic rat model [4]. Thus, promoting the angiogenesis process is beneficial to the treatment of DFUs.

Angiogenesis is a dynamic and well-regulated process by growth factors, like vascular endothelial growth factor (VEGF), as well as signaling molecules including nitric oxide (NO) [2]. NO produced by NO synthases, is a short-lived free radical that essential to angiogenesis. There are three isoforms of NO synthases in mammalian, two constitutive (neuronal and endothelial, also known as nNOS and eNOS) isoforms and one inducible isoform (known as iNOS). The expression of iNOS is induced by different signals, such as growth factor and inflammation. iNOS is usually expressed in inflammatory situations including sepsis and wound healing [5,6]. Inhibiting iNOS by competitive inhibitors reduced the deposition of collagen and breaking strength of incisional wounds, impairing the healing process [7,8]. Taken together, elevating NO level with compounds that increased iNOS level promoted the wound closure process. However, the aberrant production of NO in

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2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats, weighed 250-300 g, were purchased and housed at the animal research facility of Hunan Normal University. Rats were acclimatized to the laboratory conditions and provided free access to ad libitum food and water. The rats were acclimated for 1 week before use and maintained throughout the study in a controlled environment: 24 ± 2 °C, 50 ± 10% relative humidity, and a 12-h light/dark cycle. All experiments were conducted in accordance with the protocols approved by the Animal Care and Use Committee of the Hunan Normal University.

2.2. Cell culture

Human umbilical vein endothelial cell (HUVECs) were purchased from Shanghai Institute of Biochemistry and cultured in RPMI 1640 medium (HyClone, China) supplemented with 10% fetal bovine serum (HyClone, China) in a 5% CO₂ humidified incubator. Cells were incubated up to about 24 h and grown to about 80% confluence before experiments.

2.3. Diabetes induction

Diabetes was induced by high fat diet (HFD) together with intraperitoneal administration of streptozotocin (STZ, 50 mg/kg) in citrate buffer (pH 4.4, 0.1 M) for 5 days. For control group, rats were fed with normal diet and administrated with an equal volume of citrate buffer. Rats demonstrated a serum glucose level higher than 250 mg/dL after 5 min and washed, followed by differentiation with 1% hydrochloric acid alcohol. Then slides were staining with 0.55 eosin for 30 s, dehydrated with gradient alcohol, soaked in xylene 3 times. Finally, mounted slides with neutral gum. Slides were observed and photographed using an optical microscope.

2.4. Diabetic foot ulcer model

Excision diabetic foot ulcer was created according to the previously established method [14]. Briefly, rats anesthetized with ketamine (70 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.). The rectangular wound (2 mm × 5 mm) was created on the dorsal surface of the foot of each rat.

2.5. Hematoxylin and eosin (H&E) staining

Skin tissue was stored in 10% formalin for 24 h. The dried slices soaked in xylene, dewaxing for 10 min, rehydrated, hematoxylin rinse for 5 min and washed, followed by differentiation with 1% hydrochloric acid alcohol. Then slides were staining with 0.55 eosin for 30 s, dehydrated with gradient alcohol, soaked in xylene 3 times. Finally, mounted slides with neutral gum. Slides were observed and photographed using an optical microscope.

2.6. MTT assay

MTT assay was applied to assess the viability of MCF-7 cells. Briefly, cells were seeded in 96-well plates at a final concentration of 2 × 10⁴ cells/well. At the end of treatment, the medium was decanted and 20 μL of MTT dye solution (5 mg/ml in PBS buffer) was added per well and incubate at 37 °C for 4 h. Then supernatant was removed and replaced with 150μl DMSO. After 15 min incubation with gentle shaking at 37 °C, the absorbance of the formazan solution from the wells was measured at a wavelength of 490 nm using microtiter plate reader (Tecan, Switzerland).

2.7. Flow cytometry for cell apoptosis analysis

Flow cytometry was employed to detect the cell apoptosis. After treatment, cells were collected with trypsin digestion solution, washed twice with phosphate-buffered saline (PBS) and resuspended in 200 μL PBS. For apoptosis analysis, Annexin V/Dead Cell Apoptosis Kit (Invitrogen, CA, USA) was utilized according to the manufacturer’s instructions. Briefly, 5 μL Annexin V-FITC and 5 μL PI was added to each well and cells were incubated in the dark for 15 min. Then the cell apoptosis was measured using the FACScan flow cytometer (Becton, CA) equipped with CellQuest Software (Becton Dickinson).

2.8. In vitro wound healing assay

Scratch assay was utilized to evaluate cell migration and wound closure of HUVECs cells. Briefly, cells were seeded in 12-well plates (2 × 10⁵ cells/well) and 1% hydroxypropyl methylcellulose (HPMC) was added per well and cells were incubated in the dark for 24 h. After that, the growth of cells was measured by the T scratch analysis software [15].

2.9. RNA extraction and qPCR

Total RNA was extracted from HUVECs or rat tissues using Trizol Reagent (Invitrogen, Paisley, UK) and cDNA was synthesized by reverse transcription using PrimeScript1st Strand cDNA Synthesis Kit (Takara, Japan). The gene expression was measured by qPCR with Applied Biosystems 7500 Fast RealTime qPCR machine. The qPCR cycling conditions used were as protocol. Exponential amplification had been confirmed up to 40 cycles of the amplification. The relative gene expression levels were calculated using the 2-ΔΔCt method. All primers were purchased from Invitrogen (Paisley, UK). qPCR primers used for qPCR were synthesized by Invitrogen and sequences were shown in as follows:

Human iNOS forward primers: GCAGAATGTGACCATCAGGG, human iNOS reverse primers: ACAACCTTTGGTGTTGAAGGC;
Human VEGF forward primers: GCCTGGTTGCTGTCTACCTCAG, human VEGF reverse primers: CAAGGCACACAGGGATTIT;
mir-23a primers: GTCTATCCAGTGAGGTTTGAGGTTT;
Human IRF-1 forward primers: ACCTGGCCTAGAGATGCAGA, human IRF-1 reverse primers: GCTTTGTATCGGCCTGTGTG;
Human actin forward primers: GATGGCCACGGCTGCTTC, human actin reverse primers: TGCCTCAGGGCAGCGGAA; Rat iNOS forward primers: GACCAGAAACTGTCTCACCTG, rat iNOS reverse primers: CGAACATCGAACGTCTCACA; Rat VEGF forward primers: GGCTCTGAAACCATGAACTTTCT, rat VEGF reverse primers: GCAGTAGCTGCGCTGGTAGAC; Rat IRF-1 forward primers: GTACAACTTGCAGGTGTCGC, rat IRF-1 reverse primers: GCTGCCACTCAGACTGTTCA; Rat actin forward primers: TGCCTGACGGTCAGGTCA, rat actin reverse primers: CAGGAAGGAAGGCTGGAAG;

2.10. Western blot

Total protein was extracted and separated on 8–10% SDS-PAGE. After electrophoresis, proteins were transferred onto PVDF membranes (EMD Millipore, Billerica, USA), and blocked by 5% BSA for 1 h with shaking. Then, the membranes were incubated overnight at 4 °C with the primary antibodies. Primary antibodies against IRF-1, iNOS and VEGF were purchased from Abcam. β-actin antibodies were purchased from Sigma-Aldrich. The membranes were washed with TBST buffer and incubated with HRP–conjugated secondary antibody for 1 h. Protein bands were then visualized using the chemiluminescence detection system (Biorad). The intensity of the bands was quantified using ImageJ software tools.

2.11. Dual-luciferase reporter assay

The dual-luciferase reporter assay was utilized to determine the interaction of IRF-1/miR-23a and was conducted according to the method described by Zhang et al. [16]. Cells in 24-well plates were co-transfected with pMIR-IRF1 and miR-23a mimic, or miR-23a inhibitor, or corresponding negative controls (NC) using Lipofectamine 2000. Forty-eight hours later, cells were collected and the ratio of firefly luciferase to Renilla luciferase was detected with a Promega Glomax 2020 Single Tube Luminometer instrument (Promega) for each well, where Renilla luciferase intensity worked as internal control. Triplicates for each experiment.

2.12. Nitric oxide level assay

HUVECs were seeded in 6 well plates and the level of nitric oxide was measured level in each sample was quantified using commercially available colorimetric nitric oxide assay kit (Abcam, UK) following the manufacturer’s instructions. In brief, $2 \times 10^5$ cells per sample were harvested, washed with PBS, re-suspended in ice cold assay buffer (provided in the kit), homogenized, and then centrifuged for 2–5 min at $4 \degree C$ at top speed to remove any insoluble material. The supernatant was collected. The nitrite in the samples was converted to nitrite by using nitrate reductase and its cofactor. After an incubation time the enhancer and the Griess reagents were added and the OD of the samples was measured at 540 nm. Total nitrate (NOx) was calculated using the standard curve.

2.13. Statistics

Data were presented as mean ± SEM. For comparisons between two groups, Student’s t-test was employed. The statistical analysis between multi-groups was carried out using one-way analysis of variance (ANOVA) by Dunnett’s post-test. A two-side value of $p < 0.05$ was considered statistically significant. All statistical analyses were performed by Graphpad Prim 5 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Rg-1 facilitated the wound closure of DFUs in rats

Excision diabetic foot ulcer model was established to evaluate the effect of Rg-1 in wound closure in DFUs. After excision, diabetes rats were divided into two groups. For Rg1 group, rats received intraperitoneal administration of Rg-1 at the dose of 150 mg/kg for 14 days. Meanwhile, control group rats were administered with an equal volume of saline. The representative images for each group at Day
Fig. 2. Rg1 promoted wound closure through iNOS in HUVECs. (A). Cell proliferation of HUVECs treated with Rg1 at different doses. (B). qPCR measured mRNA level of iNOS and VEGF of HUVECs transfected with overexpression or knockdown plasmids of iNOS, followed by Rg1 treatment. (C). Cell proliferation were measured with MTT. (D). Cell apoptosis was detected with flow cytometry. (E). Angiogenesis level was visualized with microscopy. (F). Wound closure was measured with scratch assay.
0, 3, 7, 10, 14\) were presented in Fig. 1A. Animals received Rg1 treatment demonstrated a faster recovery from foot ulcers as the excision area was smaller in Rg1 group compared to the control at each time point. Fig. 1B showed the wound healing rate at corresponding time points. Rg1 group demonstrated significantly faster wound healing rate than the control group. At day 14, the size of ulcer is dramatically smaller in rats received Rg1 treatment (\(p < 0.05\)). Meanwhile, at each time point, 0.1 g of peripheral wound tissues were obtained from rats and homogenized for the detection of nitric oxide level. As shown in Fig. 1C that Rg1 treatment increased the production of NO\textsubscript{x} compared to the control group, indicating that NO production might contribute to the rapid wound closure efficacy delivered by Rg1. Taken together, Rg1 promoted the wound closure in DFUs, and NO\textsubscript{x} might contribute to the process.

3.2. Rg-1 promoted DFUs wound closure through iNOS regulation in vitro

Next, we employed high glucose cultured HUVECs as the in-vitro environment to investigate the mechanism by which Rg-1 regulated NO\textsubscript{x} under DFUs condition. Firstly, HUVECs were treated with Rg1 at different doses. MTT assay revealed that the cell number increased by Rg-1 in a dose-dependent manner (Fig. 2A). The iNOS were reported to regulate the production of nitric oxides in previous studies. And VEGF is a vital endogenous growth factor that well-known for wound healing regulation. Thus, both mRNAs were examined with qPCR. As shown in Fig. 2B, post the Rg1 treatment, mRNA level of iNOS and VEGF were increased dramatically. Based on qPCR and the previous in vivo data (Fig. 1C), we hypothesized that Rg1 promoted the wound closure of DFUs via activating iNOS and the expression of VEGF.

To validate the hypothesis, we constructed the knockdown and overexpression plasmids for iNOS. The efficacy of plasmids was validated with qPCR, as shown in Fig. 2B that pcDNA-iNOS activated the iNOS mRNA compared to NC group. And Rg1 treatment could also increase the mRNA level of iNOS. In contrast, the transfection of sh-iNOS significantly repressed Rg-1-induced iNOS in HUVECs. Meanwhile, the VEGF mRNA was positively regulated by iNOS, together with the cell proliferation (Fig. 2C). Flow cytometry revealed that Rg-1 could reduce the cell apoptosis as shown by the ratio of annexin V and propidium iodide double positive cells dropped (Fig. 2D). Moreover, more angiogenesis and faster metastasis were observed in the Rg-1 treatment group (Fig. 2E, F). The overexpression of iNOS delivered comparable anti-apoptosis, angiogenesis and pro-metastasis efficacy in HUVECs as Rg-1 treatment. However, the knockdown of iNOS abrogated the efficacy mentioned above. Taken together, we proved that Rg-1 mediated wound closure of DFUs in vitro through elevating iNOS.

3.3. Rg1 mediated miR-23a increased the expression of iNOS

Previous studies reported that miR-23a regulated the wound healing by accumulating double-strand breaks in inflammatory bowel disease [17,18]. Thus, we used miR-23a inhibitor and miR-23a mimic to investigate the role of miR-23a in wound closure of DFUs. As shown in Fig. 3A, miR-23a level was significantly higher in diabetic environment and decreased by miR-23a inhibitor in control group, Rg-1 treatment suppressed miR-23a as well. Then mRNA and protein level of iNOS and VEGF were examined with qPCR and western blot, respectively (Fig. 3B, C). qPCR revealed that miR-23a inhibitor enhanced the expression of iNOS and VEGF as Rg-1 did, but miR-23a mimics could reverse Rg1 efficacy on angiogenesis-related protein expression. (Fig. 3B). Western blot data were consistent with that of qPCR (Fig. 3C). Moreover, Rg-1-induced nitric oxide level was reversed by miR-23a mimic (Fig. 3D). These results indicated that Rg-1 induced the expression of NO\textsubscript{x} and VEGF through elevating miR-23a level.
3.4. IRF-1 was a direct target of miR-23a in HUVECs

mRNAs were known to regulate the gene expression by to specific sequences in the 3’UTR of target genes. The putative miR-23a targets were predicted with TargetScan and IRF-1 (NM_002198) (Fig. 4A). Then we confirmed it using the dual-luciferase reporter assay as previously described. miR-23a mimic significantly suppressed the luciferase activities as the fluorescence decreased by binding to IRF-1 WT. In contrast, miR-23a mimic didn’t affect luciferase activities while co-transfected with IRF-1 MUT, which confirmed the correction of
miR-23a is essential to Rg1-mediated wound closure in HUVECs. (A). HUVECs were transfected with knockdown plasmid of miR-23a, IRF-1 or iNOS, followed by the treatment of Rg1. Expression level of IRF-1, iNOS and VEGF in HUVECs was determined by western blot. (C). NO level was determined with nitric oxide assay. (D). Cell proliferation was determined by MTT assay. (E). Wound closure was measured by scratch assay. (F). Angiogenesis in HUVECs was visualized with microscopy.
A. Relative expression level of mir-23a and NOS.

B. Western blot analysis of IRF-1, NO, VEGF, and β-actin.

C. Normalized change of NOx.

D. Representative images showing the effect of Rg1 on IRF-1 and NOS.

E. Wound healing rate over time.

F. Graph showing the wound healing rate over time for different treatments.
predicted bind sites. qPCR demonstrated that suppressing miR-23a could elevate the mRNA level of IRF-1. Dual knockdown of miR-23a and IRF-1 or co-overexpression of miR-23a and IRF-1 reversed the effects of miR-23a on IRF-1, iNOS or VEGF. Western blot results were consistent with that of qPCR. Taken together, miR-23a functioned through inhibiting IRF-1 in mediating the expression of IRF-1, iNOS and VEGF (Fig. 4C and D).

3.5. Rg-1 promoted wound closure of DFUs through miR-23a/IRF-1

Then, we validated that whether Rg-1 functioned through miR-23a/IRF-1. Rg-1 treated HUVECs transfected with miR-23a mimic, or sh-IRF-1 or sh-iNOS were harvested and subjected to qPCR and western blot. Both data demonstrated that miR-23 mimic reduced the expression of IRF-1, iNOS and VEGF (Fig. 5A, B). Knockdown of IRF-1 decreased the expression of iNOS and VEGF, without affecting the expression of miR-23a, supporting that IRF-1 is the downstream of miR-23a.

Nitric oxide detection data revealed that elevating miR-23a or knockdown of IRF-1 and iNOS could efficiently reversed Rg-1-induced NO production and cell proliferation (Fig. 5C, D). Moreover, scratch assay and angiogenesis assay revealed that miR-23a overexpression and blocking the downstream IRF-1/iNOS abrogated Rg-1 mediated cell migration and angiogenesis in HUVECs (Fig. 5E, F). Taken together, Rg-1 promoted cell proliferation, migration and angiogenesis through miR-23a/IRF-1/iNOS axis, resulting in a promoted wound healing of DFUs.

3.6. miR-23a/IRF-1/iNOS axis is essential for Rg1-mediated wound healing in DFUs in vivo

To validate that Rg1 functioned through miR-23a/IRF-1 pathway in vivo, we investigated with diabetes rats model. Rats were divided into four groups and received 14-day intravenous injection of drugs. At the end of treatment, excision tissues were collected and subjected to measurement. qPCR and western blot data revealed that miR-23a mimics, sh-IRF-1 and sh-iNOS significantly reduced the transcription and expression of iNOS, IRF-1, iNOS and VEGF compared to the Rg1 group (Fig. 6A and B). The production of NOX in Rg1 treated was also suppressed by miR-23a mimic, sh-IRF-1 and sh-iNOS (Fig. 6C). Meanwhile, the results of H&E stained wound tissues reviewed that the number of blood vessel formation was also dramatically reduced in Rg1 added miR-23a, sh-IRF-1 or sh-iNOS groups (Fig. 6D and E). We measured the excision areas and found that overexpression of miR-23a, or knockdown of IRF-1 and iNOS severely impaired Rg1-mediated wound closure speed (Fig. 6F). Taken together, Rg1-mediated wound healing through miR-23a/IRF-1/iNOS axis in rats with DFUs demonstrate that Rg1 has protective efficacy in diabetes. In streptozotocin-induced diabetes rat model, the treatment of Rg1 treatment could efficiently attenuate diabetic cardiomyopathy by suppressing ER stress-induced apoptosis [9]. Here we reported that Rg1 could promote angiogenesis through regulating iNOS. Interestingly, previous studies demonstrated that eNOS, another isoform NOS could regulate angiogenesis as well [19-21]. The balance between eNOS and iNOS is intensively investigated in vivo, showing that drugs acting to restore the original balance between the two isoforms, rather than decreasing or increasing NOS, may have potential benefits for disease [22]. However, the ratio of eNOS and iNOS before/after Rg1 treatment remains further investigations.

Many micro-RNAs were also reported to contribute to Rg1 regulated angiogenesis in the past decades. For example, Chu et al. demonstrated that Rg1 activated Nrf2/ARE pathway through inhibiting miR-144, protecting neurons from ischemia/reperfusion-induced injury [23]. Yu and colleagues reported that Rg1 ameliorated chronic stress-induced depression-like behavior via tolerulating the synapse-associated factor miR-134 within the basolateral amygdala in rats [24]. These studies implied that Rg1 delivered protective effects by interacting with various micro-RNAs under different conditions. In the present study, we validated that miR-23a was a target of Rg1 as well in diabetic HUVECs. It suggested the regulation of miR-23a by Rg1 might be conserved in different cell types, thus it could expand the application of Rg1 for angiogenesis-promoting therapy.

IRF-1 was firstly discovered as an interferon regulatory factor element (IRF-E) binding protein. Its regulation efficacy on angiogenesis is controversy. IRF-1 was reported to bind directly to the IRF-E of iNOS promoter in IFN-γ-treated RAW 264.7 macrophages. Thus, IRF-1 could promoted angiogenesis through increasing the expression of iNOS [25]. In the present study, we reported that Rg1 inhibited miR-23a, subsequently removed its suppressive effects on RIF-1. Elevated IRF-1 increased the expression of iNOS, facilitating the vessel formation and wound closure in DFUs. Our study expanded the understanding of the role of IRF-1 in diabetes and suggested that inhibiting IRF-1 could be a novel strategy in promoting wound healing.

Herein, we reported that in diabetic ulcer condition, Rg1 could reduce the miR-23a level, removing its inhibitory regulation on IRF-1. Subsequently, IRF-1 elevated iNOS level and promoted angiogenesis. Uncovering the mechanism of Rg1 would benefit to find new therapeutic method for DFUs. Drug molecular controlled microenvironment through mediated the microRNA and downstream protein expression could be a novel mechanism of DFUs treatment Furthermore, the clinical efficacy of Rg1 on diabetic patients remains elusive and we will further investigate in the future.

4. Discussion

DFUs affect 15% of diabetic patients and one vital factor impeding wound closure is poor vascular flow, an environment that hampers proper wound healing process. Numerous studies highlighted that adequate vascular sufficiency and angiogenesis are essential in tissue repair. However, in diabetic wound healing, they are hypofunctional. In the present study, we validated that Rg1, a Chinese medicine monomer promoted the wound closure in DFUs via the expression of miR-23a, a microRNA that regulated the iNOS through IRF-1. Our study, provided a novel candidate for the treatment of DFUs.

Ginsenoside-Rg1 is identified as the main compound contributes to various pharmaceutical actions of ginseng. Increasing evidences

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Authors’ contributions

Ruo-Yi Liao and Hua-An Cai conceived and designed the experiments; Funding acquisition; Liang Huang performed the experiments; Li-Jun Zheng, Kun Fu and Jing Wang contributed reagents/materials/analysis tools; Feng-Dan Hu wrote the paper. All authors read and approved the final manuscript.

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