Berberine accelerated wound healing by restoring TrxR1/JNK in diabetes

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Abstract
The high disability, mortality and morbidity of diabetic ulcers indicate the urgent need to explore effective strategies for diabetic wound repair. TrxR1 plays a vital role in regulating redox homeostasis in various pathologies. In this study, the effect of berberine (BBR) on diabetic wounds was investigated by utilizing streptozotocin (STZ)-induced diabetic rats and a high glucose-induced cell model, and the mechanism of BBR on TrxR1 was elucidated. BBR treatment remarkably accelerated wound healing and enhanced extracellular matrix synthesis and significantly inhibited high glucose-induced HaCaT cell damage. Further analysis indicated that BBR activated TrxR1, suppressed its downstream JNK signaling, thereby inhibiting oxidative stress and apoptosis, promoted cell proliferation, downregulated MMP9 and upregulated TGF-β1 and TIMP1, resulting in accelerated wound healing. Importantly, the enhancement of BBR on wound repair was further abolished by TrxR1 inhibitor. Moreover, in diabetic wounds induced by a combination of STZ injection and high fat diet, BBR significantly increased wound closure rate and TrxR1 expression, and this was reversed by TrxR1 inhibitor. These data indicated that topical BBR treatment accelerated diabetic wound healing by activating TrxR1. Targeting TrxR1 may be a novel, effective strategy for restoring redox homeostasis and promoting diabetic wound healing.

Keywords: Diabetic wound; Oxidative stress; berberine; TrxR1; apoptosis
Introduction

Diabetic skin ulcers are one of the major causes of disability, mortality and morbidity in people with diabetes, resulting in a substantial economic burden worldwide. (1, 2) In contrast to normal wound repair, diabetic wound restoration intervenes with persistent inflammation, oxidative damage and dysfunction extracellular matrix remodelling (ECM), resulting in delayed wound closure. A large amount of evidence has indicated that oxidative damage in diabetes causes diverse injuries associated with cellular molecules, resulting in lipid oxidation, dysfunction and structural breakage of proteins, and DNA damage. (3) In particular, excessive reactive oxygen species (ROS) lead to a highly harmful environment that hinders the synthesis of normal ECM, delaying the normal healing process. In addition, the overexpression of ROS can cause direct oxidation of collagen and can fragment its normal helical structures. (4) ROS also induce matrix metalloproteinase (MMP) overproduction, which leads to extensive degradation of ECM, as well as the growth factors that are required for ECM maintenance and wound healing. (5, 6) ROS generation is necessary for the expression of MMPs but not for tissue inhibitors of matrix metalloproteinase (TIMPs), resulting in a vicious cycle in which ROS and MMPs promote each other. Recent studies have suggested that targeting ROS generation or enhancing antioxidant capacity are effective therapies for treating diabetic wound closures. (7-9) For example, the increase in Nrf2 activity by its activators (sulforaphane and cinnamaldehyde) accelerated wound closure by decreasing oxidative damage, (9) and the elevation of manganese superoxide dismutase (MnSOD) activity increased wound closure by approximately 15% in diabetic mice with MnSOD transfer. (10)

Thioredoxin (Trx) systems, including Trx, NADPH and thioredoxin reductase (TrxR), are among the most prominent antioxidant systems in mammalian animals and play a vital role in contributing electrons to methionine sulfoxide reductases and peroxiredoxins for the maintenance of multiple biological processes, such as DNA synthesis, antioxidant defence and apoptosis regulation. (11-13) TrxR catalyses oxidized Trx to its reduced form using electrons from NADPH, and its reductase activity largely relies on its selenocysteine (Sec) residue. (14) As the most prominent and ubiquitous isoform, thioredoxin reductase 1 (TrxR1, also named TXNRD1) is located in the cytoplasm and plays a vital role in modulating intracellular redox homeostasis, cell viability and proliferation. (11) Deletion of TrxR1 in embryos leads to early embryonic death with an obvious defect in cell proliferation. (15) In addition, DNA synthesis requires the reduction effect of TrxR1, (12) and its inhibition causes a reduction in cell proliferation and results in obvious oxidation and apoptosis. (16, 17) Furthermore, TrxR1 acts as a potent regulator of the Nrf2-keap1 system, which is a strong regulator in charge of multiple molecules that affect redox homoeostasis and cell survival. (18) There is growing evidence that the Trx/TrxR system has a beneficial effect against pancreatic β-cell injury, diabetic osteopenia and diabetic embryopathy. (19-21) Trx overexpression in β-cells not only inhibited autoimmune and streptozotocin(STZ) -induced diabetes (22) but also prevented hyperinsulinism, decreased oxidative damage and reduced β-cell dysfunction in db/db mice. (23) Recombinant human Trx-1 administration remarkably delayed type 1 diabetes by reducing β-cell damage. (24) Importantly, recent research indicated that diabetic complications caused by hyperglycaemia-induced oxidative damaged can be attenuated by enhancing TrxR1 via decreasing glucose-induced H₂O₂ generation. Furthermore, TrxR1 knockout can trigger massive death of mouse embryonic fibroblasts (MEFs), indicating a potentially essential role of TrxR1 in diabetic complications. (25) Therefore, we hypothesized that TrxR1 might play a vital role in diabetic wound healing.

Berberine (BBR) is one of the most active alkaloids extracted from Coptis Salisb. (rhizome) and has been shown to have multiple valuable effects in modulating inflammation, oxidative stress, glucose and lipid
homeostasis, and cancer growth. (26-29) Recent studies have indicated that BBR exhibits remarkable antihyperlipidemic and antihyperglycemic effects in type 2 diabetic patients, and the hypoglycemic effect was similar to metformin. (30, 31) Further investigation revealed that BBR can enhance hypoglycaemic activities and increase insulin sensitivity by upregulating insulin receptor expression in the treatment of type 2 diabetes but not type 1 diabetes. (32, 33) Growing evidence indicates that BBR suppresses ROS generation, promotes antioxidant capacity and improves diabetic complications such as nephropathy, neuropathy, endothelial and cardiovascular diseases, among others. (33, 34) For example, BBR increased the gene expression of glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase in the liver of diabetic rats. (26) BBR is widely used for the treatment of diabetes, but few studies have focused on diabetic wound healing. In fact, most studies on BBR for diabetes or its complications were investigated by oral administration. Notably, plasma levels of BBR are very low (35) and the absolute bioavailability is reported to be less than 1% (36, 37) as calculated by blood Cmax. (38) Whether in combination with other drugs or administered alone, BBR is absorbed poorly via oral administration. (35, 36, 39, 40) Moreover, vascular dysfunction in diabetes further restricts the absorption of BBR to local ulcers, resulting in a reduced therapeutic effect of oral BBR. Topical treatment can avoid these problems and allows BBR to concentrate at local diabetic wounds, which benefits wound repair. In this study, we hypothesize that topical BBR treatment can accelerate diabetic wound closures and that activation of TrxR1 by BBR restores redox homeostasis, thus attenuating dysfunction in the regenerative environment.

Materials and Methods

In vivo animal study

Male Sprague-Dawley rats weighing 170-200 g were housed and handled in compliance with the Institute of Basic Theories of Chinese Medicine, China Academy of Chinese Medical Sciences Animal Care and Use policies, and the project identification code was 2018112. They were raised in a 12-hour light/dark cycle environment and were fed adaptively for 3 days with free water at room temperature of 22-26 °C and humidity of 55%. The diabetic rats were prepared through intraperitoneal (i.p.) injection of streptozotocin (STZ, S0130), which was dissolved in sodium citrate buffer according to previous research. (41) In this study, two animal models were used, with one induced by STZ, while the other was induced by a combination of high fat diet and STZ. Recombinant human epidermal growth factor (rhEGF) obtained from Shenzhen Huashengyuan Gene Engineering Development Co., Ltd.), was used as a positive control. For the STZ-induced diabetic wounds, rats with fasting glucose levels consistently higher than 16.7 mmol were considered diabetic two weeks after 60 mg/kg STZ injection. The rats in the control group received the same sodium citrate buffer. After 2 weeks, the diabetic rats were anesthetized with sodium pentobarbital, and two wounds (diameter, 2 cm) were created on the back of each rat. Then, the diabetic rats were randomly assigned to groups that received different daily topical treatments as follows: physiological saline (STZ), BBR (STZ+BBR, 0.06 mg/mL), and rhEGF (STZ+rhEGF, 40 IU/cm²), and BBR and auranofin (STZ+BBR+Aur, 0.021mg/mL). For the high fat diet and STZ-induced diabetic wounds, after 4 weeks of high fat feeding (60%, m/m) induction, rats with random blood glucose levels between 16.7 and 33.3 mmol following a 35 mg/kg STZ injection were included. The diabetic rats received different daily topical treatments: physiological saline (hfSTZ), BBR (hfSTZ+BBR, 0.06 mg/mL), rhEGF (hfSTZ+rhEGF, 40 IU/cm²), BBR and TrxR1 inhibitor (Aurothioglucose) (hfSTZ+BBR+ATG, 0.052mg/mL), BBR and JNK activator (Allicin) (hfSTZ+BBR+ ALN, 0.062mg/mL). The rats in the control group received the same sodium citrate buffer after 4 weeks of high fat diet induction.
All wounds were fixed with a rubber ring with a diameter of 2 cm to avoid wound contraction of the loose skin. The rats in the control group received an equal volume of physiological saline through topical treatment, and all rats received drugs once per day. Photos of the wounds were taken on days 0, 6, 9, and 12 after wound generation, and the wound closures were calculated using ImageJ analysis and normalized to the original wound areas. On day 6 after wound generation, collagen growth at the front edge of the wounds was observed under a two-photon microscope (Olympus FV1000) after the rats were anesthetized with sodium pentobarbital. On day 12, the rats were anesthetized with sodium pentobarbital, and the skin at the wound site was collected for the following experiments.

**Measurement of malondialdehyde, SOD, T-AOC, ROS, GSH, TrxR activity and caspase-3 activity**

Detection kits for malondialdehyde (MDA, A003), SOD (A001), total antioxidant capacity (T-AOC, A015), GSH (A119), TrxR activity (A119) and caspase-3 activity (G015) were obtained from Nanjing Jiancheng Bioengineering Institute. ROS kits (S0033) were purchased from Beyotime Biotechnology. Measurements of MDA, SOD, T-AOC, GSH, ROS, TrxR activity and caspase-3 activity were performed on a Visible Spectrophotometer 721G (Shanghai Precision Scientific Instruments Co., Ltd.) according to the instruction manual. The levels were then normalized to the total protein in the skin tissue or HaCaT cells.

**Measurement of MMP9, TIMP1, TGF-β1 and TrxR1 by enzyme-linked immunosorbent assay (ELISA)**

ELISA kits for MMP9 (SEA553Ra), TIMP1 (SEA552Ra, SEA552Hu), TGF-β1 (SEA124Ra, SEA124Hu) and TrxR1 (SEA703Ra, SEA703Hu) were purchased from CLOUD-CLONE Corp. Harvested skin tissues or cell precipitates were used for the following measurements. Briefly, 100 μL of sample was added to 96-well plates with immobilized antibody for 1 h, and then biotinylated antibody was added for 1 h. Next, samples were incubated for 30 min with horseradish peroxidase-labelled avidin, followed by TMB substrate solution for 15 min. Finally, the reaction was stopped by stop solution, and the samples were measured at a wavelength of 450 nm using a microplate reader (Molecular Devices, USA).

**Western blotting**

The protein samples were quantified using a Beyotime Protein Assay Kit (P0013, Nanjing, China). SDS-PAGE (P0014, China) was used to separate molecules with different molecular weights. Next, the proteins were transferred onto PVDF membranes (Millipore, IPVH00010), followed by incubation with primary antibodies. Then, HRP-labelled secondary antibodies were added, and the samples were incubated with an enhanced chemiluminescence agent. In this experiment, β-actin was used as an internal control, and scanning densitometry was applied to obtain the protein signals. The primary antibodies used in these experiments were as follows: anti-thioredoxin/TRX antibody (ab109385), anti-β-actin antibody (ab8226), anti-JNK1+JNK2 (p-T183+Y185) antibody (ab131499) and anti-JNK1/2 antibody (sc-137019).

**Histology, immunohistochemistry (IHC) and immunofluorescence (IF) staining**

Harvested skin tissues were embedded in paraffin. Slides containing 6 μm sections were used for immunohistological staining after tissue dewaxing. Haematoxylin and eosin (HE) and Masson's trichrome were purchased from Nanjing Jiancheng Bioengineering Institute. Haematoxylin and eosin were used to stain the nuclei and the cytoplasm/extracellular matrix, respectively. Masson's trichrome staining was used to show collagen tissue. The Masson's trichrome and HE staining was performed according to the manufacturer's
instructions. The wound edges of the diabetic wounds were represented by the panniculus carnosus muscle gap. Photos were captured by an Olympus BX6iVS (VS120-S6-W), and the wound edges were calculated in ImageJ by normalization to the control group.

For immunohistological (IHC) staining, endogenous peroxidase in the skin sections was blocked by hydrogen peroxide followed by a trypsin solution. After the protein block was used to eliminate background absorption, the samples were incubated with primary antibody, followed by treatment with biotinylated goat anti-mouse IgG (H+L) (ab64259) or biotinylated goat anti-rabbit IgG (H+L) (ab64261). After incubation with streptavidin peroxidase, DAB staining solution was added to the sections. Haematoxylin was used for the nuclear counterstain. The antibodies used in this experiment were as follows: MMP9 (ab76003), TrxR1 (ab124954), 8-OHdG (sc-66036), TGF-β1 (ab92486), and cleaved caspase-3 (CST, 9664S). The images of scanned slides were analysed using ImageJ software. Both the dermis and epidermis of the wound tissues were included in the analysis.

For immunofluorescence (IF) staining, the cells were fixed with 10% (v/v) formalin, permeabilized with Triton X-100 (0.5% v/v) and blocked with bovine serum albumin (BSA). Then, the slides or cells were incubated with primary antibodies, including anti-MMP9 (ab76003), anti-JNK1+JNK2 (p-T183+Y185) (ab131499) and recombinant anti-Ki67 (ab92742), for 24 h at 4°C, followed by Alexa Fluor 647-conjugated secondary antibodies at room temperature for 1 h. The slides or cells were then incubated with DAPI (Sigma-Aldrich) for nuclear counterstaining for 5 min. Finally, the stained cells were observed under an LSM-880 confocal microscope (Carl Zeiss, Oberkochen, Germany).

**JC-1 and TUNEL staining**

Cell apoptosis was detected using a TUNEL detection kit (G002) obtained from Nanjing Jiancheng Bioengineering Institute and a mitochondrial membrane potential assay kit with JC-1 (C2006) obtained from Beyotime Biotechnology. The staining processes were carried out according to the instructions. Fluorescent images were observed under an LSM-880 confocal microscope (Carl Zeiss, Oberkochen, Germany).

**Cell culture**

Our *in vitro* experiments used human immortalized keratinocytes (HaCaT cells), which were obtained from the National Infrastructure of Cell Line Resource. HaCaT cells were cultured at 37°C with 5% CO₂ in DMEM supplemented with 5.55 mmol/L D-glucose (LG), 10% (v/v) FBS and 1% (m/v) penicillin/streptomycin. For the chronic hyperglycaemic experimental model, HaCaT cells were stimulated with 25 mmol/L D-glucose DMEM for 48 h. Cells were treated with 1.5625, 3.125 and 6.25 μM BBR, and auranothin was used as an inhibitor of TrxR1 following BBR treatment. Cell viability of HaCaT cells in Mannitol group (treated with 5.55 mmol/L D-glucose+19.5 mmol/L Mannitol) which was used as osmotic control for high glucose, was compared with that in 25 mmol/L D-glucose DMEM (HG). Cell viability was tested using a Cell Counting Kit-8 (CCK-8), which was purchased from Dongren Chemical Technology Co., Ltd.

**Statistical analysis**

All data are expressed as the means with standard deviations and were analysed with the Student’s t test or a one-way ANOVA with Tukey’s post hoc test. p<0.05 was regarded as significant.

**Results**
BBR inhibited HG-induced cell damage, promoted cell proliferation and activated the TrxR1/JNK pathway

The protective effect of BBR was evaluated by using a HG-induced HaCaT cell model. As indicated by Figure 1A, BBR alone did not impair cell viability but promoted keratinocyte proliferation in low glucose (LG) media. Exposure of HaCaT cells to HG media led to a significant reduction in cell viability, which was reversed by BBR treatment in a dose-dependent manner. Due to the important role of TrxR1, the effect of TrxR1 was tested by using its inhibitor auranofin. Importantly, TrxR1 inhibitor abolished BBR-induced protection in HG medium, as evidenced by the decrease in cell viability. The reduction of TrxR1 expression in HG was obviously increased by BBR and was further abolished by TrxR1 inhibitor (Figure 1B). The repressed TrxR activity in HG was reversed by BBR, whereas TrxR1 inhibitor remarkably decreased the effect of BBR on TrxR activity. However, BBR did not affect the level of Trx in HG (Figure 1C-D). In addition, BBR decreased the phosphorylation of JNK, a downstream effector of TrxR1, in HG media, and this was also reversed by TrxR1 inhibitor (Figure 1E). Because wound healing can be affected by keratinocyte proliferation, the immunofluorescent staining of Ki67 (cell proliferation marker) was studied (Figure 1F). Lower expression of Ki67 in HG medium was promoted by BBR treatment, whereas TrxR1 inhibitor abolished the enhanced expression of Ki67 induced by BBR. Collectively, these results indicated that BBR improved cell viability, enhanced cell proliferation and activated TrxR1/JNK signalling.

BBR inhibited HG-induced oxidative damage and cell apoptosis

The ROS levels, GSH levels, SOD activity, T-AOC activity, and MDA content were detected to measure the inhibitory effect of BBR on ROS generation and oxidative damage. As shown in Figure 2A-B, sustained HG stimulation caused a significant increase in ROS levels and MDA content but a decrease in antioxidant molecules such as SOD, T-AOC, and GSH, indicating increased oxidative stress and reduced antioxidant capacity. In contrast, BBR significantly enhanced GSH levels, SOD activity and T-AOC activity but reduced ROS levels and MDA content. Remarkably, these effects were all abolished by TrxR1 inhibitor, as evidenced by the obvious elevation in ROS and MDA and the sharp decreases in SOD activity, T-AOC activity and GSH levels.

Additionally, TUNEL staining and caspase-3 activity indicated that BBR decreased the TUNEL-positive rate and caspase-3 activity, whereas these indicators were increased by TrxR1 inhibitor, demonstrating that TrxR1 inhibitor abolishes the inhibition of cell apoptosis by BBR (Figure 2C and E). The decreased mitochondrial membrane potential in HG-induced HaCaT cells was inhibited by BBR as indicated by the decreased JC-1 monomer/JC-1 aggregate ratio, which was also reversed by TrxR1 inhibitor auranofin (Figure 2D).

Topical BBR treatment accelerated wound closure in diabetic rats induced by STZ injection

To investigate the protective effect of BBR, wound closure rates and histological staining were evaluated in STZ-induced diabetic wounds. As indicated in Figure 3A-B, topical BBR treatment markedly increased wound closure rates in STZ-induced diabetic rats and was comparable to that of rhEGF (positive control), whereas TrxR1 inhibitor auranofin delayed BBR-enhanced wound closure. Furthermore, stronger HE and Masson staining was observed in BBR- and rhEGF-treated wounds compared to untreated STZ wounds. Additionally, untreated STZ rats exhibited the largest wound edges, whereas BBR obviously decreased the size of the wound edge such that it was comparable to that of the control group (Figure 3C). As indicated by histological evaluations, the decreased wound edges of BBR-treated wounds were reversed by TrxR1 inhibitor. Supporting
these observations, using a two-photon microscope, more collagen growth at the front edges of the healing wounds was observed in BBR-treated wounds than in untreated STZ wounds at day 6, which was comparable to rhEGF treatment. However, this collagen growth was also reduced by auranofin (Figure 3D). These data indicated that topical BBR treatment accelerated wound closure in STZ diabetic rats and promoted extracellular matrix synthesis.

**BBR enhanced the activation of TrxR1/JNK and reduced oxidative damage and apoptosis in diabetic wounds**

To further confirm the underlying mechanism, the effect of BBR on TrxR1, Trx and their downstream effector JNK was investigated. As indicated by Figure 4A-C, topical BBR therapy increased the expression of the antioxidant protein TrxR1, which was reversed by TrxR1 inhibitor. Additionally, the decreased level of Trx in STZ rats was obviously enhanced by BBR, whereas TrxR1 inhibitor did not affect Trx. Furthermore, BBR inhibited the phosphorylation of JNK when compared with untreated STZ wounds and this was reversed by TrxR1 inhibitor auranofin. This was consistent with the results in cells in which BBR promoted TrxR1 and inhibited downstream JNK activation.

The inhibitory effect of BBR on cell apoptosis and oxidative damage was investigated in STZ-induced diabetic wound model (Figure 5A-C). The antioxidant enzymes T-AOC and SOD were markedly decreased in untreated STZ wounds, whereas topical BBR treatment significantly improved T-AOC and SOD. Supporting these observations, the increased lipid oxidative product MDA and the strong staining of 8-OHdG (a DNA oxidative marker) in untreated STZ wounds was obviously reduced following BBR treatment in untreated STZ wounds (Figure 5B-C). Importantly, TrxR1 inhibitor abolished the reduction of 8-OHdG and MDA induced by BBR, as well as the increase in T-AOC and SOD in STZ-induced diabetic wounds. In addition, increased cell apoptosis in untreated STZ wounds, as indicated by elevated activity and staining of caspase-3 and TUNEL-positive staining, was reversed by topical BBR treatment, and this reversal was remarkably absent with TrxR1 inhibitor auranofin treatment. Thus, topical BBR treatment inhibited oxidative damage and apoptosis in STZ-induced diabetic wounds and this protection could be abolished by TrxR1 inhibitor auranofin. Taken together, BBR significantly restored suppressed TrxR1 expression, inhibited activation of downstream JNK, and decreased oxidative damage and cell apoptosis in both the HG-induced HaCaT model and STZ-induced diabetic wounds; all of these effects could be abolished by treatment with the TrxR1 inhibitor auranofin.

**BBR modulated the expression of TGF-β1, TIMP1 and MMP9**

Notably, long-term oxidative injury in diabetic wounds usually causes dysfunction in tissue regeneration and matrix degradation, whereas restored redox homeostasis benefits tissue regeneration and inhibits dysfunctional tissue remodelling. Thus, we measured ECM synthesis and remodelling-related molecules such as MMP9, TIMP1 and TGF-β1 both in vitro. In STZ-induced diabetic wounds, untreated STZ wounds had higher MMP9, a greater MMP9/TIMP1 ratio, and lower TGF-β1 and TIMP1 protein levels than control wounds, whereas topical BBR treatment restored the expression of these proteins to the levels of control wounds, and this restoration were abolished by TrxR1 inhibitor (Figure 6A-B). Previous research indicated that that oxidative damage can significantly affect the function of keratinocytes and modulated its expression of MMP9 and TGF-β1, (9, 42) thus whether decreasing oxidative damage by BBR had a beneficial effect on restoring the expression of MMP9, TIMP1 and TGF-β1 in HaCaT cells, was evaluated accordingly. HaCaT cells in HG had high MMP9 levels and low expression of TIMP1 and TGF-β1, whereas BBR significantly decreased MMP9 levels while...
increasing the expression of TIMP1 and TGF-β1. Conversely, the modulation of MMP9, TIMP1 and TGF-β1 by BBR was reversed by TrxR1 inhibitor (Figure 6C-E). Taken together, topical BBR treatment elevated TGF-β1 and TIMP1 and reduced MMP9 levels in vitro and in vivo, and these effects could be abolished by TrxR1 inhibitor auranofin.

**Topical BBR treatment accelerated wound closure in type 2 diabetes through activation of TrxR1**

To further confirm the protective effect of BBR, wound closure was investigated in a diabetic model caused by a combination of STZ injection and high fat diet (hfdSTZ), which mimics the symptoms of type 2 diabetes (Figure 7). Topical BBR treatment significantly enhanced the wound closure rates when compared to hfdSTZ on day 6, and 15, which was comparable to that of rhEGF treatment. TrxR1 inhibitor (ATG) and JNK activator (ALN) obviously delayed the BBR enhanced-wound closure at day 6 and 15, highlighting the important role of TrxR1 and JNK in type 2 diabetes (Figure 7A). Notably, topical BBR therapy increased TrxR1 expression, which was reversed by TrxR1 inhibitor (ATG). Consistent with the in vivo result, TrxR1 inhibitor (ATG) and JNK activator (ALN) remarkably decreased BBR-mediated enhancement of cell viability (Figure 7B). There is no significant difference in TrxR1 expression between hfdSTZ and hfdSTZ+ JNK-IN-8, indicating that JNK inhibitor (JNK-IN-8) didn’t affect TrxR1 expression (Figure 7C). Besides, no significance observed between hfdSTZ+BBR and hfdSTZ+BBR+ALN in TrxR1 expression, suggesting that JNK activator (ALN) had no effect on TrxR1 expression (Figure 7C). These results indicated that the activation of JNK may not affect TrxR1 expression. All of these results confirmed that BBR promoted wound healing through regulating TrxR1 and JNK.

**Discussion**

In this study, in order to avoid the low bioavailability in oral administration and enhance the convenience of using BBR, topical treatment of BBR was used to promote wound healing in diabetes. We demonstrated that BBR significantly accelerated diabetic wound healing and that TrxR1 plays a vital role in this protection by restoring redox homeostasis. The therapeutic effect of BBR was demonstrated in diabetic models, as evidenced by the significantly improved wound closure rate and collagen expression and the decreases in cell apoptosis, oxidative damage and ECM remodelling dysfunction. In vitro experiments with HaCaT cells confirmed that BBR can protect against HG-induced oxidative damage, cellular apoptosis and the suppression of proliferation under hyperglycaemic conditions. TrxR1 inhibitor delayed wound healing, caused obvious oxidative damage and apoptosis, increased MMP9 and decreased TGF-β1 and TIMP1 both in vitro and in vivo, further confirming the vital role of TrxR1 in BBR protection in wound healing. The protective effect of BBR on wound healing through modulating TrxR1 was further verified in type 2 diabetes induced by both high fat diet and STZ injection.

Numerous studies have indicated that overproduction of ROS damages collagen proteins and cause DNA damage and lipid over-oxidation, triggering cell apoptosis, ECM degradation and disruption of tissue regeneration in diabetic wounds. (4, 43) Timely restoration of redox homeostasis has proven to be an effective strategy for promoting wound repair in diabetes. (7-9). Nrf2 is a central regulator in the maintenance of redox homeostasis by recognizing antioxidant response element and activates antioxidant genes and recent research indicated that using Nrf2 activators such as sulforaphane (SF) and cinnamaldehyde (CA) decrease oxidative damage, significantly accelerates wound closure, decreases MMP9 level and increases TGF-β1 level (9). In our study, BBR decreased HG-induced ROS and reduced oxidative damage which was associated with enhanced
wound closure, whereas this was reversed by TrxR1 inhibitor which increased ROS level and oxidative damage. Thus, using a free radical scavenger could decrease ROS level and reduce oxidative damage which would have a beneficial effect on diabetic wound.

Increased oxidative damage is a typical characteristic of diabetic wounds, and the increase in TrxR1 induced by BBR treatment restored impaired redox homeostasis and facilitated diabetic wound healing. TrxR1 plays a vital role in modulating intracellular redox homeostasis and can reduce multiple biomolecules, including isoforms of thioredoxin, peroxiredoxins (Prxs), and related molecules that maintain biological processes, such as intracellular redox homeostasis, cell proliferation and apoptosis regulation, by using electrons donated by NADPH. Previous studies have indicated that TrxR1 knockout in embryos results in early embryonic death with an obvious decrease in cell proliferation. (15) Additionally, inhibition of TrxR1 by auranofin and PX-12 resulted in a decrease in cell proliferation and led to apparent oxidative damage and apoptosis. (16, 17) These previous findings were consistent with our data demonstrating that increased TrxR1 induced by BBR caused a decrease in oxidative damage and apoptosis and an increase in cell proliferation, whereas inhibition of TrxR1 in BBR-treated wounds decreased cell proliferation and caused oxidative damage and apoptosis. In addition, TrxR1 deficiency was found to increase the oxidation of PTP1B, significantly influencing PDGF-β receptor tyrosine kinase signalling and affecting insulin resistance and cell apoptosis, which may also have an effect on diabetic wound healing. (46-48) Additionally, the restoration of TrxR1 by BBR accelerated keratinocyte proliferation and facilitated wound healing. Ki67 is a cell proliferation marker and the proliferation of keratinocytes is of importance since cell migration alone is insufficient to close large and full-thickness wounds. (49) Recent research has demonstrated that DNA synthesis, a primary step of cell proliferation, requires the reduction of TrxR1, (50) and the inhibition of TrxR1 results in decreased cell proliferation, (16, 51) highlighting the essential role of TrxR1 in cell proliferation. Our study showed that HaCaT cells in HG medium had a reduced proliferation (Fig.1F), consistent with other studies (52, 53) with low TrxR1 (53) levels and that restoration of TrxR1 by BBR promoted keratinocyte proliferation, which was further abolished by the inhibition of TrxR1, indicating the vital role of TrxR1 in the facilitating keratinocyte proliferation by BBR. Thus, BBR exerts a beneficial effect on wound healing through restoring TrxR1 in diabetes.

Moreover, the suppression of JNK through restoring TrxR1 plays a vital role in facilitating wound healing in BBR-treated wounds. In our study, JNK inhibitor JNK-IN-8 significantly promoted wound healing and cell viability and JNK activator ALN obviously delayed BBR-treated wound healing, indicating the vital role of JNK in diabetic wound healing. Previous researches indicated that TrxR inhibits phosphorylation of JNK and prevents downstream cell apoptosis, and suppression of TrxR results in the phosphorylation of JNK and caused cell apoptosis in various cell types such as human bronchial epithelial (HBE1) cells, DP thymocytes, and colon cancer cell, (54-56) which indicated that TrxR1 affects the phosphorylation of JNK. This was consistent with our results that increased TrxR1 was associated with decreased p-JNK/JNK and TrxR1 inhibition caused a decrease in p-JNK/JNK in STZ-induced diabetic wounds (Figure 4). Additionally, JNK inhibitor or activator didn’t affect TrxR1 expression (Figure 7). All these results showed that the enhanced wound healing by BBR was mediated by TrxR1-dependent inhibition of JNK.

Increased TGF-β1 levels promoted ECM synthesis, while high TIMP1 combined with low MMP9 expression in BBR-treated diabetic rats prevented dysfunctions in ECM remodelling, thus accelerating diabetic wound healing. Diabetic wound healing usually increases MMPs and decreases TGF-β1, which inhibits healing
in diabetic patients. TGF-β1 signalling acts as a key regulator in promoting ECM synthesis, re-epithelialization and angiogenesis. Excess ROS in diabetic wounds caused dysfunctional TGF-β1 signalling, which particularly damaged collagen synthesis and resulted in impaired wound healing. Additionally, overproduced ROS accelerated expression of MMP, which plays a vital role in ECM degradation and remodelling. MMP9 is responsible for eliminating damaged ECM and allows for skin tissue remodelling. High MMP9 levels lead to over-degradation of ECM and dysfunctional ECM remodelling, resulting in impaired wound repair, while MMP9 suppression accelerates wound healing in diabetes. MMP9 activity can be significantly inhibited by tissue inhibitors such as TIMP1, which have a high affinity for MMP9. Clinically, there is a positive correlation between the ratios of MMP9/TIMP1 and poor wound healing. Our results were consistent with these findings, demonstrating that low TGF-β1 and TIMP1, high MMP9, and a high MMP9/TIMP1 ratio were found in STZ-induced wounds. The changes in these factors were reversed by BBR treatment, which was associated with higher ECM and collagen synthesis, indicating an important role for BBR in accelerating wound healing. Importantly, the TrxR1 inhibitor auranofin abolished the BBR-induced enhancement of MMP9/TIMP1 expression and the reduction in MMP9 expression both in vitro and in vivo. These effects may be attributed to the impaired redox homeostasis and the increased ROS and oxidative damage caused by TrxR1 inhibition. All of these data highlight that TrxR1 is vital for maintaining redox homeostasis in diabetic wound healing.

In conclusion, BBR promoted wound closure, enhanced ECM synthesis, and prevented dysfunction in ECM remodelling by restoring TrxR1/JNK signalling via decreasing ROS and oxidative damage, reducing cell apoptosis and enhancing cell proliferation. These effects facilitated a healthier environment with high TGF-β1 and TIMP1 levels and low MMP9 production, allowing for the maintenance of ECM synthesis and normal remodelling (Figure 8). This study provides evidence of the efficacy of topical BBR application for the treatment of diabetic wounds, and reveals that BBR accelerated diabetic wound healing through the activation of TrxR1, and highlights a therapeutic strategy involving the targeting of TrxR1 for diabetic wound repair.

Clinical Perspectives

- Vascular dysfunction in diabetes results in reduced therapeutic effect of oral berberine (BBR). Topical treatment of berberine can concentrate berberine on local diabetic wounds, which benefits wound repair.
- BBR promoted wound healing, enhanced ECM synthesis, and prevented ECM remodelling dysfunction by restoring TrxR1/JNK signalling.
- Our results explain the specific role of TrxR1 in diabetic wounds and indicate that TrxR1 could be proposed as a potential therapeutic target for diabetic wound repair.

Author Contributions

J. Zhang conducted animal study, conceived the study and wrote the manuscript; R. Zhou performed animal study and cell related experiments; G. Cao, H. Xu, Y. Zhang, and C. Xiang performed the animal studies; H. Yang conceived and supported the study; and all authors reviewed and approved the final manuscript.

Acknowledgments

The authors would like to acknowledge the financial support from National Key R&D Plan (No. 2017YFC1702605), National Natural Science Foundation of China (Nos. 81974550), Major Science and Technology Project for “Significant New Drugs Creation” (2019ZX09201005) and Fundamental Research Funds for the Central public welfare research institutes (No. ZZ13-YQ-046).
Conflicts of Interest
The authors declare no conflict of interest.

List of Abbreviations
SOD = superoxide dismutase
MDA = malondialdehyde
ROS = reactive oxygen species
T-AOC = total antioxidant capacity
Trx = thioredoxin
TrxR = thioredoxin reductase
GSH = glutathione
Aur = auranoïn
8-OHdG = 8-hydroxy-2'-deoxyguanosine
ECM = normal extracellular matrix
MMP = matrix metalloproteinase
BBR = Berberine
TrxR1 = thioredoxin reductase 1
LG = low glucose
HG = high glucose
STZ = streptozotocin
TIMP1 = Metalloproteinase inhibitor 1
TGF-β1 = transforming growth factor-β1
MMP9 = Matrix metalloproteinase-9
ELISA = enzyme-linked immunosorbent assay
IHC = immunohistochemistry
IF = immunofluorescence
ATG = Aurothioglucose
ALN = Allicin

Figure Legends

Figure 1. BBR inhibited HG-induced damage, promoted cell proliferation and activated the TrxR1/JNK pathway.
A. Cell viability (n=6). B. TrxR1 ELISA result (n=4). C. TrxR activity (n=3). D. TRX ELISA result (n=3-4). E. Immunofluorescence staining of p-JNK (red) and DAPI staining (blue) and p-JNK/JNK ELISA result; scale bar: 50 μm. F. Immunofluorescence staining and quantitative results of Ki67 (red) (n=3-5 per group) and DAPI (blue); scale bar: 50 μm. Data are presented as the means ± SD. *p < 0.05.

Figure 2. BBR decreased oxidative damage and cell apoptosis in the HG-induced model.
A. T-AOC activity (n=3), GSH level (n=4), and SOD activity (n=4). B. ROS level and MDA content (n=6). C. Caspase-3 activity (n=3). D. JC-1 with its quantitative results (n=3-5 per group). E. TUNEL staining with its quantitative results (n=3-5 per group). Data are presented as the means ± SD. *p < 0.05.

Figure 3. Topical BBR treatment accelerated wound closure in STZ-induced diabetic rats.
A. Representative gross photographs of diabetic wounds on days 0, 6, 9, and 12 and the wound closures calculated with Image J. B. Representative images of HE and Masson staining of diabetic wounds on day 12. C. Quantitative results of the wound edge determined using Image J (n=4 for control and n=5-7 for other group); the red arrows indicate the wound boundaries and the distance between the two boundaries was considered as wound edge. D. New collagen (purple) growth at the front edge of wounds; the green indicates the background on day 6; scale bar: 70 μm. The data are presented as the means ± SD. *p < 0.05.

Figure 4. BBR enhanced TrxR1 and inhibited the activation of JNK in STZ-induced diabetic wounds on day 12.
A. Immunostaining of TrxR1 and quantitative results (n=3-5 per group); scale bar: 25 μm. B. TrxR1 ELISA result (n=5-7). C. Western blotting and quantitative results of TRX, p-JNK and JNK (n=3). Data are presented as the means ± SD. *p < 0.05.

Figure 5. BBR decreased oxidative damage and apoptosis in STZ-induced diabetic wounds on day 12.
A. T-AOC and SOD activity (n=5-7). B. MDA (lipid oxidative product) and caspase-3 activity (n=5-7). C. TUNEL staining and immunostaining for caspase-3 and 8-OHdG with quantitative results (n=3-5 animals per group); scale bar: 100 μm. Data are presented as the means ± SD. *p < 0.05.

Figure 6. BBR treatment modulated the expression of MMP9, TIMP1 and TGFβ1 in diabetic wounds in vitro and in vivo.
The regulation of MMP9, TIMP1, and TGF-β1 expression by BBR in STZ-induced diabetic wounds on day 12 (A-B) and HG-induced HaCaT cells (C-E). A. Immunostaining for MMP9, TGF-β1 and quantitative results (n=3-5); scale bar: 100 μm. B. MMP9, TGF-β1 and TIMP1 ELISA results and the calculated MMP9/TIMP1 ratio (n=5-7). C. TGF-β1 ELISA result (n=4). D. TIMP1 ELISA result (n=4). E. Immunofluorescence staining of MMP9 (red), DAPI (blue), and quantitative results (n=3-5); scale bar: 50 μm. Data are presented as the means ± SD. *p < 0.05.

Figure 7. Topical BBR treatment accelerated wound closure in type 2 diabetes.
A. Representative gross photographs of diabetic wounds on day 0, 6, and 15 and the calculated wound closure with Image J (n=8-10). B. Cell viability of BBR in HG-induced HaCaT cells (n=6). C. Immunostaining of TrxR1, scale bar: 100 μm. D. TrxR1 ELISA result (n=8), ATG: TrxR1 inhibitor; JNK-IN-8, JNK inhibitor; ALN, JNK activator; the data are presented as the mean ± SD. *p < 0.05.

Figure 8. Schematic diagram of the mechanism of how BBR accelerates diabetic wound healing by restoring TrxR1/JNK.
Oxidative damage in diabetic wound leads to delayed wound healing. BBR reduced oxidative damage and cell apoptosis and inhibited phosphorylation of JNK by restoring TrxR1, resulting in increased levels of TGF-β1 and TIMP1, increased collagen growth, and decreased MMP9, thereby accelerating wound healing. In contrast, TrxR1 inhibitor inhibited TrxR1, cancelling the effect of BBR and thus delaying wound healing.
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