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Genistein accelerates refractory wound healing by suppressing superoxide and FoxO1/iNOS pathway in type 1 diabetes☆

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Abstract

Refractory wounds in diabetic patients constitute a serious complication that often leads to amputation with limited treatment regimens. The present study was designed to determine the protective effect of the soy isoflavone genistein on diabetic wound healing and investigate underlying mechanisms. Streptozotocin (STZ)-induced type 1 diabetic mice with full-thickness excisional wounds received 0.2, 1 or 5 mg/kg/day of genistein via subcutaneous injection. Genistein dose-dependently rescued the delay of wound closure in diabetic mice. A dose of 5 mg/kg/day of genistein treatment significantly increased the mean perfusion rate, and *in vitro* treatment with genistein protected against high glucose-induced impairment of capillary tube formation in cultured endothelial cells. Diabetic conditions significantly increased superoxide anion ($O_2^{\cdot -}$) production and nitrotyrosine formation, and decreased nitrite levels in wound tissues. Genistein treatment at all doses normalized the elevated $O_2^{\cdot -}$ production and nitrotyrosine formation, and reversed the attenuated nitrite level. In diabetic wound tissues, the inducible nitric oxide synthase (iNOS) was activated, and genistein administration prevented increased iNOS activity. Moreover, genistein attenuated diabetic cutaneous silent information regulator 1 and forkhead box O transcription factor 1 (FoxO1) levels and potentiated ac-FoxO1 in a dose-dependent manner. Genistein rescued the delayed wound healing and improved wound angiogenesis in STZ-induced type 1 diabetes in mice, at least in part, by suppression of FoxO1, iNOS activity and oxidative stress.

Keywords: Genistein; Oxidative stress; Diabetes; iNOS; FoxO1; Nitrotyrosine

1. Introduction

Diabetes can interfere with the wound-healing process. This can lead to serious complications and even amputation [1]. It is estimated that every 30 s a lower limb is lost worldwide as a result of diabetes, with limited treatment regimens [2]. Diabetes restricts wound healing by interfering with the inflammatory response, decreasing granulation, and causing peripheral neuropathy and inhibition of angiogenesis [3,4]. Oxidative stress has been proposed as an important pathogenic factor in diabetic wound complications [5]. Sustained hyperglycemia-mediated superoxide anion ($O_2^{\cdot -}$) overproduction, which is the main initiator of oxidative stress, leads to the activation of several pathways involved in the pathogenesis of diabetic wound healing, including stimulation of the polyol and glucosamine pathways, activation of protein kinase C and formation of advanced glycation end products [6]. Higher concentrations of intracellular reactive oxygen species (ROS) interfere with angiogenesis and promote inflammation [5]. It also abrogates endothelial nitric oxide synthase (eNOS) and suppresses nitric oxide (NO) bioavailability, enhancing wound NO bioavailability, which could promote processes central to wound healing, including angiogenesis, migration and proliferation of fibroblasts, epithelial cells, keratinocytes, endothelial cells, and mobilization of endothelial progenitor cells [7]. Our previous study demonstrated that cutaneous gene therapy of manganese superoxide dismutase or overexpressing GTP cyclohydrolase I to retard NOS uncoupling was able to restore the delayed diabetic wound healing with suppression of wound $O_2^{\cdot -}$ as well as a concomitant augmentation of NO levels [8,9]. Accordingly, studies have shown that antioxidants accelerate diabetic wound healing due to enhanced NO bioavailability [10,11].

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Isoflavones, one of the major phytoestrogen classes, are found to be abundant in soybean and its derivative food products and are believed to have beneficial cardiovascular effects. Genistein, the primary soybean-derived isoflavone, has been shown to be the most efficacious in clinical and preclinical trials [12]. Recently, much interest has focused on beneficial effects of genistein on diabetes and diabetic complications. In rats, long-term genistein treatment has been found to preserve healthy vascular reactivity through NO- and prostaglandin-dependent pathways. It was also found to mitigate oxidative stress in the walls of the aorta [13]. Furthermore, genistein has been shown to protect against oxidative stress and inflammation, neuropathic pain, and neurotrophic and vasculature deficits in the diabetic mouse model [14]. *In vitro*, genistein protected vascular endothelial cells against high glucose- and H_2O_2-induced oxidative stress injury [15]. However, the effects of genistein on diabetic wound healing have not been previously determined.

Therefore, in the present study, we tested the hypothesis that genistein promotes diabetic wound healing, and we attempted to identify the underlying mechanism(s) by using full-thickness excisional wounds and streptozotocin (STZ)-induced type 1 diabetes in mice. Our results demonstrated that genistein could rescue the delayed wound healing in diabetic mice through suppressed inducible nitric oxide synthase (iNOS) activity and oxidative stress and that it enhanced wound angiogenesis and NO levels.

2. Methods and materials

2.1. Animals

All procedures involving animals were conducted in accordance with the European Community guidelines for the use of experimental animals and approved by the Peking University Committee on Animal Care and Use. C57BL/6 male mice at 10–12 weeks of age (20–25 g, purchased from the Animal Center of Peking University, Beijing, China) were rendered diabetic by intraperitoneal (i.p.) injection of 60 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, USA) in 50 mM sodium citrate (pH 4.5) daily for 5 days, as previously described [9]. Control mice were treated with daily injection of citrate buffer. Blood glucose was measured from the mouse tail vein using an ACCU-CHEK Aviva blood glucose monitor (Roche, Mannheim, Germany). Once blood glucose level reached above 250 mg/dl, daily measurements followed for 1 week prior to experiments [7,9].

2.2. Full-thickness excisional wound and drug administration

A full-thickness excisional wound was created as previously described [9]. Briefly, 1 week after blood glucose reached 250 mg/dl, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight), and the dorsum was clipped free of hair. Full-thickness skin was removed on the dorsomedial back of each animal using a 4-mm punch biopsy (Acuderm Inc., Fort Lauderdale, FL, USA), exposing the underlying muscle, and then the wound was covered with Bioclusive transparent dressing (Johnson & Johnson, Milpitas, CA, USA). Wound closure rate was measured by tracing the wound area every other day onto the Bioclusive dressing. The tracings were digitized, and the areas were calculated in blinded fashion with the use of a computerized algorithm (Image-pro plus 5.0; Media Cybernetics, Inc., Silver Spring, MD, USA). Diabetic mice were divided into four groups and were respectively injected subcutaneously (s.c.) with 0.2, 1 and 5 mg/kg of genistein (dissolved in 5% DMSO/95% PEG-400) once daily since the date of wounding. Nondiabetic control received an s.c. injection of genistein vehicle. To determine the effect of iNOS inhibition on wound healing in diabetic mice, iNOS selective inhibitor L-N6-(1-Iminoethyl)lysine hydrochloride (L-NIL) was used. L-NIL is now established as a potent and relatively selective iNOS inhibitor, with an IC_{50} of 5.9 μM for iNOS compared with an IC_{50} of 138 μM for eNOS and 35 μM for neuronal nitric oxide synthase (nNOS) [16]. Diabetic mice were given intragastrically 1 and 3 mg/kg of L-NIL vehicle (i.e., distilled water) once daily since the date of wounding. The doses of L-NIL were chosen based on previously published data [17,18]. Nondiabetic control received distilled water intragastrically.

2.3. Laser Doppler perfusion imaging

Tissue blood flow in regions of the dorsum wound area was measured by using a laser Doppler perfusion imager (MoorLDI, Moor Instruments Ltd., Devon, UK) on days 2, 4, 6 and 8 after surgery. This relative measure of volume flow was expressed in arbitrary perfusion units). The recorded images were analyzed using a dedicated software (MoorLDI v2.1; Moor Instruments Ltd., Devon, UK), and the perfusion value was taken and divided by the baseline measurement to give a ratio representing the change in flow.

2.4. Measurement of circulating endothelial progenitor cells

Endothelial progenitor cells (EPCs) were isolated from blood as previously described [19,20]. The quantification of circulating EPCs in the peripheral blood was also determined by flow cytometry. Peripheral blood mononuclear cells were used for fluorescence-activated cell sorter (FACS) analysis. Cells were incubated with Sca-1 antibodies (PE-conjugated rat anti-mouse Ly-6A/E, 4 μg/ml; BD Pharmingen, San Diego, CA, USA) and Flk-1 antibodies (FITC-conjugated rat anti-mouse Flk-1, 10 μg/ml; BD Pharmingen, San Diego, CA, USA) for 1 h on ice. Isotype-matched mouse immunoglobulins served as negative controls. After incubation, cells were washed with phosphate-buffered saline and fixed in 2% paraformaldehyde. To quantify the amount of Sca-1 and Flk-1 double-positive cells, the mononuclear cell fraction was gated and analyzed with a FACS Calibu and CellQuest software (Becton Dickinson, San Jose, CA, USA).

2.5. In vitro capillary tube formation assay

Human umbilical vein endothelial cells (HUVECs) were purchased from Invitrogen and maintained in supplemented Medium 200 (Invitrogen, Carlsbad, CA, USA). All experiments were performed with cells between passages 2 and 5. Matrigel-Matrix (BD Biosciences, San Jose, CA, USA) was placed in the well of a 96-well cell culture plate, and HUVECs were plated in each well at a density of 2×10^4 with Medium 200. The endothelial cells were cultured in medium containing 5.5 mM glucose, 30 mM glucose or 30 mM glucose treated with indicated concentrations of genistein. After 8 h of incubation, images of tube morphology were taken by inverted microscope (Nikon Corporation, Tokyo, Japan), and tube lengths were measured at low-power fields (magnifications×40) per sample.

2.6. Superoxide measurement

The superoxide-sensitive fluorescent dye dihydroethidium (DHE) was used to evaluate *in situ* production of $O_2^{\cdot-}$ on wound closure as described [9]. Unfixed frozen skin tissues were cut into 30-μm sections and placed on glass slides. Slides were incubated with 1 μM DHE (Invitrogen, Carlsbad, CA, USA) in a light-protected, humidified chamber at 37°C for 30 min and then coverslipped. Tissue sections were then visualized with a Zeiss 210 confocal microscope, and fluorescence was detected with a 590-nm long-pass filter. Images were collected and stored digitally.

2.7. Nitrite measurement

Wound tissue was cut into small pieces on wound closure and used to determine the nitrite level with the NO assay kit (Beyotime, Haimen, China). After incubation with Eagle's minimal essential medium (EMEM) in a CO_2 incubator for 24 h, wound tissue was weighed, and the NO stable metabolite nitrite concentration in EMEM was determined [8]. The absorbance at 540 nm was measured with a microplate reader (Thermomax, Molecular Devices Corp., Menlo Park, CA, USA). The concentrations of nitrite were calculated following the instruction of the kit.

2.8. NOS activity assay

Skin tissues were homogenized and centrifuged, and supernatants were subjected to NOS activity assay with a commercial NOS assay kit (KeyGen, Nanjing, China). Briefly, aliquots of supernatants were incubated with the working solution supplied in the kit, and after incubation at 37°C for 15 min, the reaction was terminated by adding the terminating solution provided in the kit. The absorbance at 530 nm was measured with the microplate reader. Constitutive NOS (eNOS and nNOS) activity was determined by taking the difference between total NOS activity and iNOS activity, as we previously described [9]. Results were normalized to protein content as measured by the bicinchoninic acid (BCA) assay (Thermo Scientific Pierce, Rockford, IL, USA).

2.9. Immunoblot analysis

Briefly, skin tissues were homogenized in lysis buffer [1% Triton-X100, 50 mM Tris-HCl (pH 8.0), 0.25 M NaCl, 5 mM EDTA] with a mixture of protease inhibitors (Calbiochem, San Diego, CA, USA). Homogenates were centrifuged at 12,000 *g* for 15 min at 4°C, and protein supernatants were measured. Protein concentration was measured using the BCA assay, and equal amounts of protein were analyzed by electrophoresis on a 7.5% sodium dodecyl sulfate–polyacrylamide gel and electrophoretically transferred onto nitrocellulose membranes of 0.45-μm pore size. Membranes were blocked in blocking buffer (phosphate-buffered saline plus 0.05% Tween-20 and 5% nonfat dry milk) for 1 h or more and then incubated overnight at 4°C with following antibodies: mouse anti-nitrotyrosine (1/100) (Cayman Chemicals, Ann Arbor, MI, USA), rabbit anti-silent information regulator 1 (SIRT1) (1/1000) (Beyotime, Haimen, China), rabbit anti-forkhead box O transcription factor 1 (FoxO1) (1/1000) (Cell Signaling Technology, Beverly, MA, USA), rabbit anti-ac-FoxO1 (1/1000) (Santa Cruz

Biotechnology, Santa Cruz, CA, USA) and mouse anti-actin (1/5000) (Sigma-Aldrich, St. Louis, MO, USA). After incubation with the appropriate horseradish-peroxidase-conjugated secondary antibody, blots were developed using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The staining intensity of bands was determined by densitometric image analysis with Quantity One software version 4.4.0.

2.10. Statistical analysis

Data were expressed as means ± S.E.M. The significance of differences between groups was evaluated by unpaired Student's *t* test. When more than two treatment groups were compared, one-way analysis of variance with Newman–Keuls test was used. A probability level of $P<.05$ was considered statistically significant.

3. Results

3.1. Induction of diabetes in mice

As shown in Table 1, STZ administration induced diabetes in mice as shown by hyperglycemia (blood glucose levels: 391.10 ± 21.35 vs. 143.80 ± 3.27 mg/dl in normal control mice, $P<.001$). Diabetes also resulted in a highly significant impairment of body weight gain (23.98 ± 0.77 vs. 26.28 ± 0.49 g in normal control mice, $P<.05$). Genistein treatment at doses of 0.2, 1 and 5 mg/kg did not significantly modify either blood glucose or body weight.

3.2. Effects of genistein on wound healing in diabetic mice

As shown in Fig. 1A, compared with control mice, the rate of wound closure was significantly delayed in diabetic mice (68.10% ± 2.19% vs. 40.03% ± 5.84% on day 6, $P<.01$). Genistein administration (5 mg/kg) to diabetic mice significantly accelerated the rate of wound closure by as much as 2 days and continued through day 14 (Fig. 1A and C). The percentage of wound closure on day 6 in diabetic mice treated with 5 mg/kg genistein was significantly higher than that of diabetic control mice (66.02% ± 4.75% vs. 40.03% ± 5.84%, $P<.01$) (Fig. 1B). There was no significant difference in wound healing between diabetic mice treated with genistein at dose of 0.2 mg/kg and diabetic control mice except on day 12 and 14 (53.59% ± 6.33% vs. 40.03% ± 5.84% on day 6, $P>.05$). There was no significant difference in wound healing between diabetic mice treated with genistein at dose of 1 mg/kg and diabetic control mice except on days 2, 10 and 12 (57.81% ± 9.28% vs. 40.03% ± 5.84% on day 6, $P>.05$).

3.3. Effects of genistein on perfusion around the wound in diabetic mice

The mean perfusion rate around the wound was analyzed by using laser Doppler perfusion imager at days 2, 4, 6 and 8 after surgery (Fig. 2A). The mean perfusion rate was steadily increased and peaked on day 6, then plateaued and started declining in different groups. Compared with control mice, the mean perfusion rate was significantly decreased at day 2 and continued through day 6 in diabetic mice (117.31 ± 8.52 vs. 92.35 ± 7.41 on day 6, $P<.05$) (Fig. 2A). Treatment with 5 mg/kg genistein significantly increased the mean perfusion rate on days 4 and 6 in diabetic mice (126.20 ± 7.86 vs. 92.35 ± 7.41 on day 6, $P<.05$) (Fig. 2B and C).

3.4. Effects of genistein on circulating EPC number in diabetic mice

Recently, studies have been intensively focused on bone-marrow-derived EPCs, which circulate in the blood and exhibit characteristic features consistent with endothelial cells [21]. It has been shown that EPCs can be recruited to sites of arterial injury and contribute to angiogenesis [22]. To examine the effect of genistein on circulating EPCs *in vivo*, we collected peripheral blood from each group. As shown in Fig. 3A, diabetic mice had significantly lower number of circulating Sca-1^+/Flk-1^+ EPCs compared to control (0.58% ± 0.20% vs. 1.60% ± 0.51%, $P<.05$); 5 mg/kg genistein treatment resulted in a higher level but no obvious differences in the number of circulating Sca-1^+/Flk-1^+ EPCs compared with diabetic group (1.08% ± 0.44% vs. 0.58% ± 0.20%, $P>.05$).

3.5. Effects of genistein on in vitro capillary tube formation

Next, we determined effects of genistein on angiogenesis *in vitro*. Tube formation assay on matrigel was performed to investigate the HUVECs' ability to form tubule network. As shown in Fig. 3B, high glucose evoked impairment of HUVECs tube formation capacity. Treatment with 1 μM genistein prevented the loss of tubule network, whereas 0.1 μM genistein could not significantly increase the tube formation under high-glucose conditions (Fig. 3C).

3.6. Effects of genistein on cutaneous $O_2^{\cdot -}$, nitrotyrosine formation and NO level in diabetic mice

To determine effects of genistein on cutaneous oxidative stress in diabetes, we estimated cutaneous $O_2^{\cdot -}$ production. *In situ* detection of $O_2^{\cdot -}$ by DHE staining showed that cutaneous $O_2^{\cdot -}$ production in untreated diabetic mice was increased compared to control mice ($P<.05$). Cutaneous $O_2^{\cdot -}$ production in diabetic mice treated with genistein was significantly attenuated compared with that of untreated diabetic mice (Fig. 4A).

$O_2^{\cdot -}$ and NO are known to rapidly react to form the stable peroxynitrite anion ($ONOO^-$) which is highly toxic and thought to be principal mediators of oxidative cellular damage [23]. Cutaneous $ONOO^-$ formation was determined using nitrotyrosine antibodies. As shown in Fig. 4B, nitrotyrosine was detected mainly in the protein bands of 55 kDa in skin, and levels of nitrotyrosine increased dramatically in untreated diabetic mice skin. Genistein treatment dose-dependently suppressed nitrotyrosine formation in diabetic mice skin.

We next estimated cutaneous NO level by measuring its stable metabolite nitrite. Cutaneous nitrite level was significantly reduced in

Table 1
Body weight and blood glucose level changes in mice

	Control (n = 10–16)	Diabetic (n = 8–13)	Diabetic + 0.2 mg/kg genistein (n = 10–11)	Diabetic + 1 mg/kg genistein (n = 12–13)	Diabetic + 5 mg/kg genistein (n = 8–13)
Body weight (g)					
Before treatment	26.28 ± 0.49	23.98 ± 0.77*	23.16 ± 1.01*	24.54 ± 0.49*	24.41 ± 0.72*
After treatment	29.19 ± 0.33	21.79 ± 0.59***	21.20 ± 1.34***	21.99 ± 0.97***	22.66 ± 1.32**
Blood glucose levels (mg/dl)					
Before treatment	143.80 ± 3.27	391.10 ± 21.35***	374.20 ± 24.40***	388.70 ± 26.06***	384.10 ± 23.05
After treatment	165.80 ± 8.00	403.10 ± 28.44***	366.20 ± 26.21***	420.30 ± 20.05***	427.70 ± 21.52

* Diabetic status induced by daily injection of streptozotocin for 5 days (60 mg/kg i.p.) in mice; control mice were treated with daily injections of citrate buffer. Values were mean ± S.E.M. (*$P<.05$, **$P<.01$, ***$P<.001$, compared with corresponding control).

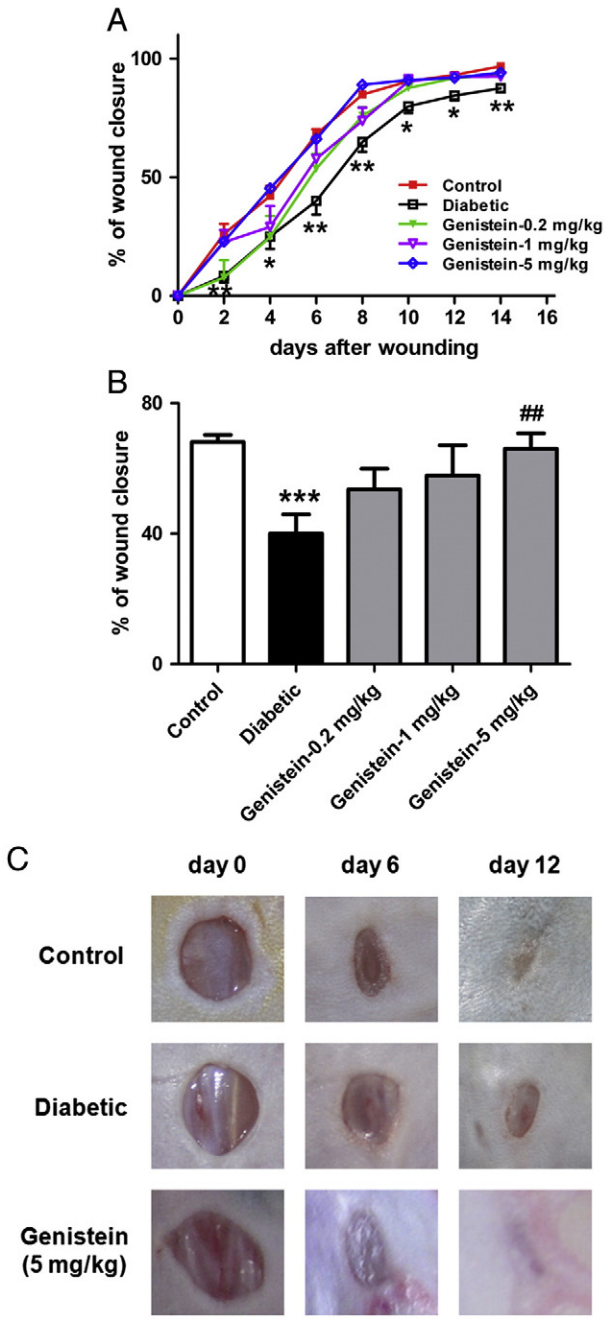

Fig. 1. (A) Wound closure rate in control, diabetic mice and diabetic mice treated with 0.2, 1 and 5 mg/kg genistein. Data were expressed as mean ± S.E.M. $n = 7–13$ for each time period. $^{*}P<.05$, $^{**}P<.01$ vs. diabetic mice treated with 5 mg/kg genistein. (B) Wound closure rate in control, diabetic mice and diabetic mice treated with genistein on day 6. Data were expressed as mean ± S.E.M. $n = 7–13$ per group. $^{***}P<.001$ vs. control mice, $^{\#\#}P<.01$ vs. diabetic mice. (C) Representative wounds on days 0, 6 and 12 after injury were shown for each group.

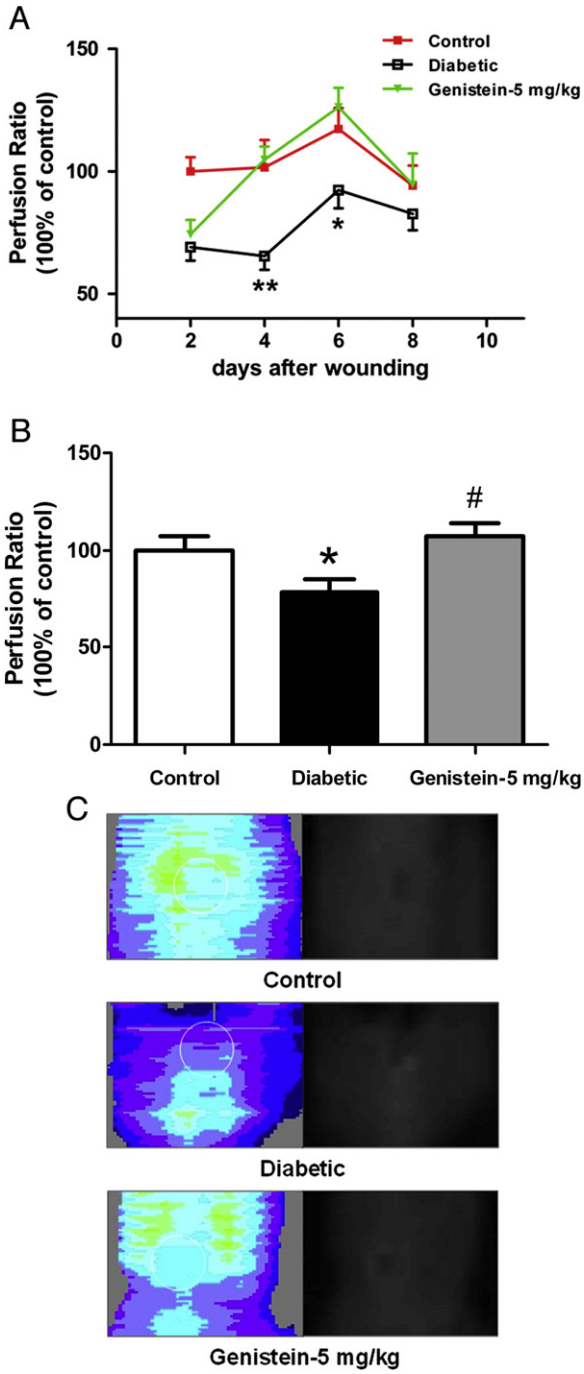

Fig. 2. (A) Wound perfusion in control, diabetic mice and diabetic mice treated with 5 mg/kg genistein was assessed by color laser Doppler on days 2, 4, 6 and 8 after wounding. Data were expressed as mean ± S.E.M. $n = 6–8$ for each time period. $^{*}P<.05$, $^{**}P<.01$ vs. diabetic mice treated with 5 mg/kg genistein. (B) Wound perfusion in control, diabetic mice and diabetic mice treated with 5 mg/kg genistein on day 6. Data were expressed as mean ± S.E.M. $n = 6–8$ per group. $^{*}P<.05$ vs. control mice, $^{\#}P<.05$ vs. diabetic mice. (C) Color scale illustrates variations in blood flow from high flow (bright pixels) to minimal flow (dark pixels); wound area was encircled. Phase-contrast images show the morphology of wound.

untreated diabetic mice compared to the nondiabetic controls. Genistein administration resulted in a significant increase of cutaneous nitrite level in diabetic mice compared to untreated diabetic mice ($P<.05$) (Fig. 4C).

3.7. Effects of genistein on NOS activity in diabetic mice

We further evaluate whether cNOS and iNOS activities were altered by genistein treatment. In untreated diabetic mice, cutaneous cNOS activity was decreased; in contrast, iNOS activity was significantly increased. Genistein treatment markedly suppressed iNOS activity in diabetic mice and led to augmentation of cNOS activity (Fig. 4D and E).

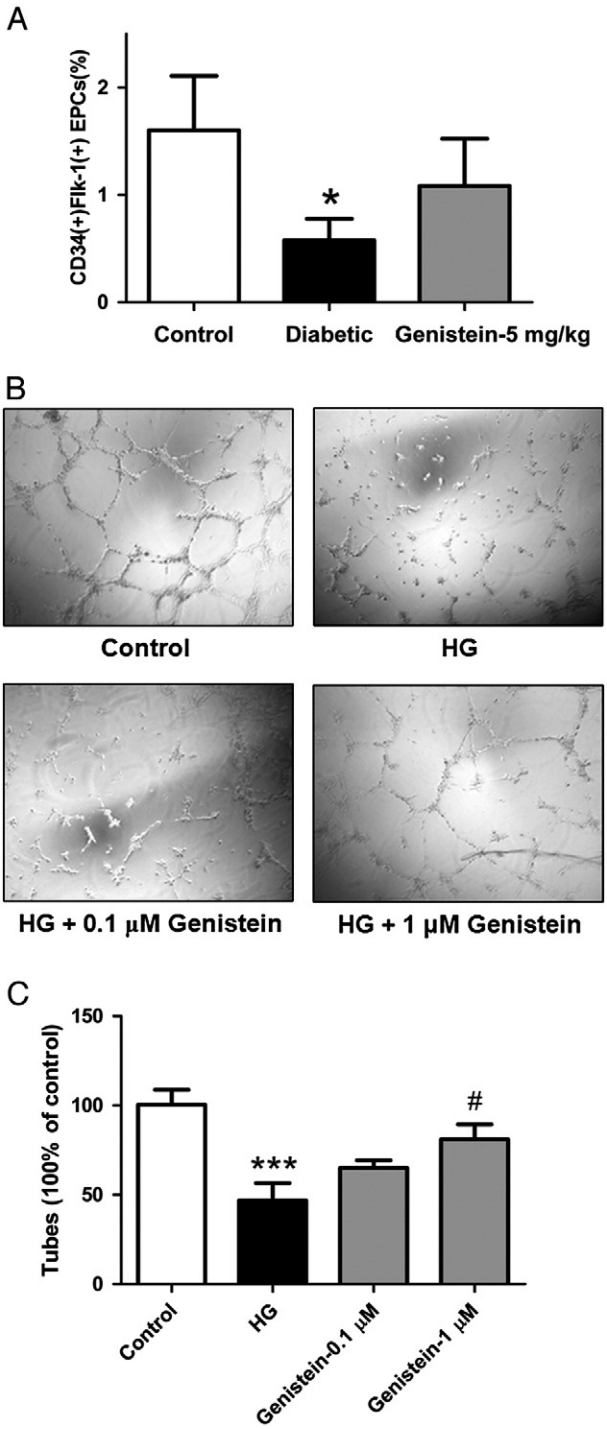

Fig. 3. (A) Circulating EPC level in control, diabetic mice and diabetic mice treated with 5 mg/kg genistein. Sca-1 and Flk-1 double-positive cells from each group were assessed by flow cytometry. Data were expressed as mean ± S.E.M. $n = 4–6$ per group. $^{*}P<.05$ vs control mice. (B) and (C) *In vitro* capillary tube formation analysis. HUVECs were incubated in medium containing 5.5 mM glucose (Control), 30 mM glucose (HG) or 30 mM glucose treated with 0.1 μM or 1 μM genistein for 8 h. Representative images were shown; statistical data were shown as histograms at the lower panels. Data were expressed as mean ± S.E.M. and were shown as a percentage of the control. $n = 5–6$ per group. $^{***}P<.001$ vs. control, $^{\#}P<.05$ vs. cells incubated with HG.

3.8. Effects of iNOS inhibition on wound healing in diabetic mice

Untreated diabetic mice showed significantly higher cutaneous iNOS activity; we next determined the effect of iNOS inhibition on impaired wound healing in diabetic mice. iNOS selective inhibitor L-NIL at 1 mg/kg and 3 mg/kg was used. Treatment of diabetic mice with L-NIL at different doses did not change either body weight or blood glucose (data not shown). As shown in Fig. 5A, compared with untreated diabetic mice, the rate of wound closure in diabetic mice treated with 3 mg/kg L-NIL was significantly higher than that of diabetic control mice beginning on day 2 and continuing through day 14 except on day 4 (66.09% ± 5.26% vs. 45.03% ± 5.64% on day 6, $P<.05$). Treatment with 1 mg/kg L-NIL significantly accelerated the rate of wound closure by 6 days and continued through day 10 (68.90% ± 3.63% vs. 45.03% ± 5.64% on day 6, $P<.05$).

3.9. Effects of iNOS inhibition on cutaneous $O_2^{\cdot -}$ and nitrotyrosine formation in diabetic mice

To determine effects of iNOS inhibition on cutaneous oxidative stress in diabetes, we estimated cutaneous $O_2^{\cdot -}$ production and nitrotyrosine formation. Cutaneous $O_2^{\cdot -}$ level and nitrotyrosine formation in diabetic mice were abolished by L-NIL treatment (Fig. 5B and C).

3.10. Effects of genistein on cutaneous SIRT1 and FoxO1 levels in diabetic mice

It has been reported that FoxO1 promotes iNOS-dependent NO-peroxynitrite generation in endothelial cells [24]. SIRT1, initially identified as a longevity gene, has recently been implicated as a novel modulator of vascular endothelial cell homeostasis, playing a key role in angiogenesis through the deacetylation of FoxO1. In untreated diabetic mice, cutaneous SIRT1 and FoxO1 levels were increased; in contrast, ac-FoxO1 was significantly decreased. Treatment with genistein suppressed the SIRT1 and FoxO1 levels but increased ac-FoxO1 in a dose-dependent manner (Fig. 6).

4. Discussion

The present study provides first evidence that *in vivo* genistein is able to (a) ameliorate the wound healing delay and impaired wound angiogenesis in STZ-induced type 1 diabetic mice, (b) significantly increase cutaneous NO and decrease $O_2^{\cdot -}$ production, and prevent the increased cutaneous iNOS activity and (c) significantly normalize SIRT1 and FoxO1 expression levels and ac-FoxO1.

The soy isoflavone genistein has various biologic functions. Recent studies suggest a potential antidiabetic role for genistein in animals and humans. It has been reported that genistein prevents diabetes onset by elevating insulin levels in nonobese diabetic mice [25]. *In vitro*, genistein, at physiologically achievable concentrations in individuals consuming soy products, potentiated rapid glucose-stimulated insulin secretion both in insulin-secreting cell lines and in mouse pancreatic islets [26]. Consistent with previous studies [27], our current result suggested that genistein could not reverse STZ-induced destruction of pancreatic β-cells since there is no influence of genistein treatment on blood glucose increase and body weight loss in STZ-induced type 1 diabetic mice.

Wound healing consists of three sequential phases: inflammation, proliferation and remodeling [28]. Inflammation and angiogenesis are two linked processes [29]. In the current study, the lower doses of genistein (0.2 and 1 mg/kg) were shown to be less effective than the higher dose (5 mg/kg) on wound healing tested before day 6 after wounding, and then the difference in the extent of wound closure between various doses of genistein was reduced. The mean perfusion rate around the wound peaked on day 6, indicating increased angiogenesis in wound tissue. Patients with diabetes are characterized by a reduction in angiogenesis, which is an important part of the proliferative phase of healing. Limited penetration of new blood

vessels into the wound would restrict entry of inflammatory cells, and high amounts of distinct angiogenic factors produced by immune cells are limited. Our present study found that genistein administration significantly accelerated the delayed wound healing in diabetic mice by improving angiogenesis. Serving as a tyrosine kinase inhibitor, genistein exerted antitumor and antiangiogenic activities. Adminis-

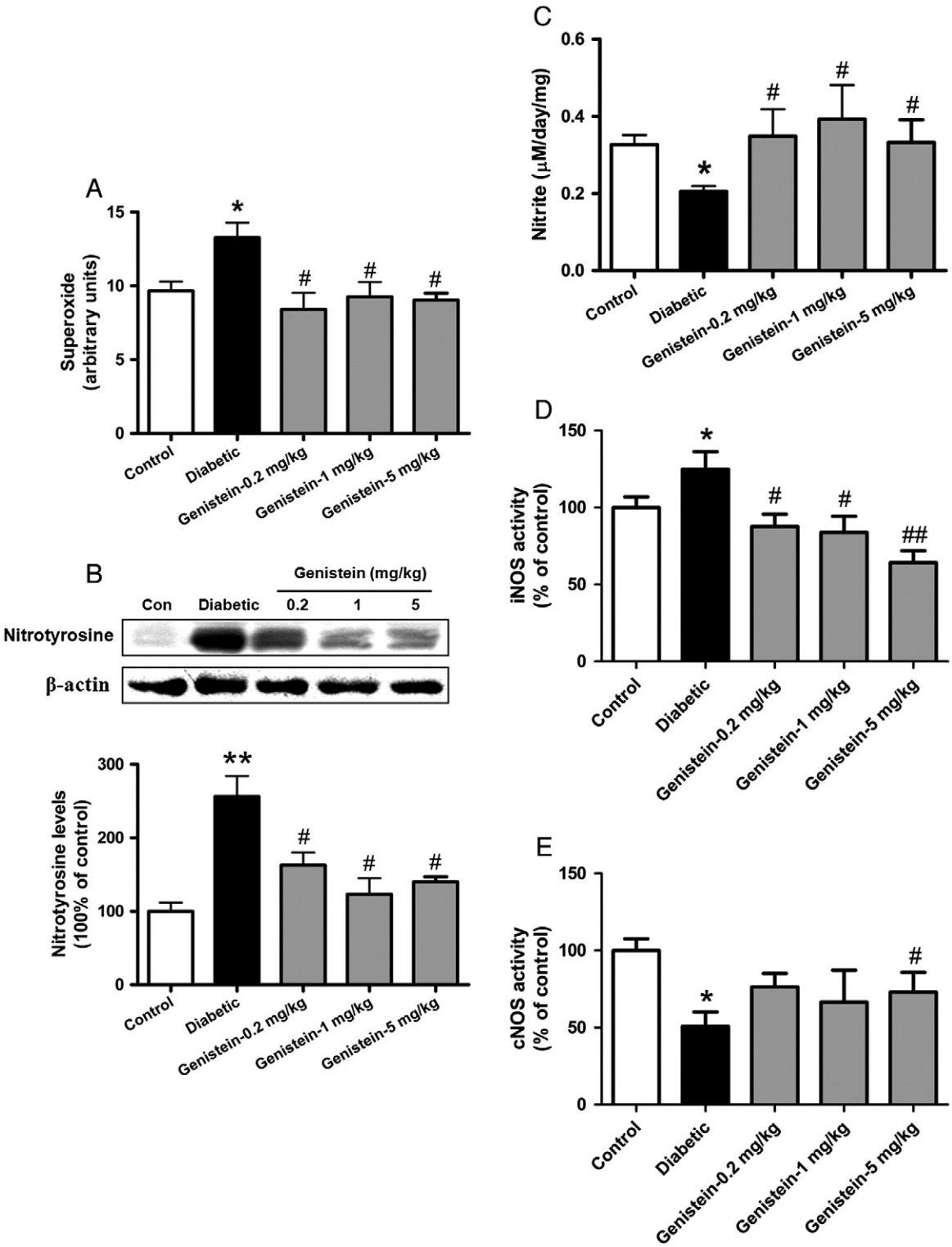

Fig. 4. (A) Effects of genistein on cutaneous $O_2^{\cdot -}$ production in diabetic mice. Statistical data of fluorescence measurement were presented as histograms. Data were expressed as mean ± S.E.M. $n = 6–9$ per group. $^{*}P<.05$ vs. control mice, $^{\#}P<.05$ vs. diabetic mice. (B) Effects of genistein on cutaneous nitrotyrosine formation in diabetic mice. Cutaneous nitrotyrosine was analyzed by Western Blot. Immunoblots of representative samples were shown; statistical data were shown as histograms at the bottom panels. Data were expressed as mean ± S.E.M. and were shown as a percentage of the control. $n = 4–12$ per group. $^{**}P<.01$ vs. control mice, $^{\#}P<.05$ vs. diabetic mice. (C) Cutaneous nitrite level on wound closure in control, diabetic mice and diabetic mice treated with genistein. Data were expressed as mean ± S.E.M. $n = 4–10$ per group. $^{*}P<.05$ vs. control mice. (D) Effects of genistein on cutaneous iNOS activity in diabetic mice. Data were expressed as mean ± S.E.M. $n = 6–11$ per group. $^{*}P<.05$ vs. control mice, $^{\#}P<.05$ vs. diabetic mice and $^{\#\#}P<.01$ vs. diabetic mice. (E) Effects of genistein on cutaneous cNOS activity. Data were expressed as mean ± S.E.M. $n = 5–11$ per group. $^{*}P<.05$ vs. control mice.

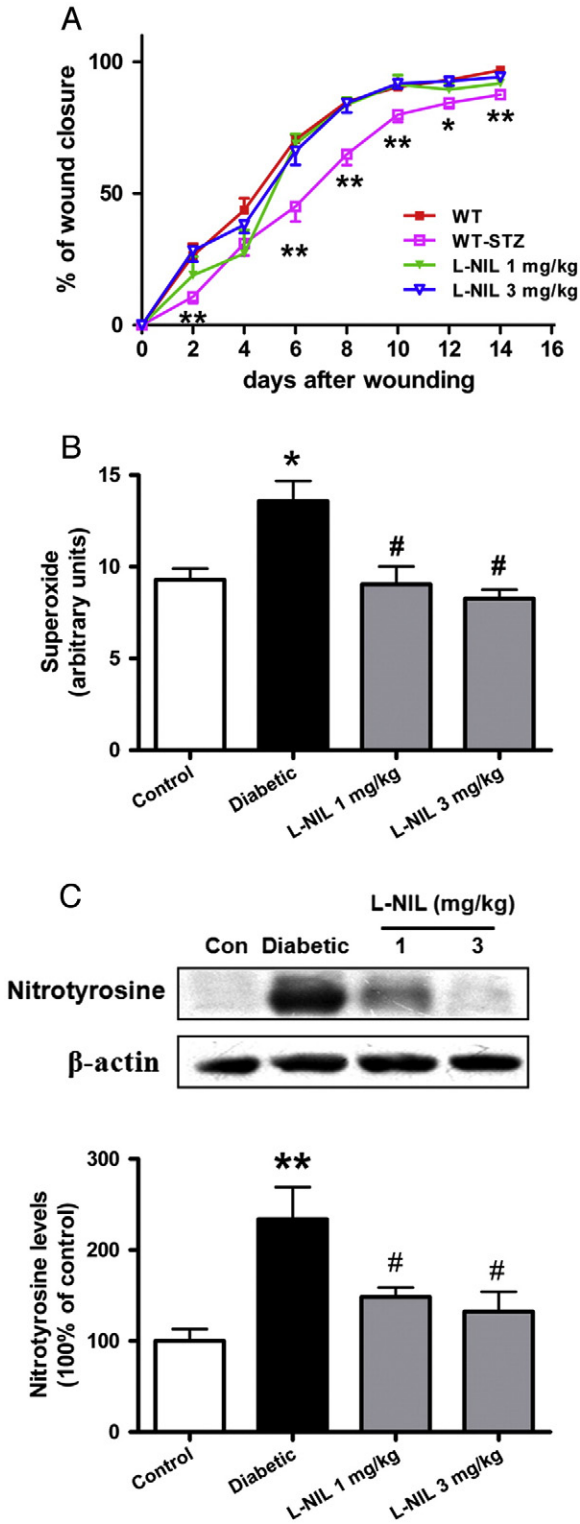

Fig. 5. (A) Wound closure rate in control, diabetic mice and diabetic mice treated with 1 and 3 mg/kg L-NIL. Data were expressed as mean ± S.E.M. $n = 11–15$ for each time period. $^{*}P<.05$, $^{**}P<.01$ vs. diabetic mice treated with 3 mg/kg L-NIL. (B) Effects of L-NIL on cutaneous $O_2^{\cdot -}$ production in diabetic mice. Statistical data of fluorescence measurement were presented as histograms. Data were expressed as mean ± S.E.M. $n = 6–10$ per group. $^{*}P<.05$ vs. control mice, $^{\#}P<.05$ vs. diabetic mice. (C) Effects of L-NIL on cutaneous nitrotyrosine formation in diabetic mice. Cutaneous nitrotyrosine was analyzed by Western Blot. Immunoblots of representative samples were shown; statistical data were shown as histograms at the bottom panels. Data were expressed as mean ± S.E.M. and were shown as a percentage of the control. $n = 5–11$ per group. $^{**}P<.01$ vs. control mice, $^{\#}P<.05$ vs. diabetic mice.

tration of genistein at a dose of 10 mg/kg/day reduced tumor-induced angiogenesis in B16 melanoma and F3II mammary carcinoma mouse models [30]. It has been reported that genistein, *in vitro*, inhibited the growth of HUVECs and tube formation at a concentration 10 μM [31]. In our current study, genistein was found to protect diabetic angiogenesis both *in vivo* and *in vitro*. One explanation for the diverse effects of genistein is that genistein induces antiangiogenic effects at high concentrations (more than 10 μM) and allows for diabetic angiogenesis at lower concentrations. EPCs, circulating in the blood, can be recruited to sites of arterial injury and contribute to vasculogenesis. However, our current data do not suggest the involvement of genistein in up-regulation of the circulating number of EPCs, which indicates that genistein-induced wound healing may be independently associated with EPC mobilization.

Genistein is also a partial agonist at estrogen receptors and binds preferentially to the β-subtype of estrogen receptor (ERβ) [32,33]. Recently, Emmerson and colleagues [34] reported that genistein substantially accelerated wound repair, which was associated with a dampened inflammatory response in the ovariectomized mouse. Co-treatment with the estrogen receptor antagonist ICI 182780 had little impact on the anti-inflammation and healing-promoting effects of genistein [34]. We have also found that protective effects of genistein on diabetic wound healing in female diabetic mice could be abolished by ICI 182,780 (data not shown), indicating that the effects of genistein on diabetic wound healing were partially mediated by estrogen receptor. Moreover, genistein has also shown antioxidant potential in abolishing hyperglycemia-induced oxidative stress. Valsecchi found that genistein dampened diabetes-induced overproduction of ROS in several tissues, including sciatic nerve, brain, thoracic aorta and liver [27]. In addition, genistein protected HUVECs against high glucose- and H_2O_2-induced oxidative stress injury via the regulation of ERβ and Bcl-2/Bax expression and also by modulation of the cell-survival signaling pathway [15]. Therefore, the protective effects of genistein on wound healing might be partially mediated by antioxidative stress via activation of ERβ.

Superoxide is generated by several metabolic and enzymatic sources within the cell under diabetic conditions, including mitochondrial respiration, xanthine oxidase, cyclooxygenases and lipoxygenases, nicotinamide adenine dinucleotide phosphate oxidases and uncoupled nitric oxide synthase(s) [35]. Our previous studies have shown in the skin of diabetic mice that when the NOS cofactor BH4 is not replete, cNOS activity and nitrite levels were decreased, paralleled by increased $O_2^{\cdot -}$ production and iNOS activity. In the absence of sufficient BH4 level, eNOS becomes uncoupled from arginine oxidation and produces $O_2^{\cdot -}$ rather than NO. Compared with cutaneous cNOS activity, iNOS activity was enhanced in diabetic mice, indicating that iNOS was another source of superoxide. Impaired endothelial function has been reported to be associated with increased expression of iNOS and nitrotyrosine and decreased expression of eNOS in STZ-diabetic rats [36]. Chronic inhibition of iNOS normalized the expression of eNOS and nitrotyrosine levels and ameliorated cardiovascular abnormalities in STZ-diabetic rats [37]. Here, we provide evidence that inhibition of iNOS accelerated impaired wound healing and attenuated cutaneous $O_2^{\cdot -}$ production and nitrotyrosine formation in diabetic mice. Genistein treatment attenuated the iNOS activation. In support of this possibility, by using the superoxide spin trap DEPMPO and the NO spin trap Fe-MGD complex, Huisman proved that BH4 cofactor-deficient iNOS is not an NO-producing enzyme but a superoxide-producing enzyme [38]. Moreover, genistein has been reported to significantly suppress iNOS in thoracic aorta of diabetic animals. One possibility is that the enhanced iNOS activation was due to inflammation, as the role of macrophage-derived iNOS is quantitatively prominent. However, an inflammatory phase was found to have occurred during the first 3

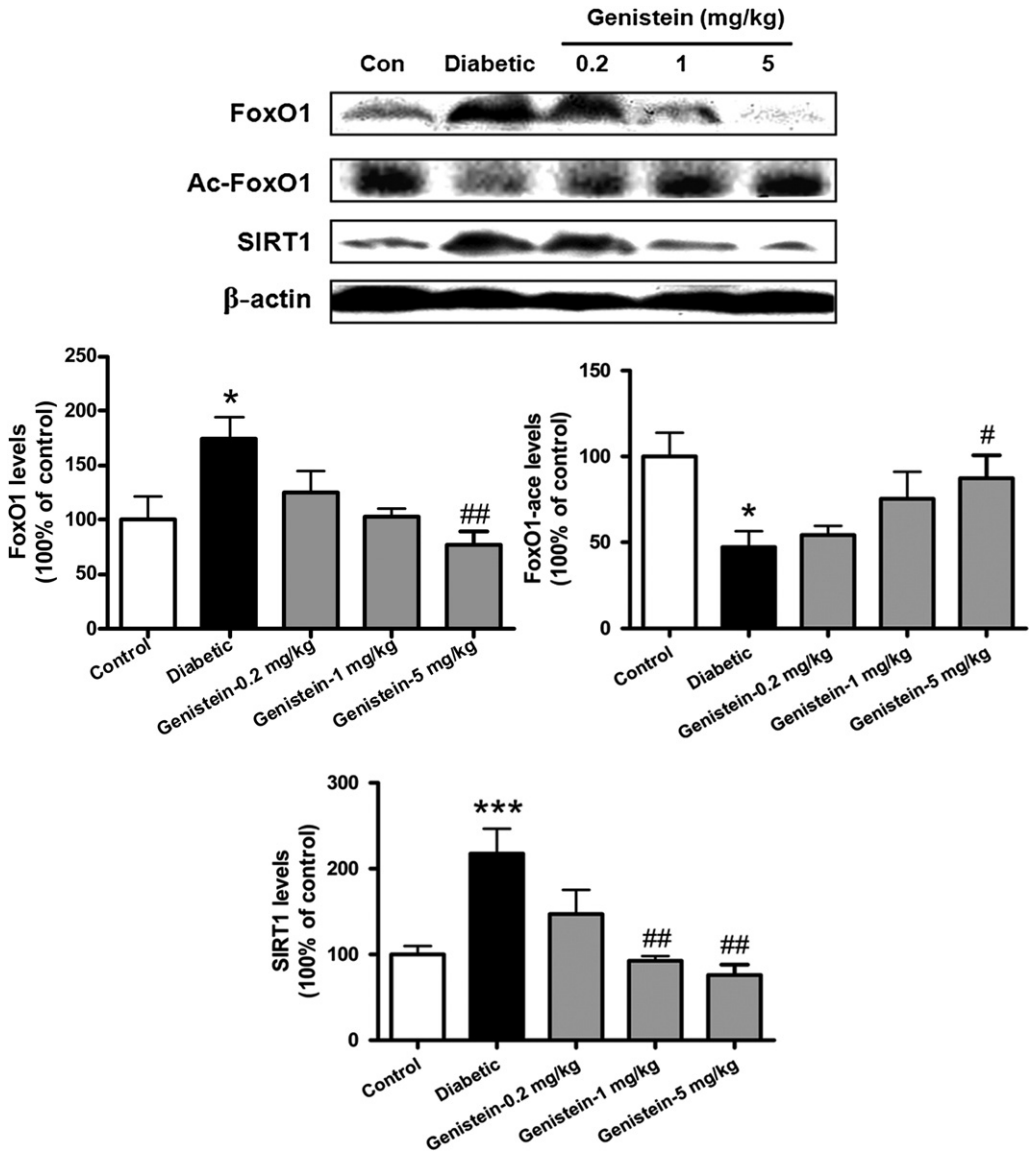

Fig. 6. Effects of genistein on cutaneous SIRT1 and FoxO1 levels in diabetic mice. Cutaneous FoxO1, ac-FoxO1 and SIRT1 were analyzed by Western Blot. Immunoblots of representative samples were shown; statistical data were shown as histograms at the bottom panels. Data were expressed as mean ± S.E.M. and were shown as a percentage of the control. $n = 5–12$ per group. $^{*}P<.05$ vs. control mice, $^{\#}P<.05$ vs. diabetic mice.

days after wound infliction, and iNOS was kept highly activated until wound closure under diabetic conditions.

Recently, it has been reported that the forkhead protein FoxO1 is activated by high glucose and oxidative stress in endothelial cells so as to promote iNOS [24]. In addition, FoxO1 activation mimics the effects of glucose and oxidative stress on ROS and nitrotyrosine production [24]. In light of this report, our results show that cutaneous FoxO1 levels increased in diabetic mice and that treatment with genistein suppressed FoxO1 levels in a dose-dependent manner, which mirrored the increase in ac-FoxO1. SIRT1, a member of the sirtuin family of NAD + -dependent protein deacetylases, mediates deacetylation of FoxO1. In the current study, we showed consistently that genistein administration led to the inhibition of SIRT1 expression levels in a dose-dependent manner. The association among oxidative stress, FoxO1 and SIRT1 is still controversial. FoxO1 has been shown to mediate an autofeedback loop regulating SIRT1 expression, and FoxO1 depletion by siRNA reduced SIRT1 expression at both the messenger RNA and protein levels in vascular smooth muscle cells and HEK293 cells [39]. In mouse embryonic stem cells, SIRT1 deficiency down-regulated the PTEN/JNK/FoxO1 pathway to block ROS-induced apoptosis [40]. Recently, SIRT1 was reported as a negative regulator of β-cell proliferation [41]. However, studies from other laboratories have demonstrated that high glucose evoked a decrease in SIRT1 expression in human endothelial cells [42], and only a few studies have investigated relationships among genistein, FoxO1 and SIRT1. Genistein reduced endogenous SIRT1 expression in PC cells [43] and increased SIRT1 expression in renal proximal tubular cells [44]. One possible explanation for this is that SIRT1 was differentially regulated during the time course of glucose exposure in different cells. SIRT1 reactivity was regulated differently in various tissues. Future studies need to address the exact molecular mechanism(s) involved in the regulation by genistein of FoxO1 and SIRT1.

In summary, results of the present study demonstrate that soy isoflavone genistein has ameliorated the wound healing delay in type 1 diabetes via suppressing cutaneous iNOS activity (Fig. 7). This finding could potentially provide a mechanistic basis for genistein and

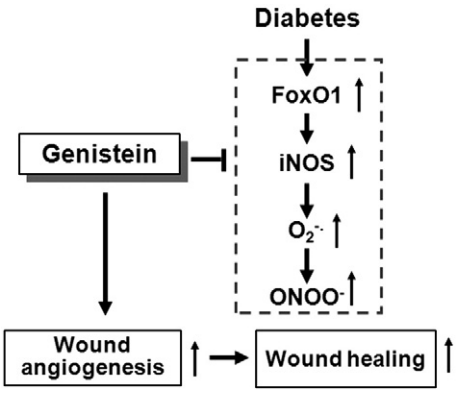

Fig. 7. Schematic illustration of possible mechanisms on genistein regulation of oxidative stress and wound healing in type 1 diabetes. Genistein ameliorated the wound healing delay by decreasing cutaneous $O_2^{\cdot-}$ production and nitrotyrosine formation and increasing wound angiogenesis. In addition, genistein maintained iNOS activities to normal with elevated NO level in cutaneous tissue of diabetic mice.

iNOS inhibition as a potential therapeutic strategy to refractory wound healing in diabetes.

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