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Folic Acid Promotes Wound Healing in Diabetic Mice by Suppression of Oxidative Stress

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Summary The aim of this study was to investigate the effects of folic acid on impaired wound healing in diabetic mice. Male mice were divided into three groups: group 1, the non-diabetic mice (control); group 2, the streptozotocin (STZ)-induced type 1 diabetic mice; and group 3, the diabetic mice that received a daily dose of 3 mg/kg folic acid via oral gavage. Full-thickness excision wounds were created with 8-mm skin biopsy punches. Each wound closure was continuously evaluated until the wound healed up. Wound healing was delayed in diabetic mice compared with the non-diabetic mice. There were significantly reduced levels of hydroxyproline content (indicator of collagen deposition) and glutathione in diabetic wounds, whereas levels of lipid peroxidation and protein nitrotyrosination were increased. Daily supplementation with folic acid restored diabetes-induced healing delay. Histopathology showed that folic acid supplementation accelerated granulation tissue formation, proliferation of fibroblasts, and tissue regeneration in diabetic mice. Interestingly, folic acid alleviated diabetes-induced impaired collagen deposition in wounds. Moreover, folic acid significantly decreased levels of lipid peroxidation, protein nitrotyrosination and glutathione depletion in diabetic wounds. In conclusion, our results indicate that folic acid supplementation may improve impaired wound healing via suppressing oxidative stress in diabetic mice.

Key Words folic acid, diabetes, wound healing, oxidative stress, collagen

The prevalence of diabetes mellitus is rapidly increasing around the world, and diabetic complications account for the majority of disease burden among diabetic patients (*1*, *2*). Refractory skin wound healing in diabetic patients represents a challenging medical and societal problem leading to a substantial reduction in quality of life (*3*). Previous studies demonstrated that sustained hyperglycemia induces excessive production of reactive oxygen species (ROS) (*4*). Excess ROS causes multiple cellular components damage, including excessive oxidation of lipids and proteins. Oxidative stress participates in the development of diabetic wound complications (5). Refractory skin wound healing occurs as a consequence of excessive ROS production. Furthermore, various factors in the diabetic wound, including inflammatory response, impaired angiogenic response, reduced quantity of granulation tissue, decreased collagen deposition and impaired vascular endothelial function, are involved in impaired wound healing (*6*). According to in vitro studies, increased oxidative stress activates collagenase and decreases fibrillar collagen synthesis in fibroblasts, which results in decreased collagen deposition (*7*, *8*). Moreover, a previous study showed that collagen deposition in acute wounds is impaired in type 1 diabetes, potentially due to a decreased fibroblast proliferation (*9*). Impaired collagen deposition is involved in delayed wound healing in diabetes (*10*, *11*).

Enhancement of antioxidant capacity plays an important role in the prevention and control of diabetic complications. Folate is a water-soluble B-complex vitamin. It is well known that exogenous folate intake is required for optimal health, growth, and development. As a cofactor of co-enzyme, folate plays key biological roles in a variety of physiological processes, particularly in one-carbon transfer reactions and the synthesis of nucleic acids and methionine regeneration (*12*). Nevertheless, other therapeutic effects of folic acid should be made the most use of. According to an early study, folic acid efficiently scavenges free radicals in vitro (*13*). A recent study showed that folic acid exhibited ability to reduce the markers of CCl4-induced liver injury through restoring oxidative stability in rats (*14*). However, whether folic acid supplementation ameliorates impaired wound healing in diabetic mice remains obscure. The aim of the

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Abbeviations: 3-NT, 3-nitrotyrosine; GSH, glutathione; ROS, reactive oxygen species; STZ, streptozotocin; TBARS, thiobarbituric acid-reactive substance.

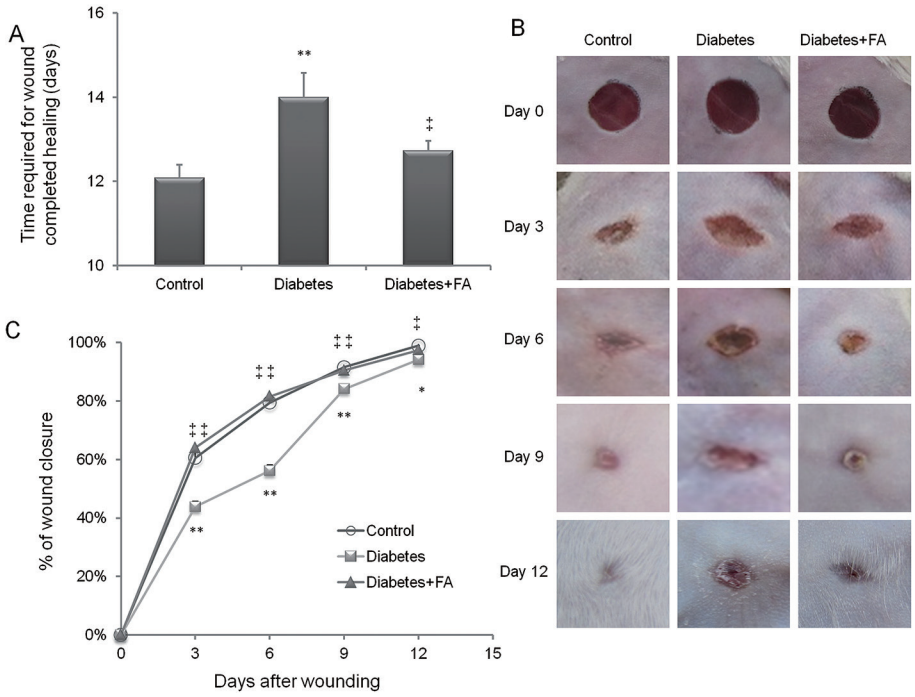

Fig. 1. Wound closure rate in control, diabetic mice and diabetic mice treated with folic acid (3 mg/kg). (A) The time required for complete wound healing. (B) Representative external photographs of the wounds at 0, 3, 6, 9 and 12 d after wounding in control, diabetic mice and diabetic mice treated with folic acid (3 mg/kg). (C) Percentage of wound closure at 3, 6, 9 and 12 d after wounding. The wound closure rate is presented as the ratio of the wound area to the initial wound size. Data are presented as means±SE. $n=16$ mice per group. $^{**}p<0.01$ vs control mice. $^{‡}p<0.05$, $^{‡‡}p<0.01$ vs diabetic mice.

present study was to investigate the effects of folic acid supplementation on impaired wound healing in diabetic mice. Our results showed that folic acid supplementation improves impaired wound healing at least partially through its anti-oxidative stress effects in diabetic mice.

MATERIALS AND METHODS

Animals. The ICR male mice (6–8 wk old) were purchased from Beijing Vital River, whose foundation colonies were all introduced from Charles River Laboratories, Inc (Wilmington, MA). The animals were maintained on a 12:12 h light/dark cycle and provided with food and water ad libitum. All procedures on animals were in strict accordance with Chinese legislation on the use and care of laboratory animals. This study protocol was approved by the Ethics Committee for Laboratory Animals of Anhui Medical University (LLSC20150347).

Experimental design. Mice were randomly assigned to three weight-matched experimental groups: (1) the first group (Control) remained as a wounded non-diabetic control group and was given phosphate buffered saline (PBS) intragastrically, (2) the second group (Diabetes) was a wounded diabetic group receiving PBS intragastrically, and (3) the third group (Diabetes+FA) was a wounded diabetic group intragastrically supplemented with 3 mg/kg of folic acid. The folic acid dosing regimen was based on a previous study exploring the effects of folic acid supplementation on oxidative stress in mice (*15*).

Sixteen mice from each group were continuously evaluated for wound healing until the wound healed completely. In addition, six mice from each group were sacrificed at days 0, 3, 6, 9, and 12 after wounding. Skin tissues around the wounds were collected for histopathological analysis and measurement of hydroxyproline concentration. Moreover, six mice from each group were sacrificed at days 6 after wounding. Skin tissues around the wounds were collected for measurement of glutathione (GSH), 3-nitrotyrosine (3-NT) and thiobarbituric acid-reactive substance (TBARS). Livers were collected for measurement of GSH.

Diabetic and wound models. For induction of type 1 diabetes, the animals were injected intraperitoneally with streptozotocin (STZ; Sigma Chemical, St. Louis, MO) dissolved in sterile citrate buffer (0.05 mol/L sodium citrate, pH 4.5, 50 mg/kg). STZ was administered for 5 d, as previously described (*16*, *17*). Blood glucose was measured from the mouse tail vein using an ACCU-CHEK Aviva blood glucose monitor (Roche, Mannheim, Germany). Diabetes was confirmed in mice by measuring the fasting blood glucose level (above 250 mg/dL) before use in this study.

The full thickness excisional wounds were created as previously described (*18*). Briefly, 1 wk after blood glucose reached 250 mg/dL, mice were anesthetized with a 10% chloral hydrate, and the dorsum was depilated with 8% Na_2S. Full-thickness dermal excisional wounds were placed on the dorsomedial back of each animal using an 8-mm punch biopsy instrument. Mice were divided into three groups and were respectively given intragastrically

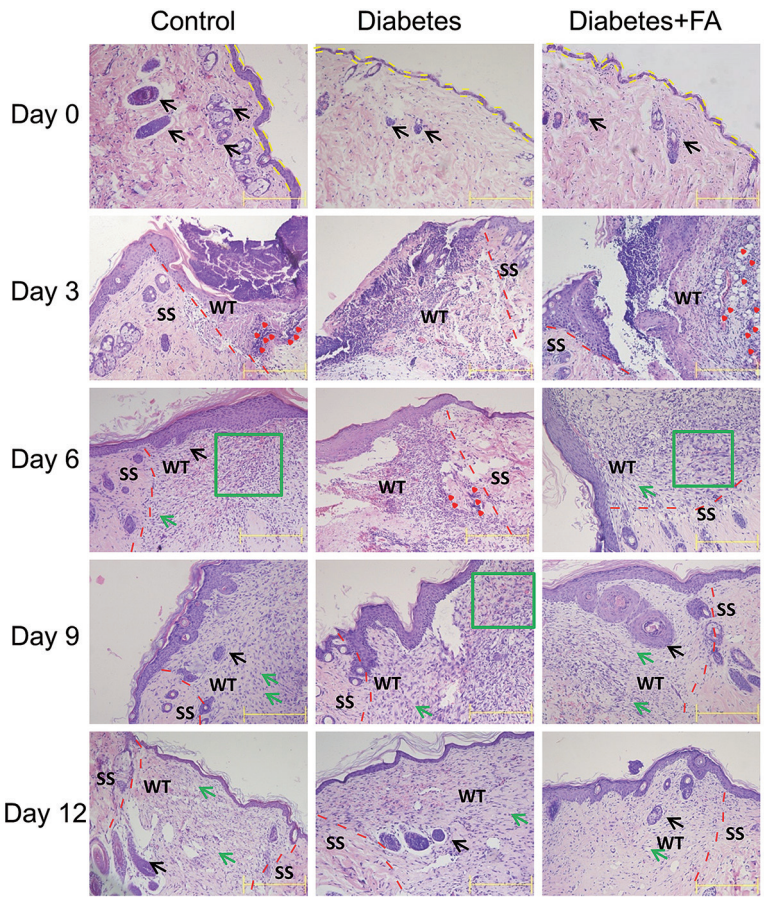

Fig. 2. Representative photomicrographs of cutaneous histological specimens by H&E staining at 0, 3, 6, 9 and 12 d after wounding in control, diabetic mice and diabetic mice treated with folic acid (3 mg/kg). Yellow dotted lines indicate the edge of the epidermis layer. Red dotted lines indicate the boundary between the region of wound tissue (WT) and the region of surrounding skin (SS). Black arrows indicate the skin appendage. Green arrows indicate the fibroblast. Red triangles indicate massive inflammatory cells. Green boxes indicate granulation tissue. Original magnification, ×100. Scale bar=500 μm.

3 mg/kg of folic acid or PBS vehicle once daily after the date of wounding.

Evaluation of wound healing. Wound healing was evaluated until the wound healed completely. The time required for complete wound healing was recorded. The procedure for measuring closure was previously described (*19*). Wounds were digitally photographed every 3 d. Image-Plus Pro 6.0 software was used to calculate the wound areas. The initial wound area was recorded and used as a reference. The wound closure rate is presented as the ratio of the wound closure area to the initial wound area.

Histopathological analysis. Six mice from each group were anesthetized and sacrificed at days 0, 3, 6, 9, and 12. Skin tissues from the affected area and 3 mm of the surrounding skin were excised in full depth and collected for histopathological analysis by hematoxylin and eosin (H&E) staining. At day 0, normal skin tissues of three groups were obtained using an 8 mm biopsy punch for comparisons. Skin tissues were fixed in 4% paraformaldehyde and embedded in paraffin according to the standard procedure. Skin tissue slides (5 μm) were stained with H&E for histopathological analysis. Computational analysis was performed for quantitative assessment. The thickness of the epidermis layer, and the number of skin appendages, inflammatory cells and fibroblasts were measured in three different areas at both ends as well as in the middle of the dermis. These quantitative counts were made independently by two observers blinded to treatment, and the counts were compared to ascertain that no more than a 10% difference existed between counts from the two observers on the same tissue sections.

Assay for hydroxyproline content. Six mice from each group were anesthetized and sacrificed at days 3, 6, 9, and 12. Skin tissues around the wounds were excised in full depth and collected for measurement of hydroxyproline concentration, which was directly related to the total collagen content in skin tissue. Hydroxyproline content was detected with a commercial hydroxyproline detection kit (Jiancheng Institute of Biotechnology, Nanjing, China) following its manufacturer's instructions.

Lipid peroxidation assay. Lipid peroxidation was quantified by measuring TBARS as described previously (*20*). Skin tissues were homogenized in 50 mmol/L Tris-HCl buffer (pH 7.4) containing 180 mmol/L KCl,

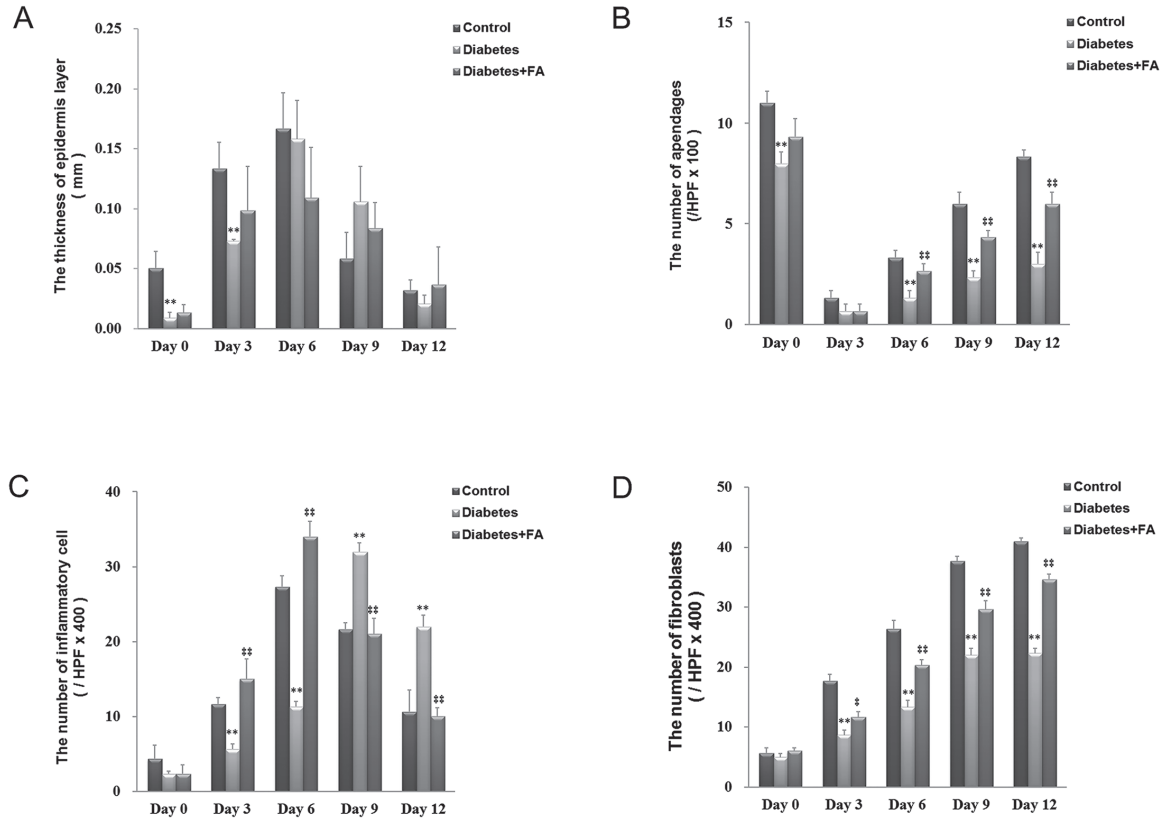

Fig. 3. Histological quantitative analysis of wound healing at 0, 3, 6, 9 and 12 d after wounding in control, diabetic mice and diabetic mice treated with folic acid (3 mg/kg). (A) The thickness of the epidermis layer. (B) The number of skin appendages per high power field (HPF) (×100). (C) The number of inflammatory cells per HPF (×400). (D) The number of fibroblasts per HPF (×400). $n=6$ per group at 0, 3, 6, 9 and 12 d after wounding, respectively. $^{*}p<0.05$, $^{**}p<0.01$ vs control mice. $^{‡}p<0.05$, $^{‡‡}p<0.01$ vs diabetic mice.

10 mmol/L EDTA, and 0.02% butylated hydroxytoluene. To 0.2 mL of the tissue homogenate, 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid, 1.5 mL of 0.9% thiobarbituric acid, and 0.6 mL of distilled water were added and vortexed. The reaction mixture was placed in a water bath at 95˚C for 1 h. After cooling on ice, 1.0 mL of distilled water and 5.0 mL of butanol/pyridine mixture (15 : 1, volume/volume) were added and vortexed. After centrifugation at 10,000 ×g for 10 min, absorbance of the resulting lower phase was determined at 532 nm. The TBARS concentration was calculated using 1,1,3,3-tetraethoxypropane as a standard and presented as nanomole TBARS per milligram of protein.

Immunoblot. Skin tissues were homogenized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mM PMSF) supplemented with a 1% cocktail of protease inhibitors (P8340, Sigma) and centrifuged at 14,000 ×g for 10 min at 4˚C. The supernatant was collected and protein concentrations were determined with the BCA protein assay reagents. For immunoblot, the same amount of protein (30–50 μg per lane) was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% defatted milk in Dulbecco phosphate-buffered saline (DPBS) for 2 h at room temperature. The membranes were washed and incubated with mouse anti-3-NT (1 : 1,000 dilution) for 3 h. Beta-actin was used as a loading control. The membranes were washed with DPBS containing 0.05% Tween-20 and then incubated with horse radish peroxidase-linked secondary antibody in 5% defatted milk in DPBS, 1 : 50,000 dilutions of goat anti-mouse antibody (Millipore, Billerica, MA) for 2 h. The membranes were washed again and blots were developed using an enhanced ECL detection kit. Relative quantification of protein was calculated after normalization to loading control protein by densitometric analysis with Image-Pro Plus 6 software.

Assay for glutathione content. Samples of liver and skin tissue were homogenized in iced-cold normal saline. The homogenates were centrifuged at 4,000 ×g for 10 min at 4˚C. The supernatants were collected and protein concentrations were determined with the BCA protein assay reagents. Proteins of 0.4 mL homogenates were precipitated by the addition of 0.2 mL of 20% trichloroacetic acid. The protein precipitate was separated from the remaining solution by centrifugation at 4,000 ×g at 4˚C for 10 min. The supernatant (0.1 mL) was combined with 4.4 mL of 300 mM Na_2HPO_4 buffer. Then 0.5 mL dithio-bis-nitrobenzoic acid (DTNB) (0.04%, w/v; 40 mg DTNB in 100 mL of 1% sodium

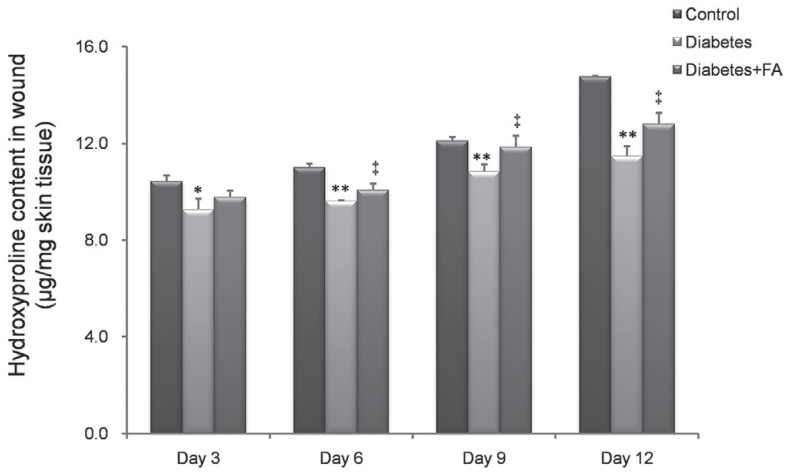

Fig. 4. Effects of folic acid on hydroxyproline content in diabetic mice. The hydroxyproline contents of skin tissue around the wounds were measured at 0, 3, 6, 9 and 12 d after wounding in control, diabetic mice and diabetic mice treated with folic acid (3 mg/kg). Data are presented as means±SE. $n=6$ mice per group. $^{*}p<0.05$, $^{**}p<0.01$ vs control mice. $^{‡}p<0.05$ vs diabetic mice.

citrate) was added to the blank and sample. Absorbance of the sample was read against the blank at 412 nm within 5 min. GSH content was determined using a calibration curve prepared with a reference standard. All GSH assay experiments were performed in triplicate. GSH values are expressed as nanomole GSH per milligram of protein.

Statistical analysis. Data are presented as means± SEM. Statistical analysis of the data was performed using ANOVA and the Student-Newmann-Keuls test. Values of $p<0.05$ were considered statistically significant.

RESULTS

Effects of folic acid on wound healing in diabetic mice

As expected, diabetes resulted in wound healing delay in mice. As shown in Fig. 1A, the time required for wound healing was significantly prolonged in diabetic mice when compared with the control mice (14.0±2.0 d vs. 12.1±1.1 d, $p<0.01$). The rate of wound healing in diabetic mice was also significantly lower than that in normal control mice at 3, 6, 9, and 12 d after wounding (Fig. 1B and 1C). Remarkably, folic acid accelerated wound healing in diabetic mice. The time required for wound healing was significantly reduced to 12.7±0.8 d when diabetic mice were administered folic acid. Correspondingly, the rate of wound healing was increased at each time point in diabetic mice treated with folic acid.

Histopathological characteristics of the wounded skin at 0, 3, 6, 9, and 12 d after wounding are shown in Fig. 2 and Fig. 3. The thickness of epidermis layer and the number of skin appendages were obviously decreased in diabetic mice compared with control mice (day 0). The skin tissue obtained from the diabetic mice exhibited a thinning epidermis layer and loose arrangement of dermal tissues. On day 3, the control group exhibited massive inflammatory cell infiltration and fibroblast proliferation in the dermis layer. However, those were not observed in diabetic mice until day 6 after wounding. Folic acid supplementation improved the deferred

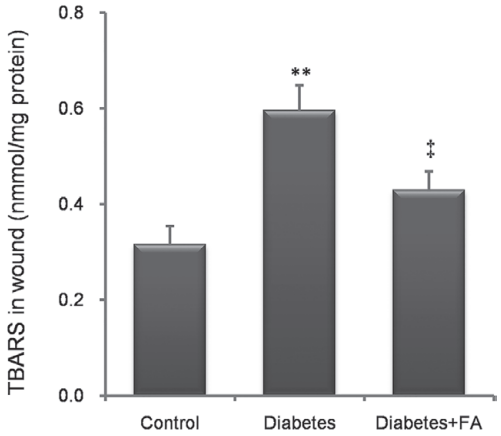

Fig. 5. Effects of folic acid on lipid peroxidation in diabetic mice. The levels of TBARS in skin tissues around the wounds were measured at 6 d after wounding in control, diabetic mice and diabetic mice treated with folic acid (3 mg/kg). Data are presented as means±SE. $n=6$ mice per group. $^{**}p<0.01$ vs control mice. $^{‡}p<0.05$ vs diabetic mice.

inflammatory cell infiltration and fibroblast proliferation in diabetic mice. Moreover, granulation tissue formation was obvious in the wounds of control mice and diabetic mice treated with folic acid at day 6 and in the wounds of diabetic mice at day 9. In summary, folic acid supplementation accelerated fibroblast proliferation, granulation tissue formation, and tissue regeneration in diabetic mice (Fig. 2 and Fig. 3).

Effects of folic acid on wound collagen content in diabetic mice

Hydroxyproline concentration is directly related to total collagen content. The effects of folic acid on wound hydroxyproline in diabetic mice were analyzed. As shown in Fig. 4, the content of wound hydroxyproline was gradually increased in normal control mice after establishment of the wound model. As expected, the wound hydroxyproline concentration in diabetic mice

was significantly lower than that in normal control mice at 3, 6, 9, and 12 d after wounding. Interestingly, a significant increase in wound hydroxyproline concentration was observed in diabetic mice treated with folic acid at 6, 9, and 12 d after wounding when compared with the diabetic mice.

Effects of folic acid on lipid peroxidation in diabetic mice

The effects of folic acid on the TBARS level around the wounds on day 6 after wounding are presented in Fig. 5. Results showed that the TBARS level was significantly increased in the skin tissues of diabetic mice when compared with those of the normal control mice. Interestingly, supplementation with folic acid inhibited an increase of TBARS level around the wounds in diabetic mice.

Effects of folic acid on 3-NT in diabetic mice

3-NT is a specific marker for protein nitrotyrosination. As shown in Fig. 6, the level of 3-NT in skin tissue around the wounds was significantly increased in diabetic mice. Folic acid supplementation significantly attenuated diabetes-induced protein nitrotyrosination in skin tissue around the wounds.

Effects of folic acid on GSH depletion in diabetic mice

The effects of folic acid on GSH depletion on day 6 after wounding are presented in Fig. 7. The levels of GSH in skin tissue around the wounds and in the liver were significantly reduced in diabetes mice. Interestingly, folic acid supplementation significantly inhibited diabetes-induced GSH depletion in skin and liver tissue.

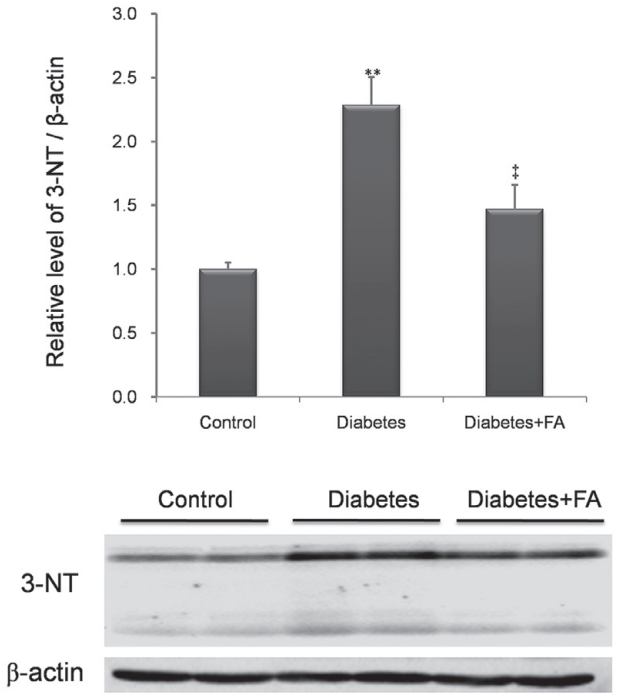

Fig. 6. Effects of folic acid on 3-NT in diabetic mice. The levels of 3-NT in skin tissues around the wounds were determined by immunoblot at 6 d after wounding. All experiments were repeated three times. Data are presented as means±SE. $n=6$ mice per group. $^{**}p<0.01$ vs control mice. $^{‡}p<0.05$ vs diabetic mice.

DISCUSSION

The present study investigated whether folic acid supplementation improved wound healing in diabetic mice. The results showed that diabetes can delay wound healing in mice. Interestingly, folic acid supplementation significantly increased the wound healing rate and reduced the time required for wound healing. These data demonstrate that folic acid supplementation accelerated wound healing in diabetic mice.

Wound healing is a complex process of tissue repair following injury, including the hemostasis and inflammatory phase, formation of granulation tissue and angiogenesis, proliferation of fibroblasts and collagen deposition, and finally remodeling by collagen cross-linking and scar maturation (*21*). Histological observations showed that formation of granulation tissue, proliferation of fibroblasts, and finally remodeling by collagen cross-linking were deferred in the wound area of diabetic mice. We further investigated the effects of folic acid on the wound healing process. Meaningfully, folic acid supplementation accelerated fibroblast proliferation, granulation tissue formation, and tissue regen-

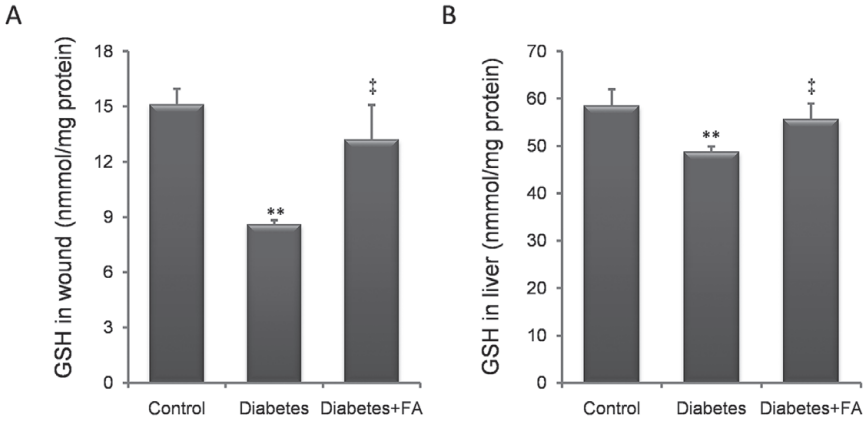

Fig. 7. Effects of folic acid on GSH depletion in diabetic mice. GSH in skin tissues around the wounds and in the liver were measured at 6 d after wounding in control, diabetic mice and diabetic mice treated with folic acid (3 mg/kg). (A) GSH in skin tissues around the wounds. (B) GSH in the liver. Data are presented as means±SE. $n=6$ mice per group. $^{**}p<0.01$ vs control mice. $^{‡}p<0.05$ vs diabetic mice.

eration. Collagen fiber is the framework of the dermis. Collagen is the major structural protein of the dermis and provides strength and integrity to the tissue matrix (*22*). The net deposition and stabilization of collagen in the wound area play a major role in wound healing. In this study, we found that the wound collagen content in diabetic mice was significantly lower than that in control mice at the same time points. Interestingly, a significant increase of wound collagen content was observed in diabetic mice treated with folic acid. Our results suggested that folic acid supplementation may promote wound healing in diabetic mice through accelerating collagen synthesis.

Increasing evidence demonstrates that hyperglycemia-induced overproduction of superoxide (O_2^-) is the first and key event in the activation of all pathways involved in the pathogenesis of diabetes complications (*23*). Increased ROS can overwhelm antioxidant defenses and then cause damage to DNA mutations/breaks, protein nitrotyrosination and lipid peroxidation. Oxidative damage can compromise cell survival, proliferation, differentiation, and metabolism; reduce antioxidants; and induce apoptosis (*24*). Additionally, according to an in vitro study, increased O_2^- formation can induce collagen degradation by up-regulating matrix metalloproteinases in fibroblasts and keratinocytes (*7*, *25*). Moreover, oxidative stress decreases collagen synthesis in fibroblasts (*8*, *26*). Previous studies demonstrated that oxidative stress was involved in impaired collagen deposition in diabetes-induced slow-healing wounds (*27*, *28*). In the present study, our results suggest that the impaired collagen deposition in the diabetic mice is associated with a significant increase in the lipid peroxidation level and protein nitrotyrosination.

Indeed, folic acid also exerts direct antioxidant effects. According to an in vitro study, folic acid can not only scavenge thiyl radicals efficiently but also repair these thiols at physiological pH. Subsequently, folic acid significantly inhibits radical damage in the cell (*13*). Recent studies also suggest that folic acid has potent biological effects, such as prevention of ethanol-induced kidney damage, carbon tetrachloride-induced hepatic injury, and diabetes-induced angiopathy, via its suppression of lipid and protein damage (*14*, *29*, *30*). We further investigated whether folic acid inhibits oxidative stress in wound tissue to increase collagen deposition. Interestingly, our data showed that folic acid supplementation significantly inhibited the elevation of lipid peroxidation and 3-NT, a marker of protein nitrotyrosination, in wound tissue. These data indicate that the protective effect of folic acid on diabetes-induced impaired collagen deposition can be partly attributed to its inhibition of oxidative stress.

Increased oxidative stress in diabetes is due to an imbalance between the production of ROS and the protection by cellular antioxidants (*4*, *5*, *31*). GSH plays important roles in antioxidant defense. GSH deficiency contributes to oxidative stress and is expected to exert a significant impact on physiological and metabolic functions of cellular membranes (*32*). Our data showed that GSH content was significantly decreased in wound and liver tissue in diabetic mice. Furthermore, in consistence with our previous results, the present study showed that folic acid supplementation significantly alleviated diabetes-induced GSH depletion in wound and liver tissue (*15*). These fingings further explain the antioxidant effects of folic acid in improving wound healing in diabetes.

CONCLUSIONS

The present study indicates that folic acid supplementation significantly ameliorates diabetes-induced impaired wound healing. Moreover, folic acid improves impaired collagen deposition in diabetes. Furthermore, the protection of folic acid against diabetes-induced impaired wound healing might be attributed to its antioxidative effects. We conclude that supplementation with folic acid has potential preventive and therapeutic utilities for preventing diabetes-induced impaired wound healing.

Acknowledgments

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Author contributions

M. Zhao and J. Zhou contributed equally to this work.

REFERENCES

1) GBD 2015 Mortality and Causes of Death Collaborators. 2016. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **388**: 1459–1544.

2) GBD 2015 Disease and Injury Incidence and Prevalence Collaborators. 2016. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **388**: 1545–1602.

3) Hicks CW, Selvarajah S, Mathioudakis N, Perler BA, Freischlag JA, Black JH, Abularrage CJ. 2014. Trends and determinants of costs associated with the inpatient care of diabetic foot ulcers. *J Vasc Surg* **60**: 1247–1254, 1254.e1–2.

4) Lan CC, Wu CS, Huang SM, Wu IH, Chen GS. 2013. High-glucose environment enhanced oxidative stress and increased interleukin-8 secretion from keratinocytes: new insights into impaired diabetic wound healing. *Diabetes* **62**: 2530–2538.

5) Pitocco D, Tesauro M, Alessandro R, Ghirlanda G, Cardillo C. 2013. Oxidative stress in diabetes: implications for vascular and other complications. *Int J Mol Sci* **14**: 21525–21550.

6) Brem H, Tomic-Canic M. 2007. Cellular and molecular basis of wound healing in diabetes. *J Clin Invest* **117**: 1219–1222.

7) Brenneisen P, Briviba K, Wlaschek M, Wenk J, Scharffetter-Kochanek K. 1997. Hydrogen peroxide (H2O2) increases the steady-state mRNA levels of collagenase/MMP-1 in human dermal fibroblasts. *Free Radic Biol Med* **22**: 515–524.

8) Siwik DA, Pagano PJ, Colucci WS. 2001. Oxidative stress regulates collagen synthesis and matrix metalloproteinase activity in cardiac fibroblasts. *Am J Physiol Cell Physiol* **280**: C53–60.

9) Black E, Vibe-Petersen J, Jorgensen LN, Madsen SM, Agren MS, Holstein PE, Perrild H, Gottrup F. 2003. Decrease of collagen deposition in wound repair in type 1 diabetes independent of glycemic control. *Arch Surg* **138**: 34–40.

10) Tan Q, Chen B, Yan X, Lin Y, Xiao Z, Hou X, Dai J. 2014. Promotion of diabetic wound healing by collagen scaffold with collagen-binding vascular endothelial growth factor in a diabetic rat model. *J Tissue Eng Regen Med* **8**: 195–201.

11) O'Loughlin A, Kulkarni M, Creane M, Vaughan EE, Mooney E, Shaw G, Murphy M, Dockery P, Pandit A, O'Brien T. 2013. Topical administration of allogeneic mesenchymal stromal cells seeded in a collagen scaffold augments wound healing and increases angiogenesis in the diabetic rabbit ulcer. *Diabetes* **62**: 2588–2594.

12) Fox JT, Stover PJ. 2008. Folate-mediated one-carbon metabolism. *Vitam Horm* **79**: 1–44.

13) Joshi R, Adhikari S, Patro BS, Chattopadhyay S, Mukherjee T. 2001. Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity. *Free Radic Biol Med* **30**: 1390–1399.

14) Ebaid H, Bashandy SA, Alhazza IM, Rady A, El-Shehry S. 2013. Folic acid and melatonin ameliorate carbon tetrachloride-induced hepatic injury, oxidative stress and inflammation in rats. *Nutr Metab (Lond)* **10**: 20.

15) Zhao M, Chen YH, Chen X, Dong XT, Zhou J, Wang H, Wu SX, Zhang C, Xu DX. 2014. Folic acid supplementation during pregnancy protects against lipopolysaccharide-induced neural tube defects in mice. *Toxicol Lett* **224**: 201–208.

16) Seifter E, Rettura G, Padawer J, Stratford F, Kambosos D, Levenson SM. 1981. Impaired wound healing in streptozotocin diabetes. Prevention by supplemental vitamin A. *Ann Surg* **194**: 42–50.

17) Tie L, Yang HQ, An Y, Liu SQ, Han J, Xu Y, Hu M, Li WD, Chen AF, Lin ZB, Li XJ. 2012. Ganoderma lucidum polysaccharide accelerates refractory wound healing by inhibition of mitochondrial oxidative stress in type 1 diabetes. *Cell Physiol Biochem* **29**: 583–594.

18) Luo JD, Wang YY, Fu WL, Wu J, Chen AF. 2004. Gene therapy of endothelial nitric oxide synthase and manganese superoxide dismutase restores delayed wound healing in type 1 diabetic mice. *Circulation* **110**: 2484–2493.

19) Ebaid H, Salem A, Sayed A, Metwalli A. 2011. Whey protein enhances normal inflammatory responses during cutaneous wound healing in diabetic rats. *Lipids Health Dis* **10**: 235.

20) Xu DX, Wang H, Zhao L, Ning H, Chen YH, Zhang C. 2007. Effects of low-dose lipopolysaccharide (LPS) pretreatment on LPS-induced intra-uterine fetal death and preterm labor. *Toxicology* **234**: 167–175.

21) Diegelmann RF, Evans MC. 2004. Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci* **9**: 283–289.

22) Ruszczak Z. 2003. Effect of collagen matrices on dermal wound healing. *Adv Drug Deliv Rev* **55**: 1595–1611.

23) Tabak O, Gelisgen R, Erman H, Erdenen F, Muderrisoglu C, Aral H, Uzun H. 2011. Oxidative lipid, protein, and DNA damage as oxidative stress markers in vascular complications of diabetes mellitus. *Clin Invest Med* **34**: E163–171.

24) Altavilla D, Saitta A, Cucinotta D, Galeano M, Deodato B, Colonna M, Torre V, Russo G, Sardella A, Urna G, Campo GM, Cavallari V, Squadrito G, Squadrito F. 2001. Inhibition of lipid peroxidation restores impaired vascular endothelial growth factor expression and stimulates wound healing and angiogenesis in the genetically diabetic mouse. *Diabetes* **50**: 667–674.

25) Kim KC, Kang SS, Lee J, Park D, Hyun JW. 2012. Baicalein attenuates oxidative stress-induced expression of matrix metalloproteinase-1 by regulating the ERK/JNK/AP-1 pathway in human keratinocytes. *Biomol Ther (Seoul)* **20**: 57–61.

26) Qin Z, Robichaud P, He T, Fisher GJ, Voorhees JJ, Quan T. 2014. Oxidant exposure induces cysteine-rich protein 61 (CCN1) via c-Jun/AP-1 to reduce collagen expression in human dermal fibroblasts. *PLoS One* **9**: e115402.

27) Sveen KA, Dahl-Jørgensen K, Stensaeth KH, Angel K, Seljeflot I, Sell DR, Monnier VM, Hanssen KF. 2015. Glucosepane and oxidative markers in skin collagen correlate with intima media thickness and arterial stiffness in long-term type 1 diabetes. *J Diabetes Complications* **29**: 407–412.

28) Deveci M, Gilmont RR, Dunham WR, Mudge BP, Smith DJ, Marcelo CL. 2005. Glutathione enhances fibroblast collagen contraction and protects keratinocytes from apoptosis in hyperglycaemic culture. *Br J Dermatol* **152**: 217–224.

29) Ojeda ML, Barrero MJ, Nogales F, Murillo ML, Carreras O. 2012. Oxidative effects of chronic ethanol consumption on the functions of heart and kidney: folic acid supplementation. *Alcohol Alcohol* **47**: 404–412.

30) Shukla N, Angelini GD, Jeremy JY. 2008. The administration of folic acid reduces intravascular oxidative stress in diabetic rabbits. *Metabolism* **57**: 774–781.

31) Mendes JJ, Leandro CI, Bonaparte DP, Pinto AL. 2012. A rat model of diabetic wound infection for the evaluation of topical antimicrobial therapies. *Comp Med* **62**: 37–48.

32) Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. 2004. Glutathione metabolism and its implications for health. *J Nutr* **134**: 489–492.