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

Mei Zhao1,*, Jun Zhou2, Yuan-hua Chen3, Li Yuan1, Man-man Yuan1, Xin-qiong Zhang1, Yan Hu1 and Huan Yu1

1 School of Nursing, Anhui Medical University, Hefei 230032, China
2 Huainan Vocational Technical College, Huainan 232001, China
3 Department of Histology and Embryology, Anhui Medical University, Hefei 230032, China
(Received July 27, 2017)

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

To whom correspondence should be addressed.
E-mail: zhaomei@ahmu.edu.cn

Abbreviations: 3-NT, 3-nitrotyrosine; GSH, glutathione; ROS, reactive oxygen species; STZ, streptozotocin; TBARS, thiobarbituric acid-reactive substance.
The 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 deplated with 8% Na2S. 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...
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. 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.](image)
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 x 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 x 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 x g at 4˚C for 10 min. The supernatant (0.1 mL) was combined with 4.4 mL of 300 mM Na2HPO4 buffer. Then 0.5 mL dithio-bis-nitrobenzoic acid (DTNB) (0.04%, w/v; 40 mg DTNB in 100 mL of 1% sodium phosphate buffer) was added. Absorbance of the sample was recorded at 412 nm using a spectrophotometer.
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 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 (3 mg/kg). 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 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. \( p<0.05 \), \( **p<0.01 \) vs control mice. \( \dagger p<0.05 \) vs 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.

**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-
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 thyl radicals efficiently but also repair these thiol 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, by 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 findings 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

This work was supported by National Natural Science Foundation of China (81671471 and 81471467) and Key University Science Research Project of Anhui Province (KJ2016A360). The authors gratefully acknowledge Prof. De-xiang Xu for advice on experimental design and technical assistance.

Author contributions

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

REFERENCES


