ORIGINAL RESEARCH

Folic Acid Protects Against Kidney Damage in Mice with Diabetic Nephropathy by Inhibiting M1 Macrophage Polarization via Nuclear Factor-k-gene Binding Pathway

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ABSTRACT

Kidney damage is one of the most common complications of diabetes, and inflammation caused by macrophage infiltration plays an important role. Folic acid (FA), a water-soluble vitamin, was previously found to affect inflammation by regulating macrophage polarization. In our study, we aimed to investigate the effect of FA on renal injury in mice with diabetic nephropathy (DN). We found that FA treatment ameliorated diabetic metabolic parameters in mice with DN, including reducing 24-hour food consumption, 24-hour urine volume and 24-hour water intake and increasing body weight and serum insulin. Of note, FA treatment improved renal functional and structural damage in mice with DN. In addition, FA treatment significantly reduced the number of renal infiltrating M1 macrophages, inflammatory cytokine FA stimulation significantly reduced the increase in F4/80+CD86+ cell ratio, inflammatory factor content and p-p65/p65 protein expression induced by high glucose exposure in RAW264.7 cells.

All in all, our results indicated that FA protects against kidney damage in mice with DN by inhibiting M1 macrophage polarization, and its mechanism may be related to the inhibition of nuclear factor-k-gene binding (NF-kB) signaling pathway. (*Altern Ther Health Med.* 2023;29(6):274-279).

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INTRODUCTION

Diabetes mellitus is a metabolic disease whose main feature is hyperglycemia, and long-term hyperglycemia can lead to chronic damage and dysfunction of various tissues, especially the eyes, kidneys, cardiovascular system and nerves.[1,2] Diabetic nephropathy (DN) is one of the most common microvascular lesions in patients with diabetes, and approximately one-third of patients with diabetes will eventually develop DN.[3] The pathogenesis of DN is complex, and multiple links are involved in the kidney damage of DN, such as metabolic environment disorder, obesity, hemodynamic changes, hormone effects, pro-inflammatory effects, oxidation and stress, protein kinase activity and genetics.[3,4] Recently, more and more studies have confirmed that chronic immune inflammation plays an important role in the pathogenesis of DN, and innate immunity plays a key role.[5,6] Macrophages are the most important innate immune cells in DN kidney tissue, and they number many more than other types of immune cells. And macrophage polarization caused by renal microinflammation plays a decisive role in the occurrence and development of DN, and activated macrophages promote the deterioration of DN by secreting pro-inflammatory mediators.[7,8] Therefore, targeted regulation of macrophage polarization has become a research hotspot in the prevention and treatment of DN.

Folic acid (FA) is a water-soluble vitamin B that is essential for the renewal of red blood cells and other cells in the human body and is an essential vitamin. As an organic substance necessary to maintain the normal life process of organisms, FA becomes at least 5 active coenzymes after being absorbed by the body.[9,10] These coenzymes are involved in the transfer of one-carbon units in the body, and play a particularly important role in the biosynthesis of purines, pyrimidines, nucleic acids and proteins, as well as cell division and growth.[11,12] Lack of FA in the human body is related to the occurrence and development of many diseases, such as neonatal malformations,[13] macrocytic anemia[14] and cardiovascular and neurological diseases.[9,10]

There is a good deal of clinical evidence that has shown that serum FA levels in patients with diabetes are lower than

in healthy people,[15] and FA deficiency in patients with type 2 diabetes (T2D) increases the risk for diabetic nephropathy.[16] Of note, FA supplementation can not only improve blood sugar control in patients with diabetes,[14] but also help reduce the risk for gestational diabetes and diabetic nephropathy.[17]

However, the exact molecular mechanism by which FA supplementation prevents and treats DN remains unknown. A previous study suggested that FA can inhibit the lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages by suppressing MAPKs and nuclear factor-k-gene binding (NF-kB) activation.[18] In our study, we aimed to investigate the effect of FA on renal injury in mice with DN, and to explore its mechanism related to macrophage polarization and the NF-kB pathway.

MATERIALS AND METHODS

Animals and Drug Administration

All animal experiments complied with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and the European Union Directive 2010/63/EU for animal experiments. We purchased 28 C57/BL6 female mice (6 to 8 weeks old; weight, 20 to 22 g) for this study from Shanghai Lingchang Biotechnology Co., Ltd. After 1 week of adaptive feeding under laboratory conditions, the mice were used for follow-up studies. As previously described,[19] we randomly selected 21 mice in which we induced diabetes via a high-fat diet and intraperitoneal injection of streptozotocin (STZ) (40 mg/kg); the remaining 7 mice were fed a normal diet and received intraperitoneal injection of the same amount of normal saline as controls (the control group). After 2 weeks, we defined those mice with fasting blood glucose >11.1 mmol/L as mice as having diabetes. Subsequently, the mice were randomly divided into 3 groups (n = 7 each): the DN group, a folic acid (FA) group (5 mg/kg) and an FA group (10 mg/kg). Mice in the 2 FA groups were given different doses (5 or 10 mg/kg) of FA (59-30-3; MilliporeSigma, Merck, Shanghai China) by gavage daily for 8 weeks. After 8 weeks of FA treatment, we housed each mouse individually in order to measure 24-hour food consumption, 24-hour urine volume, 24-hour water intake, 24-hour urine protein fasting blood glucose and weight. Blood was collected and serum creatinine (Scr), blood urea nitrogen (BUN) and serum insulin levels were measured using colorimetric kits.

Kidney Histology and Tissue Immunofluorescence

Researchers euthanized the mice by having them inhale excess carbon dioxide and then removed their kidneys. Mouse kidney tissue was fixed with 4% paraformaldehyde (P1110; Solarbio Science and Technology, Beijing, China) for 24 hours at 4°C, and then sliced into 4-μm sections for hematoxylin-eosin (H&E) staining according to the manufacturer's instructions (SL7070; Coolaber Science & Technology, Shanghai, China). In addition, 4-μm sections of kidney tissue were deparaffinized, dehydrated and washed with phosphate-buffered saline (PBS) buffer. Antigen retrieval was performed with citrate after removal of endogenous peroxidase with 3% H_2O_2. The antibody of alexa fluor 488 Anti-CD86 antibody (1:100, ab290990, Abcam Biotechnology, Cambridge UK) was incubated overnight at 4°C. Finally, all samples were analyzed by confocal microscopy (TCS SP5; Leica Biosystems, Wetzlar, Germany).

Cell Culture and Flow Cytometry Analysis

RAW264.7 cells were purchased from American type culture collection (ATCC, Manassas, Virginia USA), and were cultured in RPMI-1640 medium (A1049101, Gbico, Thermo Fisher Scientific, Waltham, Massachusetts USA) with 10% fetal bovine serum (10099141; Gbico) added at 37°C with 5% CO_2.

High glucose exposure protocol. After RAW264.7 cells were stimulated to differentiate into macrophages by phorbol-12-myristate-13-acetate (PMA), we exposed them to 30 nM glucose medium for 12 hours.

FA treatment protocol. After completion of high glucose exposure, macrophages derived from Raw246.7 cells were treated with different concentrations of FA (10 ug/mL or 20 ug/mL) for 48 hours. RAW264.7 cells in the control group received only phorbol 12-myristate 13-acetate (PMA) induction without high glucose exposure and FA treatment. After completing all treatments, we collected cells to analyze the F4/80+CD86+ cell ratio via flow cytometry with staining F4/80 antibody (ab60343; Abcam) and CD86 antibody (ab275357; Abcam).

Enzyme Linked Immunosorbent Assay (ELISA)

Levels of TNF-α, IL-1β and IL-6 in mouse kidney tissue homogenate and cell culture medium were detected using mouse IL-6 ELISA kit (PI326; Beyotime Biotechnology, Shanghai, China; mouse TNF-α ELISA kit (PT512; Beyotime) and mouse IL-1β ELISA kit (EK0502, Signalway Antibody, Greenbelt, Maryland USA).

Western Blot

Immunoblotting was used to measure the protein expression in the mouse kidney tissues and Raw246.7 cells. First, a radioimmunoprecipitation assay (RIPA) lysis buffer (R0010; Solarbio) was used to extract total protein from the kidney tissues of mice and TCMK-1 cells. After detecting the protein concentration using a BCA kit (BC0020; Solarbio), 40 μg total protein was separated by 10% SDS-PAGE. After being transferred to PVDF membrane and blocked with 5% nonfat milk at room temperature for 1 hour, primary antibody against CD86 (1:500, ab220188; Abcam), p65 (1:1000, ab32536; Abcam) and p-P65 (1:500, ab76302; Abcam) were incubated at 4°C overnight. After being incubated with secondary antibody at room temperature for 1 hour, the proteins were visualized with enhanced chemiluminescence solution (ECL) (WBKLS0100; Beijing Xinjingke Biotechnologies Co., Ltd, China), followed by densitometry analysis using ImageJ 3.0 (National Institutes of Health [NIH], Bethesda, Maryland USA). β-actin was loaded as the control.

Statistical Analysis

Data in this study were analyzed with IBM® SPSS 19.0 software (IBM, Armonk, New York USA). One-way ANOVA with Tukey's test was used *post hoc* to compare the difference between multiple groups; $P < .05$ indicated a significant difference.

RESULTS

Folic Acid Ameliorates Diabetic Metabolic Parameters in Mice with DN

Compared with the normal mice in the control group, the diabetic model mice in the DN group had increased food and water intake and urination, tarnished fur and were restless, irritable, prone to conflict, etc. These symptoms were gradually alleviated after FA treatment (Figure 1).

Quantitatively, after 8 weeks of FA treatment, we found that the level of 24-hour food consumption (Figure 2A), 24-hour urine volume (Figure 2B), 24-hour water intake (Figure 2C) in the mice with DN in the DN group were all significantly higher than in normal mice in the control group, and FA treatment could significantly decrease the level of 24-hour food consumption, 24-hour urine volume and 24-hour water intake in mice with DN in the FA group. At the same time, the body weight of mice in the DN group was significantly lower than in the control group, while FA treatment significantly increased body weight of mice with DN (Figure 2D). In addition, the level of fasting blood glucose in mice in the DN group was significantly higher than in the control group, while FA treatment significantly decreased the level of fasting blood glucose in mice with DN (Figure 2E). In addition, the level of serum insulin in mice in the DN group was significantly lower than in the control group, while FA treatment significantly increased the level of serum insulin in mice with DN (Figure 2F).

FA Improves Renal Functional and Structural Damage in Mice with DN

Kidney damage is one of the most common complications of diabetes. In our study, we found that blood urea nitrogen (Figure 3A), serum creatinine (Figure 3B) and 24-hour urine protein (Figure 3C) in mice with DN in the DN group were all significantly higher than in normal mice in the control group, while FA treatment could significantly decrease the blood urea nitrogen, serum creatinine and 24-hour urine protein in mice with DN. In order to perform pathological analysis, we euthanized all mice after 8 weeks of FA treatment to obtain kidney tissue and performed pathological evaluation of the kidneys using hematoxylin and eosin (H&E) staining. As shown, the glomerular area of mice with DN in the DN group was significantly higher than in the normal mice in the control group, while FA treatment could significantly decrease the glomerular area in mice with DN (Figure 3D). Moreover, H&E staining also showed that the DN model mice developed inflammatory cell infiltration (green arrow), pathological changes such as glomerular congestion and enlargement, collapse and necrosis of the renal tubules (blue arrow) and FA treatment significantly improved these changes (Figure 3E).

Figure 1. Chemical formula of folic acid.

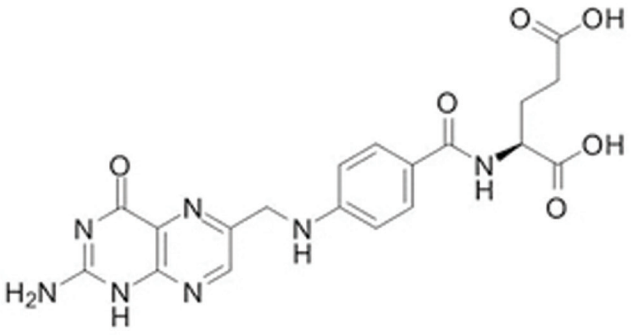
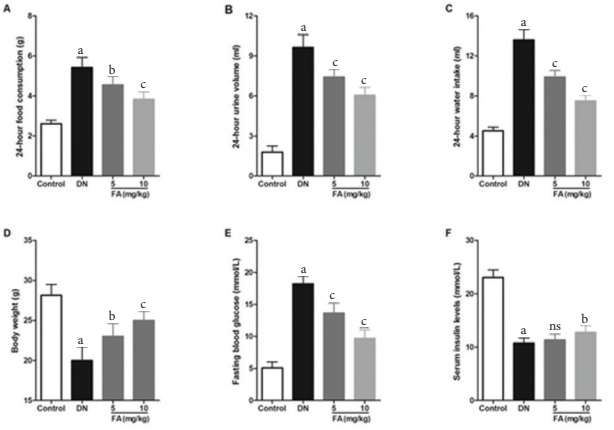


Figure 2. Effect of FA on mice with DN. After 8 weeks of FA treatment, we measured 24-hour food consumption (**2A**), 24-hour urine volume (**2B**), 24-hour water intake (**2C**), body weight (**2D**), fasting blood glucose (**2E**) and serum insulin levels (**2F**) in mice in each group. There were 7 mice in each group, and the *P* value was calculated by post hoc comparisons.

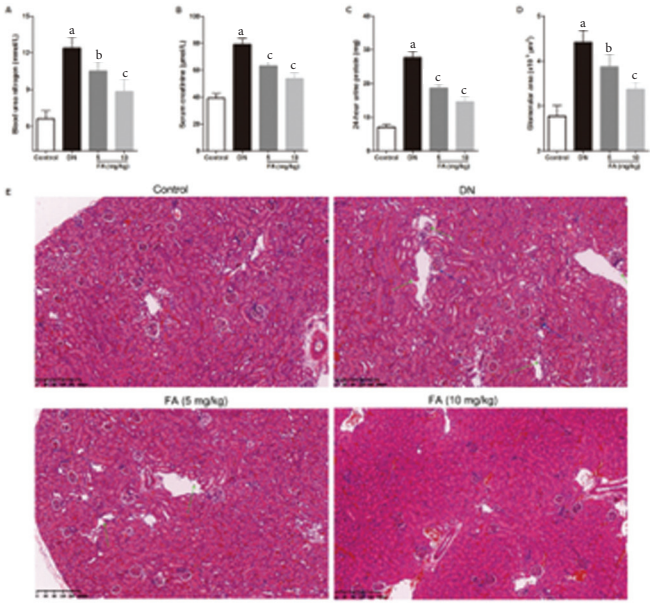


***$P < .001$ vs control group; ns $P > .05$.
\#\#$P < .01$
\#\#\#$P < .001$ vs DN group.

Abbreviations: DN, diabetic nephropathy; FA, folic acid.

As shown, we used tissue immunofluorescence to stain CD86 (M1 macrophage marker) in mouse kidney tissue (Figure 4A), and found that the number of CD86 positive cells infiltrated in the kidney tissue of mice with DN was significantly higher than in the control group, while FA treatment significantly decreased the number of CD86 positive cells infiltrated into the kidney tissue in mice with DN (Figure 4B). In a similar fashion, we also used western blot to detect the expression of CD86 protein in the kidney tissue of mice (Figure 4C), and found that the expression of CD86 protein in the kidney tissue of mice with DN was significantly higher than in the control group, while FA treatment significantly decreased the expression of CD86 protein in the kidney tissue of mice with DN (Figure 4D).

Figure 3. Effect of FA on kidney injury in mice with DN. After 8 weeks of FA treatment, we measured blood urea nitrogen (**3A**), serum creatinine (**3B**), 24-hour urine protein (**3C**) and glomerular area (**3D**) of mice in each group. (**3E**) Representative mouse kidney pathology detected with H&E staining (10 X). Bar is 10 μm. There were 7 mice in each group, and the *P* value was calculated by post hoc comparison.



[a]*P* < .001 vs control group.
[b]*P* < .01 and
[c]*P* < .001 vs DN group.

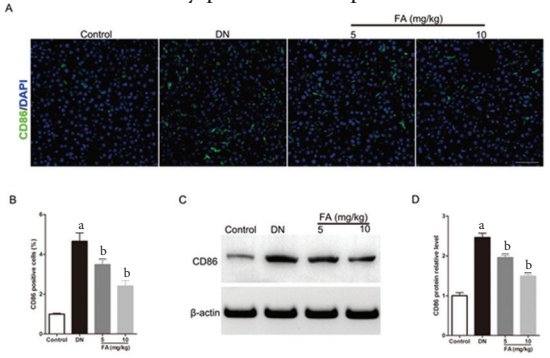
Note: Green arrow: inflammatory cell infiltration; blue arrow: glomerular congestion and enlargement, collapse and necrosis of renal tubules.

Abbreviations: DN, diabetic nephrology; FA, folic acid.

FA Inhibits Inflammation and the NF-kB Pathway in Kidneys of Mice with DN

Further, we evaluated the presence of inflammatory factors in the supernatant of mouse kidney tissue homogenate via ELISA kit after 8 weeks of FA treatment, and found that the expression of tumor necrosis factor alpha (TNF-α) (Figure 5A), interleukin (IL)-1β (Figure 5B) and IL-6 (Figure 5C) in the kidney tissue of mice with DN was significantly higher than in the control group, while FA treatment significantly decreased the expression of TNF-α, IL-1β and IL-6. At the same time, we also detected the expression of p65 and p-p65 protein, a key protein in the NF-kB pathway, in the kidney tissue of mice via western blot (Figure 5D), and found that the expression of p-p65/p65 protein in the kidney tissue of mice with DN was significantly higher than in the control group, while FA treatment significantly decreased the expression of p-p65/p65 protein in the kidney tissue of mice with DN.

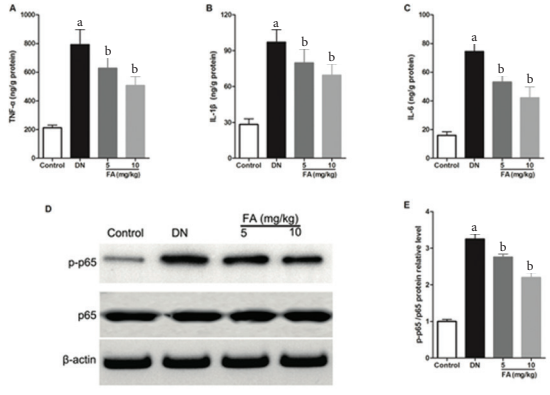
Figure 4. Effect of FA on the polarization of M1 macrophages in mouse kidney. After 8 weeks of FA, we euthanized mice in each group and obtained kidney tissue. (**4A**) Representative CD86 protein (green) staining in mouse kidney tissue using tissue fluorescence staining (20 X). Bar is 50 μm. (**4B**) Comparison of CD86 positive cells in different groups of mouse kidneys. (**4C**) Representative CD86 protein brands in mouse kidney tissue using western blotting. (**4D**) Comparison of gray value of CD86 protein bands in different groups of mouse kidneys. There were 7 mice in each group, and the *P* value was calculated by post hoc comparison.



[a]*P* < .001 vs control group.
[b]*P* < .001 vs DN group.

Abbreviations: DN, diabetic nephrology; FA, folic acid.

Figure 5. Effect of FA on the levels of inflammatory factors and the NF-kB pathway in mouse kidneys. After 8 weeks of FA treatment, we euthanized the mice in all groups and obtained kidney tissue in order to detect the level of TNF-α (**5A**), IL-1β (**5B**) and IL-6 (**5C**). (**5D**) Representative p-p65 and p65 protein brands in mouse kidney tissue using western blotting. (**5E**) Comparison of gray ratio value of p-p65 / p65 protein bands in different groups of mouse kidneys. There was a total of 7 mice in each group, and the *P* value was calculated by post-hoc comparison.



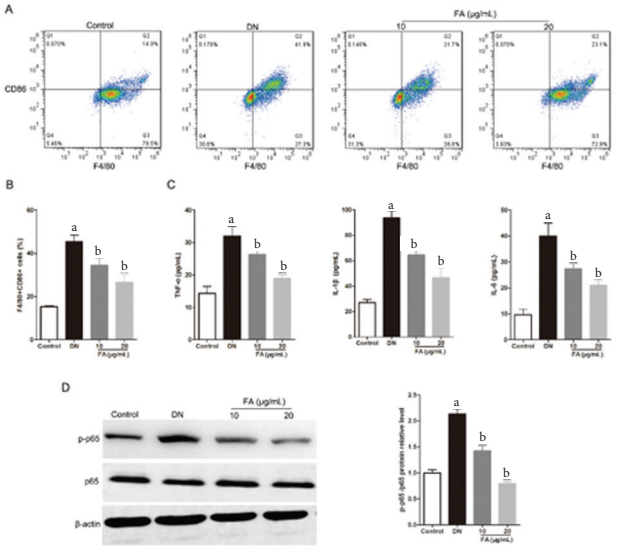
[a]*P* < .001 vs the control group.
[b]*P* < .001 vs the DN group.

Abbreviations: DN, diabetic nephropathy; FA, folic acid; IL, interleukin; NF-k, nuclear factor-k-gene binding; TNF-α, tumor necrosis factor alpha.

FA Inhibits M1 Macrophage Polarization, Inflammatory Factor Secretion and NF-kB Pathway Transduction in RAW264.7 Cells

In vitro, we used RAW264.7 cells to study the effect of FA on macrophage polarization, inflammatory cytokine secretion and the NF-kB pathway. First, results of flow cytometry analysis showed that high glucose exposure can significantly increase M1 macrophage polarization from RAW264.7 cells, while FA stimulation could significantly decrease M1 macrophage polarization induced by high glucose exposure (Figure 6A and Figure 6B). At the same time, we used ELISA kits to detect the content of inflammatory factors in the cell medium, and found that high glucose exposure can significantly increase the secretion of TNF-α, IL-1β and IL-6 from RAW264.7 cells, while FA stimulation could significantly decrease the secretion of TNF-α, IL-1β and IL-6 induced by high glucose exposure (Figure 6C). Furthermore, immunoblotting results indicated that high glucose exposure can significantly increase the expression of p-p65/p65 protein in RAW264.7 cells, while FA stimulation could significantly decrease the expression of p-p65/p65 protein induced by high glucose exposure (Figure 6D).

Figure 6. Effect of FA on macrophages polarization, inflammatory factor secretion and NF-kB pathway transduction in RAW264.7 cells. (**6A**) Representative flow cytometry plots to analyze M1 macrophage polarization from RAW264.7 cells (F4/80+CD86+ cells). (**6B**) Statistical comparison of the percentage of F4/80+CD86+ cells from RAW264.7 cells. (**6C**) Levels of TNF-α, IL-1β and IL-6 in the cell medium. (**6D**) Representative p-p65 and p65 protein brands inRAW264.7 cells using western blotting. (**6E**) Comparison of gray ratio value of p-p65/p65 protein bands in different groups of RAW264.7 cells. There was a total of 3 independent repetitions of each test. *P* value was calculated by post-hoc comparisons.



[a]*P* < .001 vs control group.
[b]*P* < .001 vs DN group.

Abbreviations: FA, folic acid; IL, interleukin; TNF-α, tumor necrosis factor alpha.

DISCUSSION

In this study, we found that FA not only ameliorated diabetic metabolic parameters, but also improved renal functional and structural damage in mice with DN. Our findings in the present study are consistent with previous findings that FA exhibits metabolic regulation and renal protection in animal models of DN.[17,20]

We also found that FA treatment significantly reduced the number of renal infiltrating M1 macrophages in the kidney tissues of mice with DN. Macrophages are generally divided into 2 subgroups based on their function: M1 macrophages and M2 macrophages.[21]

M1 macrophages are pro-inflammatory macrophages activated by lipopolysaccharide and/or inflammatory cytokines such as interferon-gamma, interleukin-12 and interleukin-2. M1-type pro-inflammatory macrophages are involved in a variety of infectious diseases by mediating host defense immunity against a variety of bacteria and viruses.[2,23] In addition, M1 macrophages play an important role in the development of chronic diseases, especially chronic inflammatory diseases, including DN.[24,25] Macrophage infiltration has been shown to be responsible for interstitial hyperplasia and irreversible pathological changes in renal tissue in patients with DN,[18,26] while M1 macrophage-induced inflammation is the direct cause of high glucose-induced renal injury.[27,28] Therefore, regulation of renal macrophage polarization has become a hot topic in the treatment and prevention of DN.

Inflammation caused by immune cell infiltration is a key factor in diabetes-induced kidney injury, especially macrophages.[29,30] After finding that FA significantly reduced the number of M1 macrophages in the kidneys of mice with DN, we further investigated the effect of FA on the expression of inflammatory factors in the kidneys of mice with DN. Our study results showed that FA treatment significantly reduced inflammatory cytokine secretion (TNF-α, IL-1β and IL-6) and p-p65/p65 protein expression in kidney tissues of mice with DN, suggesting that FA treatment significantly reduced inflammation by inhibiting the NF-kB pathway in kidney tissues of mice with DN.

The NF-κB pathway is one of the important pathways for macrophages polarization and function.[31] In DN animal models, that NF-κB pathway is found to be activated, M1 macrophages are activated and inflammatory factors are released, triggering the immune inflammatory response.[8] Multiple drugs were found to inhibit the polarization of M1 macrophages by inhibiting the NF-kB pathway, ultimately reducing kidney inflammation in mice with DN.[32,33]

To exclude the effect of *in vivo* confounding factors, like Wang, et al.[19], we investigated the effect of FA on the polarization of M1 macrophages induced by high glucose exposure *in vitro*. We found that FA significantly reduced the

increase in F4/80+CD86+ cell ratio, inflammatory factor content and p-p65/p65 protein expression induced by high glucose exposure in RAW264.7 cells. Therefore, our results indicate that FA protects against kidney damage in mice with DN by inhibiting M1 macrophage polarization via the NF-kB pathway.

ETHICS APPROVAL

The Ethics Committee of First Central Hospital Baoding City approved this study.

CONFLICT OF INTEREST

None.

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