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KIDNEY DISEASE

Reno-protective Effects of *Coreopsis Tinctoria* Flavonoids in db/db Mice Models Through the AGE/RAGE Pathway

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Introduction. The study investigates the renal protective effects of flavonoids derived from *Coreopsis tinctoria* Nutt in type 2 diabetes using *db/db* mice.

Methods. With the *db/m* mice born in the same litter as the non-diabetic normal group, 8-week-old *db/db* mice were divided into four groups: a diabetes model group, a low-dose (0.4 g/kg) flavonoids group, a high-dose (1.2 g/kg) flavonoids group, and a metformin positive control group. Mice were treated daily for 8 weeks.

Results. After 8 weeks of continuous gavage feeding, the high-dose group of flavonoids significantly improved fasting blood glucose levels in 16-week-old *db/db* mice, and both dosages of flavonoids reduced average daily water intake. The levels of serum creatinine (CRE) and BUN in *db/db* diabetic mice were significantly reduced by both high and low dosages of flavonoids, mitigating the loss of glomerular cells under hyperglycemic conditions, inhibiting glomerular hypertrophy and mesangial matrix hyperplasia, and demonstrating protective effects on renal function damage. The levels of AGEs in *db/db* mice kidneys were elevated compared to *db/m* mice, but significantly decreased with flavonoid treatment. Flavonoids also reduced RAGE protein expression and NF-κB activation. Additionally, both doses of flavonoids lowered MDA and IL-1β levels, and enhanced antioxidant enzyme activities.

Conclusions. The study concludes that flavonoids from *Coreopsis tinctoria* Nutt can inhibit the accumulation of AGEs and binding to RAGE in kidneys, improve oxidative stress and inflammation, and protect against renal damage induced by hyperglycemia. This suggests their potential of flavonoids from *Coreopsis tinctoria* Nutt as functional food and medicine for diabetic nephropathy.

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INTRODUCTION

DM is one of the major diseases that seriously endangers human health. The prevalence of diabetes and impaired glucose tolerance among adults worldwide has been increasing year by year. According to the International Diabetes Federation, it is estimated that 463 million adults aged 20 to 79 suffer from DM in the world in 2019. It is expected

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that the number of such individuals will reach 700 million by 2045. Diabetic nephropathy (DN) refers to chronic kidney disease that is caused by diabetes, making it one of the most common and severe microvascular complications associated with diabetes.[1] Its clinical features include persistent increase in urinary albumin excretion and/or progressive decrease in glomerular filtration rate, eventually leading to the development of ESRD. Its pathological features include thickening of the basement membrane, disappearance of foot processes, dilation of mesangial matrix, sclerosis of glomerular tuberosity, and fibrosis in the tubulointerstitial area.[2] Studies have found that DN is the primary cause of ESRD, and approximately 30 to 50% of ESRD cases worldwide are attributed to DN.[3] Furthermore, patients with DN have a mortality rate that is 50% higher than that of patients with type 2 diabetes alone.[4] Therefore, ameliorating nephropathy induced by diabetes is of significant importance as it not only preserves renal function and enhances the quality of life for diabetic patients but also reduces the escalating healthcare costs associated with ESRD.

The pathogenesis of DN is intricate and multifactorial, initiated and maintained by four causal factors that involve metabolic derangements, hemodynamic changes, growth factors, and inflammatory or profibrotic elements.[5] Numerous clinical studies have demonstrated that hyperglycemia is one of the primary causes of DN. The generation of advanced glycation end products (AGEs), which are closely associated with hyperglycemia, plays a pivotal role in the pathological mechanism of DN.[6] AGEs are large molecules formed through non-enzymatic glycation reactions between amino acids, proteins, and lipids with reducing sugars such as glucose.[7] Oxidative stress and inflammation are the primary damages caused by AGEs.[8] Recent evidence suggests that the expression of the receptor for AGE (RAGE) is increased in aging kidneys and DN, and this increased RAGE expression mediates the activation of oxidative stress and inflammation pathways such as the nuclear factor-κB (NF-κB) signaling pathway.[9,10] On the contrary, under diabetic conditions, AGEs-RAGE induces oxidative stress and inflammation, further promoting the formation of AGEs. The positive feedback mechanism between AGEs and downstream signaling pathways mediated by RAGE leads to a vicious cycle in DN.[11] Therefore, inhibiting the production of AGEs and preventing the activation of the AGE-RAGE axis are particularly important for the prevention and treatment of DN.

Flavonoid compounds (FCs) are an important class of plant secondary metabolites and serve as one of the primary active ingredients in traditional Chinese medicinal herbs. They possess anti-inflammatory properties, increase insulin sensitivity, and improve insulin resistance, which have been proven to be effective in the treatment of diabetes and its complications.[12] It was found that rutin alleviates insulin resistance in HepG2 cells induced by AGEs by inhibiting SOCS3/IRS1 and activating the PI3K/AKT signaling pathway.[13] Oleanolic acid can effectively inhibit the formation of AGEs.[14] The protective effects of apigenin and baicalein were associated with inhibiting the AGEs/RAGE/NF-κB pathway and improving oxidative damage.[15] The screening of natural plant FCs as inhibitors of AGEs, with their safety and minimal side effects, has become a prominent topic in current research.

Coreopsis tinctoria Nutt, commonly known as Golden Tickseed or Snow Chrysanthemum, originates from North America and has since spread worldwide. In China, it is mainly distributed in the Kunlun Mountains area of the northwest region at an altitude of about 3 km. Over 120 chemical constituents have been identified in *Coreopsis tinctoria* Nutt, with flavonoids being the predominant bioactive compounds, accounting for more than 20% of its dry weight, which indicates high nutritional and medicinal values.[16,17] Various solvent extracts of *Coreopsis tinctoria* Nutt have been shown to significantly improve kidney damage in multiple types of diabetic animals. Sprague-Dawley (SD) rats, treated with a high fructose and fat diet combined with a single intraperitoneal injection of 35 mg/kg streptozotocin (STZ), developed characteristics of DN. The inflammatory and fibrotic responses in the kidney were mitigated by a 4-week gavage of the ethyl acetate extract from *Coreopsis tinctoria* Nutt.[18] In the model of glomerular mesangial cell injury induced by high glucose and palmitic acid,

as well as in the *db/db* mice model, it was observed that the alcohol extract of *Coreopsis tinctoria* Nutt could protect against diabetic renal injury by inhibiting renal oxidation, inflammation, and fibrosis through the RhoA/ROCK and NF-κB/TGF-β/Smad signaling pathways.[19] The alcohol extract of *Coreopsis tinctoria* Nutt can also improve the degree of renal fibrosis in *db/db* mice through the miR-192/miR-200b and PTEN/AKT, as well as ZEB2/ECM pathways.[20] Currently, the research on the anti-DN mechanism of *Coreopsis tinctoria* Nutt is becoming increasingly profound. Furthermore, it remains to be demonstrated whether the total flavonoids of *Coreopsis tinctoria* Nutt (TFC) can effectively inhibit the formation and accumulation of AGEs in vivo, thus potentially playing a renal protective role.

In this study, eight-week-old *db/db* mice with type 2 diabetes mellitus were continuously administered gavage for 8 weeks. The control group consisted of *db/m* mice born in the same litters, and metformin was used as a positive drug. The sample groups were given low and high doses of TFC from *Coreopsis tinctoria* Nutt. Based on the basic metabolism, blood glucose levels, kidney pathological injury, oxidative stress, inflammatory factors, and the AGEs/RAGE/NF-κB signaling pathway in mice from each group were evaluated to investigate the efficacy and mechanism of TFC in improving DN injury. This study provides a theoretical basis and data support for the application and development of *Coreopsis tinctoria* Nutt in preventing complications associated with diabetes.

MATERIALS AND METHODS

Chemicals and Materials

Aminoguanidine (AG), bovine serum albumin (BSA), aqueous solutions of methylglyoxal (40%) and glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). High performance liquid chromatography (HPLC)-grade acetonitrile was purchased from Fisher (Fisher Scientific, Waltham, MA, USA). Metformin hydrochloride tablets (0.5 g/tablet) were purchased from Sino-American Shanghai Squibb Pharmaceuticals Ltd (Shanghai, China).

Preparation of the TFC

TFC were kindly supplied by the Department of Food Science at Rutgers University. The TFC powder was produced from dried flowers of *Coreopsis tinctoria* Nutt using the following extraction technology: soaking in ethanol, water extraction (using a tea-to-water ratio of 1:8 at 100 °C), filtering, concentrating, and freeze-drying. The powder was dissolved in DMSO to a concentration of 250 mg/mL and subjected to ultrasound for 30 min to accelerate dissolution. Subsequently, the solution was filtered using a 0.22 μm Millipore membrane filter (Millipore, Bedford, MA) and then diluted with sterilized PBS as required.

Fractionation of the TFC Using HPLC

The TFC were determined using an HPLC system 2695 (Waters Corp, Milford, MA, USA) equipped with a degasser, an autosampler, quaternary pump and coupled with a 2996 photodiode array detector (PDA), as well as a phenomenex luna C18 (150 mm × 4.6 mm, 3 μm) ID column. The extracted samples were injected at a flow rate of 1 mL min-1, with 10 μl being used. Separation was carried out at 35 °C, and the detection wavelength was set at 280 and 385 nm. The mobile phase consisted of A (0.1% trifluoroacetic acid solution) and B (CH3CN), using gradient elution conditions as follows: 0 to 10 min, 0 to 12% B; 12 to 20 min, 12 to 25% B; 20 to 25 min, 40% B; 25 to 30 min, 40-12% B. Samples were also filtered through 0.22 μm PTFE (polytetrafluoroethylene) membrane filters (Millipore, Madrid, Spain) before injection. For identification and quantification, the retention times of each peak were compared to external standards and stored.

Animals and Experimental Design

Six-week-old male C57BL/KsJ-Lepr$^{db/db}$ diabetic mice (db/db) and their non-diabetic lean heterozygous littermates (db/m) were purchased from the Model Animal Research Center (MARC) (Nanjing, Jiangsu, China). After a two-week acclimatization period, the mice were divided randomly into five groups (n = 8 /each group) as follows: *db/m* mice normal control group (db/m); *db/db* mice DN group (db/db); metformin group (*db/db* mice + metformin, 300 mg/kg/d) (Met); TFC low dose group (*db/db* mice + TFC, 40 mg/kg/d) (FL); TFC high dose group (*db/db* mice + TFC,

120 mg/kg/d) (FH). The *db/m* mice in the normal control group and the *db/db* mice in the DN group were administered sterile distilled water by gavage for 8 weeks. All drugs were dissolved in sterile distilled water and administered to *db/db* mice by gavage for 8 weeks. The animals were housed and provided with unrestricted access to food and water in the SPF barrier environment at 21 ± 2 °C, with humidity ranging from 40 to 50%, following a 12h light:12h dark cycle. Approval for animal studies was obtained from the Institutional Animal Care and Use Committee of Beijing Normal University.

Collection of Serum Samples and Assessment of Biochemical Parameters

During oral administration, body weight, food intake, and water intake were recorded consecutively every week. Blood was collected from the mice's tail vein once a week, and glucose readings were taken using a glucose meter. This included measurements of fasting and non-fasting blood glucose levels. Fasting blood glucose was measured approximately 8 hours after overnight fasting, while non-fasting blood glucose was measured at 21:00 on the same day. After 8 weeks of intervention, mice were anesthetized with pentobarbital sodium (30 mg/kg), and their blood samples were taken after a fasting period of 6 hours. The blood was centrifuged at a rotational speed of 3500 rpm for 15 minutes. The serum was carefully collected and subsequently frozen at -80 ℃ for future use. Biochemical parameters of serum creatinine (CRE) and BUN were measured using ELISA kits following the manufacturer's protocols.

Collection of Kidney Samples and Assessment of Biochemical Indices

After blood extraction, the right kidneys of mice were collected and frozen in a liquid nitrogen tank for protein extraction, while the left kidneys were fixed with 4% paraformaldehyde for pathological and immunohistochemical analyses.

The Expression Levels of RAGE and NFκB-p65 in the Kidney by Using Western Blot

Proteins extracted from kidneys were separated using 10% SDS-PAGE and then blotted with primary antibodies against RAGE (1:500, Santa Cruz), phospho-NF-κB P65, total NF-κB P65 (1:1000, Cell Signaling, Beverly, MA, USA), and β-actin (C4) (1:2000, Santa Cruz). β-actin was used as a loading control. The photo density analysis was quantified using a gel image analysis system (Odyssey, LI-COR, Lincoln, NE, USA).

The Levels of AGEs and IL-1β in the Kidney by Using An ELISA

The renal AGE levels were determined using commercially available ELISA kits purchased from Cell Biolabs (San Diego, CA, USA). IL-1β levels in the kidneys were measured using ELISA kits (R & D, Minneapolis, USA) according to the manufacturer's instructions. All of the results were normalized to total protein concentrations.

Detection of Oxidative Stress Related Indexes

A certain amount of mouse kidney tissue was cut on ice. A 10% homogenate of the kidney tissue was prepared by adding normal saline at a ratio of 1:9 (m/v). The mixture was then centrifuged at 4 °C and 2500 r/m for 20 minutes, and the resulting supernatant was collected. The total protein concentration was measured using a BCA kit, with a dilution ratio typically ranging from 80 to 100 times. The levels of MDA, SOD, CAT, and GSH-PX were determined strictly following the instructions provided in the kit.

Histopathological Examination

The left kidney tissues of mice were initially preserved in a 4% paraformaldehyde solution for 24h. After that, they underwent dehydration using alcohol and were then embedded in paraffin. Subsequently, sections of the tissue, each measuring 3 μm in thickness, were prepared using a microtome. These sections were later stained with H&E. Glomerular cell number and glomerular area were analyzed using Leica image analysis software (Leica, Buffalo Grove, IL, USA) through light microscopic examination. Renal morphometric changes were observed at a magnification of 400×, and 20 randomly selected glomeruli per mouse (n = 3 mice per group) were quantified.

Statistical Analysis

The results are expressed as the mean ± SEM.

Student's t-test and one-way ANOVA using SPSS 20 were performed to compare the experimental and control groups. A significance level of $P < .05$ was considered.

RESULTS

The Phytochemicals of TFC

HPLC was used to analyze the main chemical composition in FC. As shown in Figure 1 and Table 1, six compounds; including marein, flavanomarein, okalin, isokalin, 3,5-o-dicaffeoylquinic acid, and chlorogenic acid were identified. It can be observed that the TFC content is 50.06%, with Marein having the highest content of 343.2 mg/g, followed by flavanomarein at 107.5 mg/g, and isokalin at 10.8 mg/g.

Table 1. The Flavonoid Compounds and Their Content Changes in TFC

Items	TFC
Total flavonoids (%)	50.06
Marein, mg/g	343.2
Flavanomarein, mg/g	107.5
Okalin, mg/g	-
Isokalin, mg/g	10.8
3,5-o-dicaffeoylquinic acid, mg/g	21.6
Chlorogenic acid, mg/g	17.5

Effects of TFC on Body Weight, Daily Food Intake and Water Intake in *db/db* Mice

The effects of TFC on the basic signs of mice were evaluated by observing changes in appearance and body weight. The five groups of mice received intragastric administration once a day starting from 8 weeks old, and their weight was measured weekly. After 8 weeks of feeding, the mice's weight, food intake, and water intake were recorded in Table 2.

The results are shown in Table 2. The weight of mice in all groups increased with increasing age each week. At the age of 8 weeks, the weight of mice in the diabetes model group (db/db group) was significantly higher than that in the normal control group (db/m group), indicating early-onset obesity. By the 16th week, *db/db* mice weighed approximately 46 to 50 g, while *db/m* mice only weighed about 22 g. After continuous oral administration for 8 weeks, both TFC (FL and FH groups) and metformin (MET group) improved the hair color and appearance of *db/db* mice. However, despite drug intervention, the body weight of all *db/*

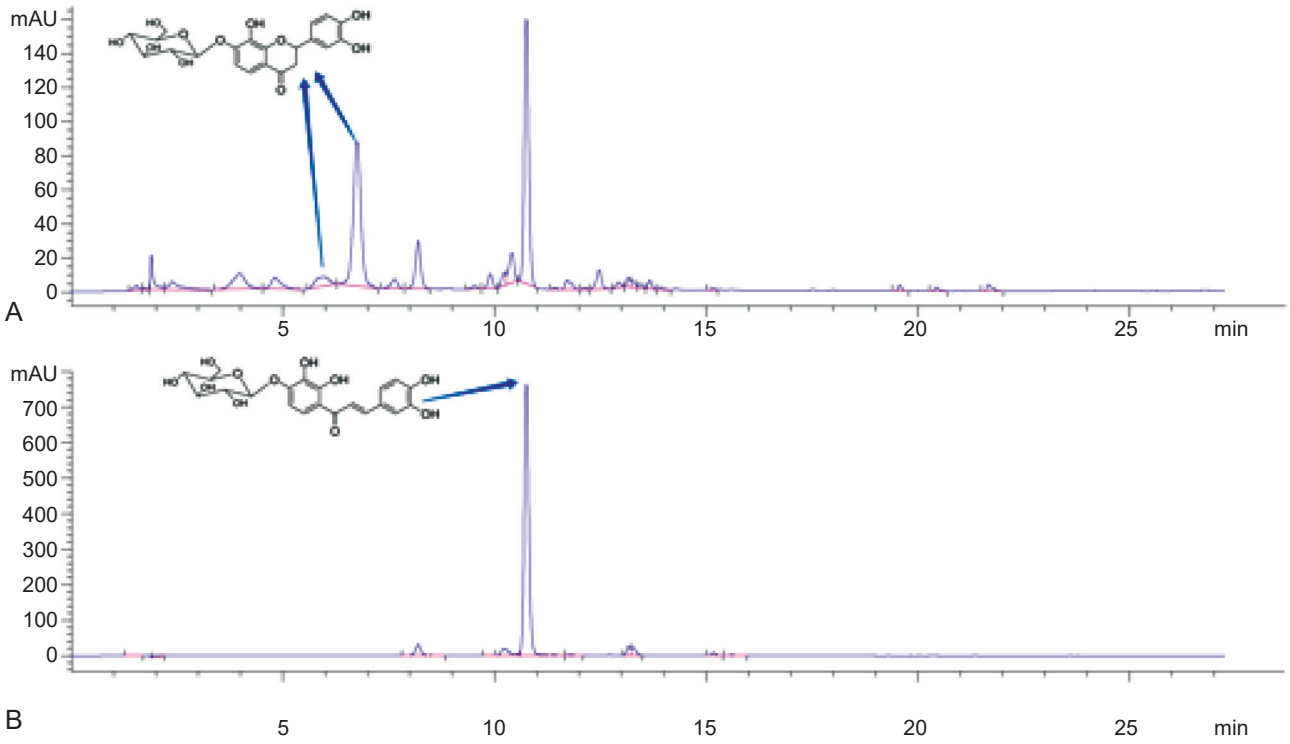

Figure 1. Analysis of TFC by HPLC

Table 2. Body Weight, Daily Average Food Intake and Water Intake in Each Group of Mice During Intragastric Administration

Parameters(g)	db/m	db/db	FL	FH	MET
Body weight (8 weeks)	19.39 ± 0.69	35.10 ± 0.75	35.04 ± 0.75	33.96 ± 0.79	35.03 ± 0.72
Body weight (12 weeks)	21.45 ± 0.69	44.95 ± 0.75	45.29 ± 0.75	42.10 ± 0.79	44.08 ± 0.72
Body weight (16 weeks)	22.78 ± 0.69	46.40 ± 0.75	50.70 ± 0.75	46.11 ± 0.79	48.60 ± 0.72
daily average food intake	3.21 ± 0.19	7.79 ± 0.81	7.35 ± 0.61	7.24 ± 0.39	7.71 ± 0.24
daily food intake/ body weight	0.192 ± 0.006	0.187 ± 0.009	0.173 ± 0.009	0.196 ± 0.011	0.176 ± 0.006
daily average water intake	4.07 ± 0.38	25.61 ± 1.89	19.86 ± 2.02	17.44 ± 1.56	15.24 ± 2.67
daily water intake/ body weight	0.179 ± 0.017**	0.553 ± 0.039	0.416 ± 0.049*	0.344 ± 0.033**	0.319 ± 0.065**

Note. The data are presented as the mean ± SD (*P < .05, **P < .01; respectively, compared to the *db/db* mice in the model group (n = 8)).

db mice remained relatively stable and consistent.

During the 8-week feeding period, there was no significant difference in the average daily food intake among all of the db/db group (n = 8, P > .05). However, after adjusting for body weight differences, there was a significant difference in average daily water intake. Compared to the model group's *db/db* mice, both the high dose group (FH) and positive control group (MET) showed a significant decrease in drinking water (P < .01), while the low dose group's *db/db* mice also exhibited a significant decrease in drinking water (P < .05).

Effects of TFC on Blood Glucose in *db/db* Mice

The changes in non-fasting blood glucose and fasting blood glucose were recorded at 0, 2, 4, 6, and 8 weeks after the intragastric administration of TFC. At the beginning of the 8-week administration period, there was a significant difference in non-fasting blood glucose levels between *db/db* mice in the model group and *db/m* mice in the negative control group (P < .05), as shown in Figures 2A and 2C. The fasting blood glucose level of *db/db* mice in the model group also exhibited a significant difference compared to that of *db/m* mice (P < .05, Figures 2B and 2D). After continuous intragastric intervention for 8 weeks, the fasting blood glucose level of *db/db* mice in the FH group significantly decreased compared to that in the model group (P < .05). Meanwhile, non-fasting blood glucose levels were consistent between the FH group and model group. These results indicate that TFC can improve to some extent the increase in fasting blood glucose levels of *db/db* diabetic mice. However, neither the low-dose nor high-dose groups receiving TFC via gavage over an 8-week period showed a significant inhibition of non-fasting blood glucose increase in *db/db* mice. Nevertheless, both the high-dose group (FH) and positive control group (MET) exhibited a significant improvement in fasting blood glucose levels of *db/db* mice (P < .05).

Effects of TFC on Renal Histology in *db/db* Mice

According to Table 2, the CRE content of *db/db* mice in the diabetic model group showed a significant increase, with an average value of 45.4625 ± 2.6938 μmol/L, which was 2.2 times higher than that of *db/m* mice in the normal control group (20.6625 ± 1.4469 μmol/L). After 8 weeks of continuous gavage, the CRE content of diabetic mice in the FL and FH groups decreased significantly to 35.7125 ± 2.6487 and 33.80 ± 1.6197 μmol/L, respectively (Table 3). In the positive control group (MET), the CRE content of *db/db* mice decreased to 29.7125 ± 2.2321 μmol/L after 8 weeks of continuous gavage with metformin, and the difference was extremely significant (P < .01).

BUN levels of *db/db* mice in the model group at 16 weeks old were significantly increased to 11.13 ± 0.46 mmol/L, which is 1.6 times higher compared to *db/m* mice (6.97 ± 0.72), respectively (Table 3). After continuous gavage intervention for 8 weeks, BUN levels of diabetic mice in the low and high dose groups (FL and FH) were significantly decreased (P < .01), measuring 8.88 ± 0.35 mmol/L and 7.52 ± 0.21 mmol/L, respectively. BUN content

Table 3. Serum Creatinine and Urine Nitrogen in Each Group of Mice

Group	CRE (μmol/L)	BUN (mmol/L)
db/m	20.6625 ± 1.4469**	6.97 ± 0.72**
db/db	45.4625 ± 2.6938	11.13 ± 0.46
FL	35.7125 ± 2.6487*	8.88 ± 0.35**
FH	33.8000 ± 1.6197**	7.52 ± 0.21**
MET	29.7125 ± 2.2321**	6.99 ± 0.30**

Note. The data are presented as the mean ± SD (*P < .05 and **P < .01, respectively; compared to the *db/db* mice in the model group (n = 8)).

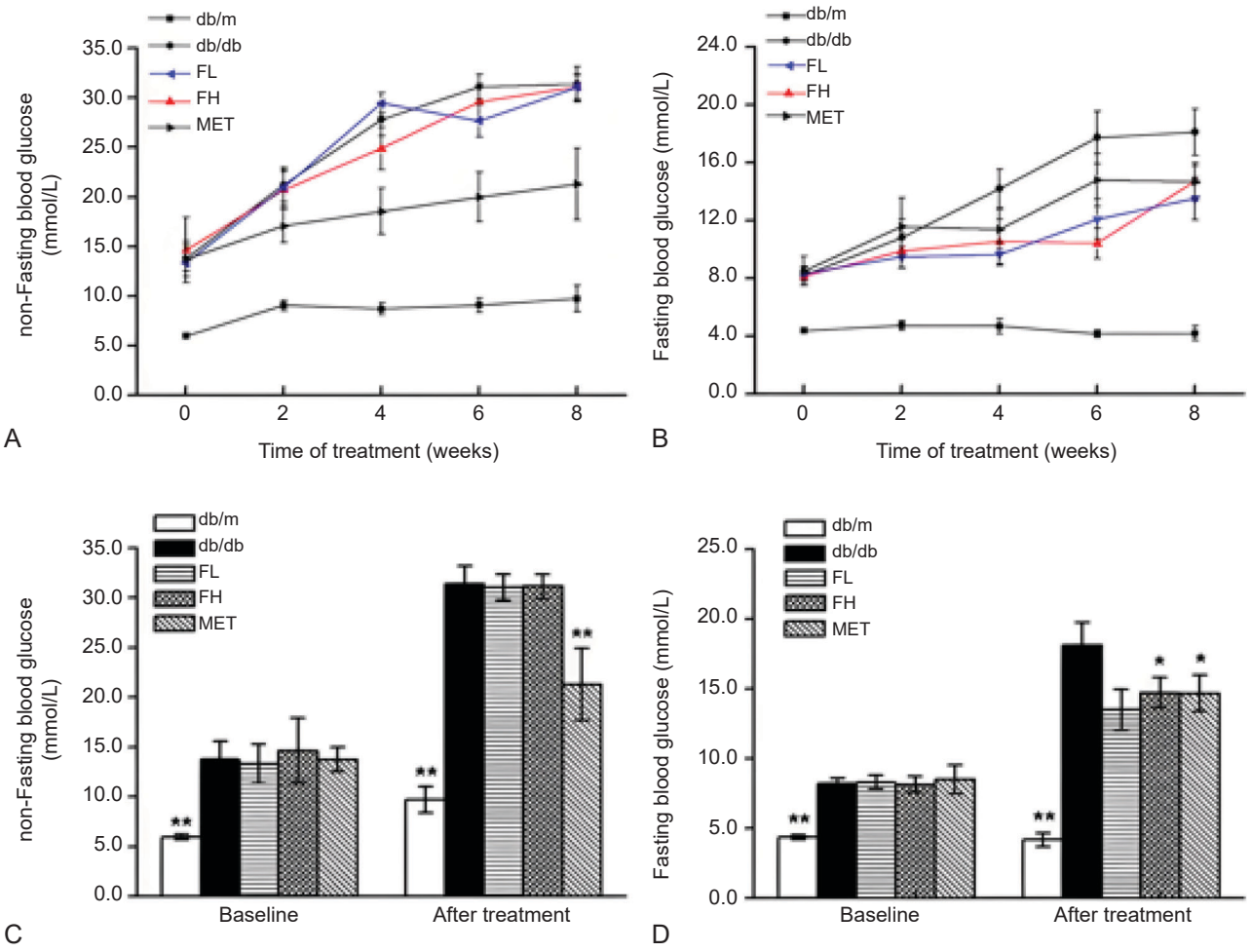

Figure 2. Changes in blood glucose levels during intragastric administration were observed in each group of mice. A) The trend of non-fasting blood glucose was measured every two weeks, B) The trend of fasting blood glucose was measured every two weeks, C) non-fasting blood glucose value of each group after the administration of TFC, and D) Fasting blood glucose value of each group after the administration of TFC. There were 8 mice in each group (The data are presented as the mean ± SD) (*P < .05 and **P < .01, respectively; compared to the *db/db* mice in the model group).

in the positive control group (MET) decreased to 6.99 ± 0.30 mmol/L (P < .01).

The results of histological examinations using H&E staining in Figure 3A reveal renal cell damage, which was found to be higher in the kidneys of *db/db* vehicle mice compared to those of *db/m* mice. In the control group, the structure of renal tissue appeared normal with no evident pathological changes. However, in the db/db model group, glomerular hypertrophy was noticeable and mild to moderate proliferation and glycogen accumulation were observed in some glomerular mesangial cells and matrix. Vacuolar degeneration occurred in renal tubular epithelial cells, along with individual thickening of the glomerular basement membrane. Nevertheless, after administering high and low doses of TFC (FH and FL groups) as well as metformin (MET group) for 8 weeks, there was a clear reduction in the degree of glomerular matrix hyperplasia and tubular vacuolar degeneration observed in *db/db* mice compared to that seen in the model group (db/db).

The results of H&E and PAS staining of mouse glomerular tissue sections were shown in Figure 3B, respectively. In the normal control group (db/m), the kidney tissue structure was normal, and no obvious lesions were observed. The model group consisting of *db/db* mice exhibited glomerular hypertrophy. For instance, there was mild to moderate hyperplasia in some mesangial cells and stroma, thickening of the glomerular basement membrane to a certain extent, as well as rare vacuolar degeneration and glycogen accumulation

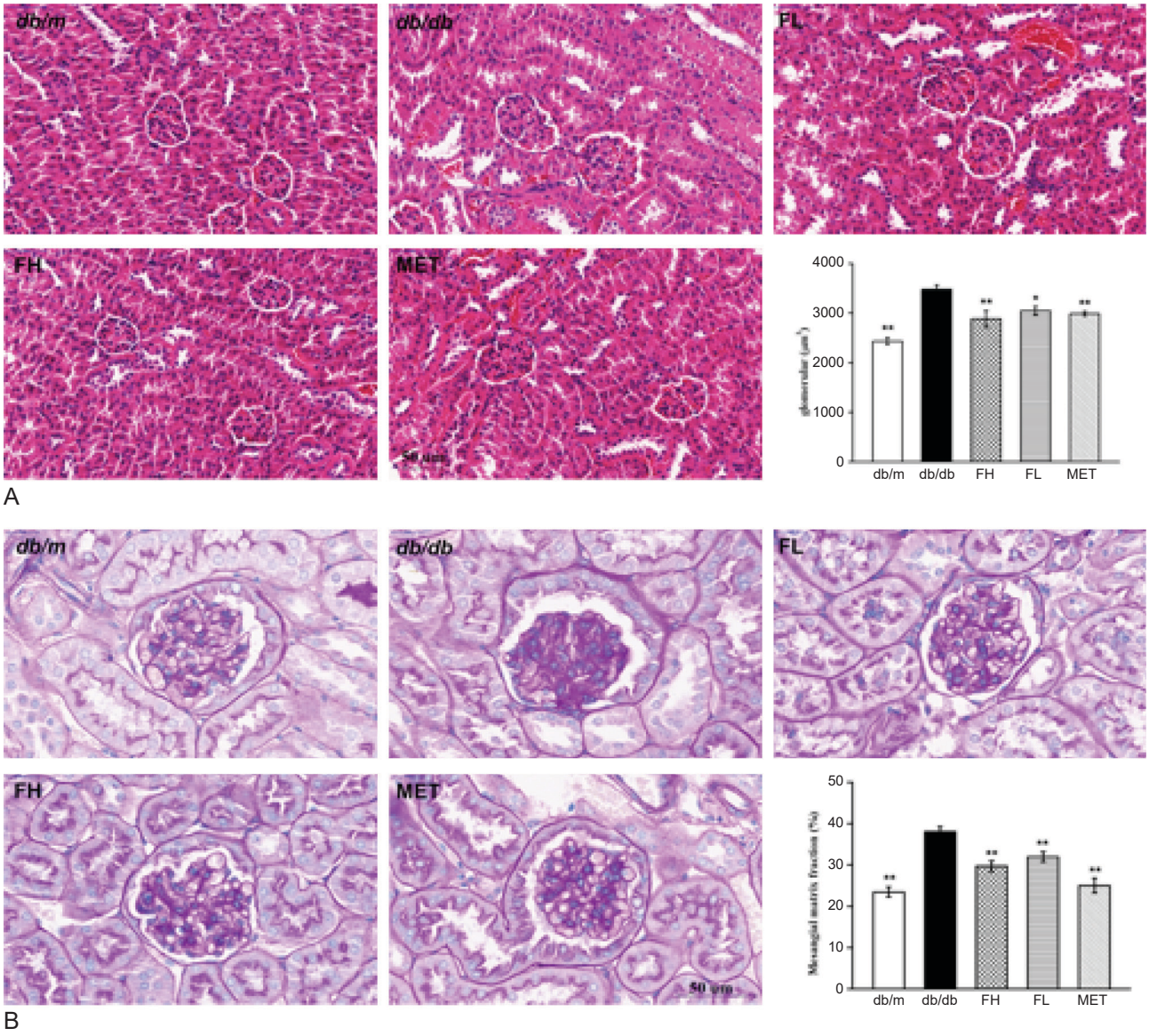

Figure 3. Effect of TFC on the Glomerular Area and the Expansion of Glomerular Extracellular Matrix in db/db Mice. A) Pathological features of renal tissue in db/m mice and db/db mice were observed using HE staining, B) Pathological features of renal tissue in *db/m* mice and *db/db* mice were observed using PAS staining. Three mice were randomly selected from each group, and 10 to 15 glomeruli were randomly chosen from each mouse for statistical quantification (original magnification, ×400))

in tubular epithelial cells. After an 8-week intervention with low-dose (FL) and high-dose (FH) TFC or metformin (MET), the proliferation degree of glomerular matrix decreased along with reduced renal tubule vacuolar degeneration, leading to relatively relieved mesangial matrix proliferation.

TFC Inhibits the Expression of AGE/RAGE in the Kidneys of *db/db* Mice

Compared with the normal control group (db/m), the levels of AGEs in the kidney tissues of diabetic mice in the 16-week-old diabetic model group (db/db) were significantly increased (4.39 ng/mg, $P < .01$, Figure 4). This increase was nearly 1.6 times that of the former (2.82 ng/mg). The level of AGEs in the kidney tissue effectively decreased in the treated group, with the high-dose group (FH) being only 75.3% of that in the model group (db/db), indicating an extremely significant difference ($P < .01$). A similar effect was observed in the positive control group (MET) with a decrease to 73.5%. The content of AGEs also decreased by 86.6% in the low dose

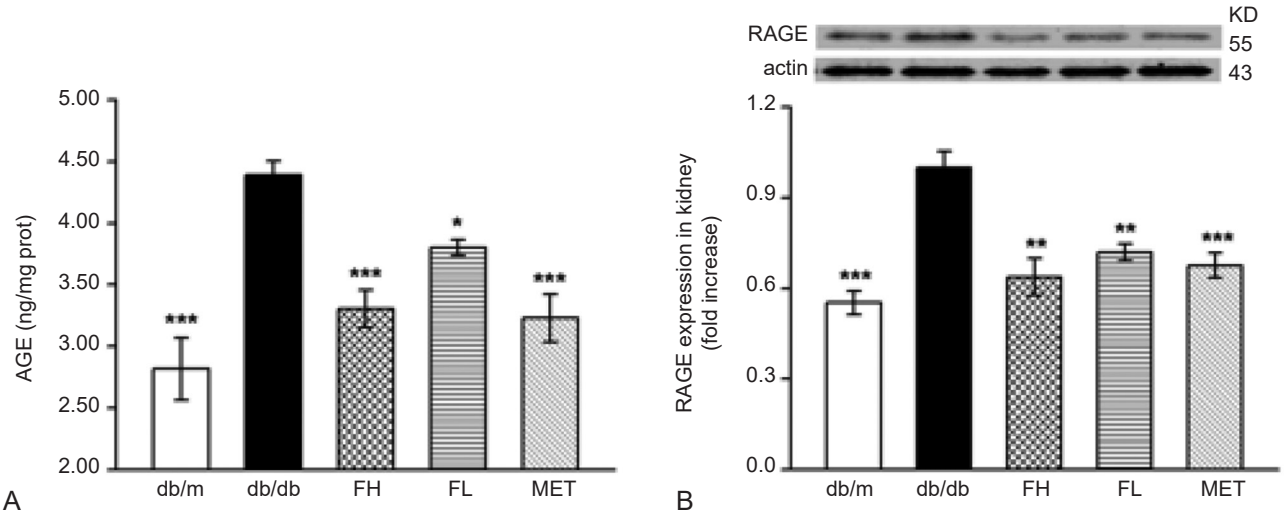

Figure 4. TFC inhibits the expression of AGE/RAGE in the kidneys of *db/db* mice. A) Protein expression and analysis of the AGE by ELISA assay, B) Protein expression and analysis of the RAGE by western blot, GAPDH was used as the internal control) (The data are presented as the mean ± SD (*$P < .05$ and **$P < .01$, respectively; compared to the *db/db* mice in the model group (n = 3)).

group (FL), and this difference was significant as well ($P < .05$). The protein expression of RAGE in kidney tissues of diabetic mice in the 16-week-old model group (db/db) was increased, approximately 1.8 times that of normal *db/m* mice, and this trend was consistent with the level of AGEs (as shown in Figure 4). After an 8-week intervention, RAGE expression decreased significantly by 28 and 36.2% in the low-dose and high-dose groups (FL and FH), respectively ($P < .01$), and these changes were consistent with the levels of AGEs.

TFC Suppresses AGE/RAGE to Prevent Renal Inflammation in *db/db* Mice

The *db/db* mice exhibited up-regulation of nuclear NF-κB p65 protein compared to *db/m* mice, but TFC treatment down-regulated these increased protein expressions in a concentration-dependent manner ($P < .05$). Additionally, IL-1β protein expressions in vehicle-treated *db/db* mice (db/db) were significantly higher than those in *db/m* mice. TFC-treated *db/db* mice at 120 mg·kg-1 (FH) showed significantly lower expressions of these proteins compared to vehicle-treated *db/db* mice (db/db), even lower than that of metformin-treated *db/db* mice (MET) ($P < .05$) (Figure 5).

TFC Suppresses AGE/RAGE to Prevent Renal Peroxidation in *db/db* Mice

As shown in Figure 6, kidney tissue MDA levels were found to be significantly higher in the db/db group when compared with the control db/m group ($P < .01$), while the values of SOD, CAT, and GSH-PX were significantly lower. On the other hand, TFC caused a significant decrease in the level of MDA in both treatment groups (FL and FH) compared to vehicle-treated *db/db* mice. Additionally, TFC was able to ameliorate the levels of SOD, CAT, and GSH-PX in both treatment groups (FL and FH) compared to the *db/db* model group.

DISCUSSION

In the state of hyperglycemia, osmotic diuresis leads to excessive water loss and intracellular dehydration in kidney tissue, resulting in stimulation of the thirst center and increased water intake. Therefore, there is a proportional relationship between increased urination and higher water consumption during hyperglycemia. This experiment investigated the appearance, body weight, food intake, water intake, and blood glucose levels of *db/db* mice. The results showed that compared to *db/m* mice in the control group, 16-week-old *db/db* mice (model group) had a significant difference in body weight, as well as significantly increased water intake and fasting blood glucose levels. These findings are consistent with previous literature reports.[21] After 8 weeks of consecutive TFC intervention, there were no significant changes observed in food intake or

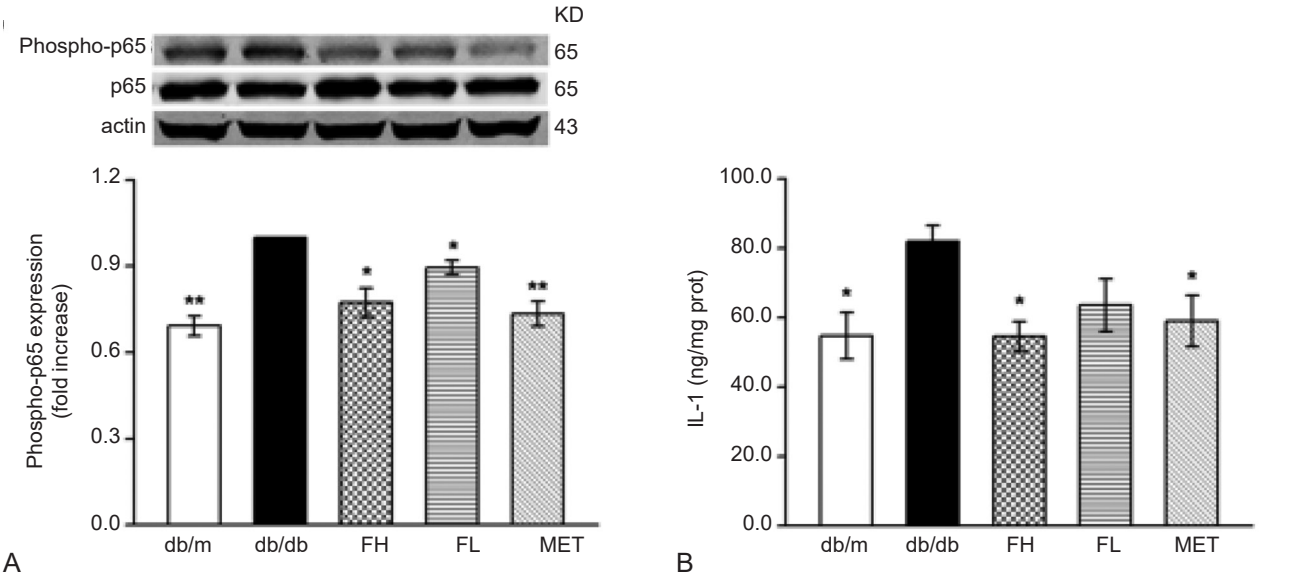

Figure 5. TFC inhibits the expression of NF-κB p65 and IL-1β in the kidneys of *db/db* mice. A) Protein expression and analysis of the NF-κB p65 by western blot, GAPDH was used as the internal control, B) Protein expression and analysis of the IL-1β by ELISA assay). The data are presented as the mean ± SD (**P* < .05 and ***P* < .01; respectively, compared to the *db/db* mice in the model group (n = 6).

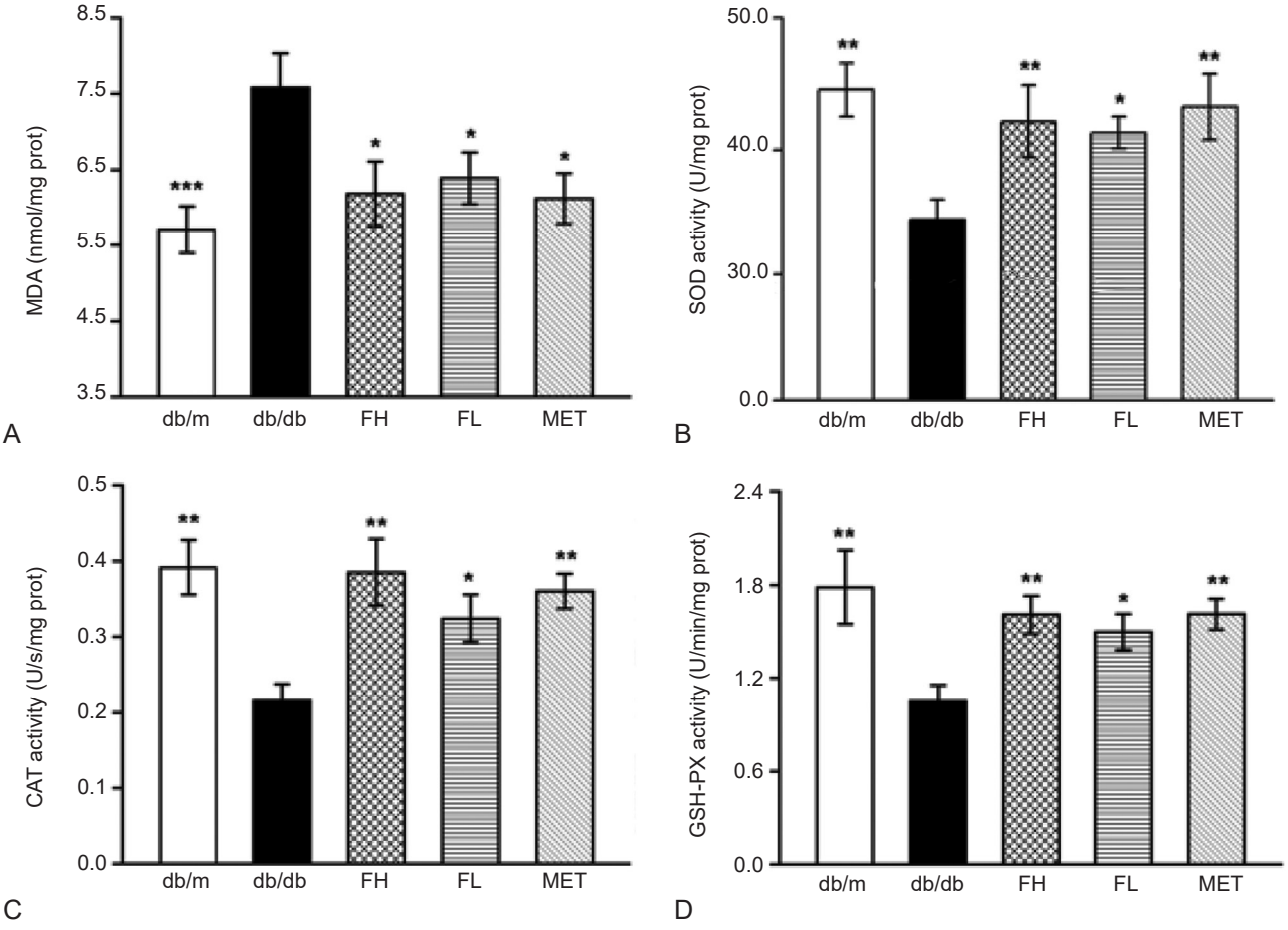

Figure 6. TFC inhibits the levels of MDA and improves the activities of SOD, CAT, and GSH-PX in the kidneys of *db/db* mice. A) The levels of MDA, B) The activity of SOD, C) The activity of CAT, and D) The activity of GSH-PX). The data are presented as the mean ± SD (**P* < .05 and ***P* < .01, respectively; compared to the *db/db* mice in the model group (n = 6)).

body weight of *db/db* mice (model group), but their water intake decreased significantly. The difference between low-dose and high-dose groups (FL and FH) of *db/db* mice reached a significant level, indicating an improvement in diabetes symptoms to some extent.

Glomerular hypertrophy, slight thickening of the basement membrane, and excessive deposition of the glomerular extracellular mesangial matrix are characteristic pathological changes in early DN. If not addressed, this condition progresses to glomerular sclerosis and renal interstitial fibrosis, which leads to a decrease in the glomerular filtration rate and ultimately results in renal failure.[22] *db/db* mice typically exhibit early symptoms of DN at around 2 months of age, making them an ideal model for studying the mechanisms underlying DN.[23] It has been reported that the flavonoids of cymbidium have a certain renal protective effect on diabetic animals, and its ethyl acetate extract (0.15 g/kg) can reduce the levels of blood glucose and lipids in diabetic SD rats, as well as effectively decrease serum CRE and uric acid levels.[24] In this study, 16-week-old *db/db* diabetic mice exhibited significant widening of the mesangial region, increased mesangial matrix, and glycogen deposition in renal tubules. Following TFC intervention, glomerular hypertrophy, mesangial matrix hyperplasia, and basement membrane thickening in kidney tissues were inhibited to a certain extent. Both low and high doses of TFC (FL and FH) effectively interfered with the increase of glomerular area and significantly decreased serum CRE and BUN levels for 8 weeks, which was consistent with the inhibitory effect observed in the MET group. These results indicate that TFC has an effect on improving renal lesions in DN.

Under pathological conditions, the combination of AGEs and RAGE can lead to increased oxidative stress in the kidney, resulting in inflammation and podocyte damage, thereby promoting the progression of kidney disease. Considering the specific mechanism of action of the AGE-RAGE axis, scholars are continuously searching for drugs that can reduce AGE levels in order to alleviate kidney damage caused by AGEs through blocking the signal transduction pathway of the AGE-RAGE axis. It has been found that pyridoxamine and aminoguanidine, as carbonyl scavengers, can inhibit the formation of AGEs, maintain the integrity of mesangium and renal tubules, and reduce oxidative stress in experiments.[25,26] In streptozotocin-induced diabetic mouse models, kidney damage was mitigated by neutralizing RAGE antibody therapy.[27] Additionally, gliclazide alleviates the damage to mesangial cells and tubular epithelial cells by inhibiting the RAGE-P22phox-NF-κB pathway.[28] Recently, it has been discovered that some proprietary Chinese medicine components also have therapeutic effects such as curcumin, ginger, cinnamon, and clove which can inhibit glycosylation in vitro. Their anti-glycosylation potential is related to their polyphenol content.[29] Luo Y *et al.* reported that natural tea polyphenols, especially catechins, regulate the expression of RAGE and subsequent MAPK and TGF-β pathways by blocking the formation of AGEs, significantly alleviating multiple diseases such as aging, DN, and retinopathy.[30] Feng Guo[19] found that continuous intragastric administration of TFC for 8 weeks interfered with and induced oxidative stress, inflammation, and fibrosis in the kidneys of *db/db* mice through the NF-κB/TGF-β/Smad signaling pathway. Marein may be the main component responsible for the anti-inflammatory and anti-renal fibrosis effects of TFC.[31]

In this study, protein glycation damage was observed in the kidney tissues of 16-week-old diabetic mice (db/db), accompanied by oxidative stress and inflammation. The levels of AGEs in the kidneys of the db/db group were significantly increased, along with activation of its cell membrane receptor RAGE and NFκB. Additionally, there was a decrease in antioxidant enzyme activity and an increase in lipid peroxidation product MDA levels, as well as up-regulation of IL-1β expression. After 8 weeks of intragastric administration, both dosage groups (0.4 and 1.2 g/kg) of TFC effectively reduced the levels of AGEs in the kidneys of diabetic *db/db* mice and down-regulated inflammatory signaling mediated by AGE-RAGE binding. Furthermore, NFκB activation was significantly inhibited while MDA levels decreased in the kidney. Partial restoration was observed in SOD, CAT, and GSH-PX activities. These results suggest a certain improvement on DN in *db/db* mice.

CONCLUSIONS

Following 8 weeks of continuous oral administration of TFC to diabetic mice, there was a notable reduction in CRE and BNU levels, indicating improved kidney function. While fasting blood glucose levels were positively impacted, postprandial blood glucose levels did not show a significant decrease. TFC treatment effectively reduced the loss of glomerular cells in diabetic mice, inhibited the enlargement of glomeruli and the proliferation of mesangial matrix, leading to a significant improvement in the pathological structure of the kidneys. Additionally, TFC treatment resulted in decreased expression of AGEs and RAGE in the kidneys, along with a reduction in downstream oxidative stress and inflammation. These findings suggest that TFC holds promise as a potential therapeutic agent for the prevention and treatment of DN. It can be inferred that TFC intervention may down-regulate renal inflammatory signaling mediated by the AGEs-RAGE pathway in response to hyperglycemia, thereby ameliorating kidney damage in *db/db* mice.

COMPLIANCE WITH ETHICS GUIDELINES

Limin Guo: Conceptualization, Formal analysis, Funding acquisition, Investigation, Resources, Validation, Visualization, Writing-original draft, Writing-review and editing.

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Wensheng Zhang: Conceptualization, Resources, Writing-review and editing, Supervision, Project administration.

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