

Mori cortex prevents kidney damage through inhibiting expression of inflammatory factors in the glomerulus in streptozocin-induced diabetic rats

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ABSTRACT

Objective(s): It has been widely reported that *Mori cortex extract* (MCE) is used for the treatment of diabetes mellitus in traditional medicine. The present study was designed to investigate its mechanism of action in the treatment of diabetic nephropathy (DN). We assessed whether MCE preventive treatment ameliorates kidney damage in high-fat diet and streptozotocin (STZ)-induced type 2 diabetic rats.

Materials and Methods: Rats were fed a high-fat diet and injected with STZ. MCE was given to rats daily at 10 g/kg. Fasting blood glucose (FBG) and postprandial plasma glucose were measured. Blood and urine biochemical parameters, renal tissue morphology, and inflammation were investigated.

Results: Prevention with MCE significantly decreased FBG and homoeostasis model assessment (HOMA) of IR (HOMA-IR) levels and increased insulin levels in diabetic rats. MCE prevention significantly decreased levels of KW/BW, BUN, Cr, and 24 hr urinary protein. MCE inhibited glomerular basement membrane thickening, tubular epithelial cell hypertrophy, and glomerular capillary dilation. MCE also prevented the disappearance of Bowman's space and renal tubular lumen and decreased collagen deposition in rat kidney. Moreover, MCE reduced the levels of inflammatory factors (MCP-1 and TNF- α) and fibrosis factors (collagen IV and fibronectin).

Conclusion: MCE prevents DN through inhibition of inflammation and fibrosis in a rat model. It might provide a safe and effective way to prevent DN.

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Introduction

Diabetic nephropathy (DN) is a microvascular complication caused by diabetes, and can severely affect patients' quality of life (1). DN is currently one of the most common causes of end-stage renal disease globally. Unfortunately, up to 40% of people with diabetes may develop kidney complications globally (2). In China, the incidence of DN was 33.6% among diabetic patients (3). The data from International Diabetes Federation showed that more than 415 million people worldwide had diabetes in 2015 and this number was expected to reach 642 million by 2040 (4). Thus there is an urgent need to develop new strategies for the prevention of DN, and in-depth studies of the etiology and pathogenesis of DN are of great significance. There is increasing evidence that inflammation is a cardinal pathogenic mechanism in

DN (5). Extracellular matrix damages, glomerulosclerosis, and renal failure are also associated with the development of DN (6).

Tumor necrosis factor (TNF- α) and monocyte chemoattractant protein-1 (MCP-1) are two cytokines that are promising targets for future clinical applications. In a high glucose environment, the tubular epithelium in the kidney secretes large amounts of a variety of cytokines, including TNF- α , and MCP-1 (7). MCP-1 is a cytokine that plays a central role in tubular fibrosis and glomerulosclerosis, as it promotes renal tissue hypertrophy, stimulates extracellular matrix (ECM) synthesis, inhibits the activity of the proteolytic enzymes and matrix degradation enzymes in the ECM, and promotes ECM deposition (8). TNF- α is a pleiotropic inflammatory cytokine secreted by resident renal cells and infiltrating macrophages. However, TNF- α can be cytotoxic to renal cells and directly cause

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kidney damage. An increase in serum TNF- α concentration induces production of nitric oxide, vasodilation, and hypotension, which may cause ischemia and hypoxia, and ultimately acute tubular necrosis and renal failure (9). Research using isolated rat glomeruli revealed that TNF- α activates NADPH oxidase through PKC/phosphatidylinositol 3-kinase (10). TNF- α also induces apoptosis (11), changes glomerular hemodynamics, and induces kidney cells to produce a variety of reactive oxygen species (10). The diverse biological activities of TNF- α have an important role in the development of diabetic kidney damage.

ECM is mainly composed of collagen and fibronectin (12). In DN, collagen IV is the most abundant collagen, which usually results in a progressive decline in renal function with time when normal tissue is replaced with scar tissue, and it promotes the development of diabetic kidney disease (13). Fibronectin plays an important role in cell adhesion, migration, differentiation, and proliferation (14-16). A long-term high glucose condition stimulates mesangial cells to up-regulate the expression of genes encoding ECM proteins and increase the synthesis rate of these proteins, including fibronectin, laminin, and collagen IV, leading to renal fibrosis (17).

Currently, there is no ideal strategy for the prevention or treatment of DN. Therefore it is desirable to find therapeutic agents that can prevent both the initiation and progression of DN, with few side effects. *Mori cortex* Extract (MCE) is the extract of the root bark of *Moraceae* plants. Recent studies have shown that MCE can improve insulin resistance and lower blood glucose and lipid (17, 18). MCE may protect pancreatic beta cells from degeneration and reduce serum lipid peroxidation (19). MCE can prevent early peripheral neuropathy in diabetic rats, and the overall curative effect is better than that of methylcobalamin (20). However, the mechanisms of MCE's pharmacological function have not been studied in-depth at the cellular and molecular levels. In this study, a DN rat model created with a high-fat diet combined with a low-dose streptozotocin was used to study the protective effect of MCE on renal function and the associated molecular mechanisms. And we report here for the first time that MCE prevents DN.

Materials and Methods

Drugs and chemicals

The raw medicinal herb *Mori cortex* was obtained from the Shanghai Yan-He-Tang Traditional Chinese Medicine Company. The raw herb was botanically authenticated by Prof Yiming Li in the School of Pharmacy, Shanghai University of Traditional Chinese Medicine (Shanghai, China). The voucher specimen (No. MC001) was deposited at the Herbarium of the Department of TCM Chemistry, School of Pharmacy, Shanghai University of

Traditional Chinese Medicine. MC was extracted separately using the following process: one kilogram of *Mori cortex* was reflux extracted with five kilograms of 70% alcohol twice, 90 min each time. The extract solution was allowed to stand overnight, then filtered. The filtrate was concentrated to a small volume under reduced-pressure evaporation and dried by lyophilization. The extract yield after freeze drying was 11.6% and was named MCE. STZ was purchased from Sigma-Aldrich Chemicals Pvt Ltd (USA). All primary antibodies were from Abcam (UK) except for the β -actin primary antibody, which was from Biosynthesis Biotechnology CO, LTD (China). Secondary antibodies were HRP-labeled Goat Anti-Rabbit IgG from Beyotime (China). All other chemicals used were of analytical grade.

Animal experiment and generation of experimental diabetic rats

Male Sprague-Dawley rats aged 8 weeks (weight 250 ± 15 g) were obtained from the Laboratory Animal Center of the Academy of Zhejiang Medical Sciences (Zhejiang, Certificate No 0012371). Experimental protocols were approved by the Animal Care and Use Ethics Committee of the Academy of Zhejiang Medical Sciences. All rats were housed in standard polypropylene cages in a room of 23 ± 1 °C, humidity $50 \pm 10\%$, and a 12-hr light/dark cycle. Rats were used for experiments after one week of acclimation. A schematic diagram of the experimental protocol is given in Figure 1.

The rats were divided into four groups as follows: (1) control (the normal group), (2) MCE (the normal plus MCE group), (3) DM (the diabetes group), and (4) DM+MCE (diabetes plus MCE group). Control and MCE rats were given the standard diet, and DM and DM+MCE rats were fed the high-fat diet during the entire experimental period, which lasted 24 weeks. MCE was given by intragastric gavage at 10 g/kg-d during the 8th-24th weeks, and for comparison, groups control and DM were given normal saline through the same route. Rats in the DM and DM+MCE groups were fasted overnight and then injected a single dose of freshly prepared STZ (40 mg/kg, intraperitoneally) in 0.1 mol/L citrate phosphate buffer (pH 4.5) on the first day of the 8th week; for comparison, control and MCE rats were injected with the citrate buffer. The development of hyperglycemia in rats was confirmed by the measurement of fasting serum glucose 72 hr after STZ injection. Rats with fasting serum glucose levels above 13.89 mmol/l were considered diabetic. By the end of the experimental period, rats were anesthetized with diethyl ether and sacrificed by cervical dislocation. A kidney was removed from each rat and weighed, and half of the kidney was frozen in liquid nitrogen for RNA and protein extractions, while the other half was fixed in 10% formalin for histopathological assessments.

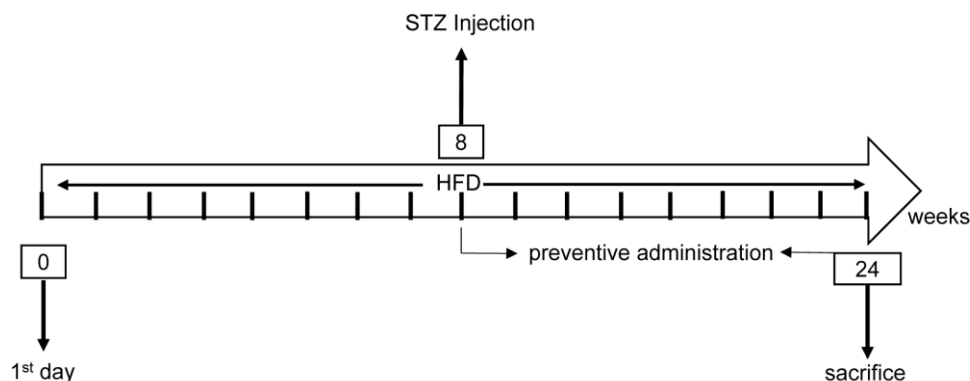


Figure 1. Diagram of the experimental procedure

Measurement of the body weight, blood-glucose, and insulin resistance

Body weight was measured once a month. The FBG, measured after a 12-hr fast, and 2 hr postprandial blood glucose (PBG) were measured once a week from the tail vein using One Touch Ultra test strips and a glucometer (One Touch; Johnson and Johnson, New Brunswick, NJ, USA) in different days, respectively.

Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was performed at the end of the study. Overnight-fasted rats were given glucose (2 g/kg) through oral gavage and subsequently, blood was drawn from the tail vein to measure blood glucose levels at 0, 30, 60, and 120 min using a glucometer. A glucose tolerance curve was created and the area under the curve (AUC) was calculated.

Serum and urine biochemical measurements

Prior to sacrifice, rats were kept individually in metabolic cages and 24-hr urine samples were collected. Blood samples were collected from the retro-orbital sinus. The levels of serum insulin were measured with a Rat Insulin ELISA kit (Millipore, Darmstadt, Germany) using a Scientific Multiskan FC Microplate Photometer (Thermo Scientific, MA, USA). Renal function parameters creatinine (Cr), blood urea nitrogen (BUN), and 24-hr urinary protein were analyzed with a MODULAR P800 Automation Biochemist Analyzer (Roche, Basel, Switzerland). HOMA-IR was calculated by the HOMA method using the following equation: IR (HOMA-IR) = fasting glucose (mmol/l) * fasting insulin (μ IU/ml))/22.5.

Quantitative real-time pcr

Total RNA was extracted from frozen renal tissues using the TRIzol reagent (Invitrogen, USA). cDNA was synthesized from RNA using a commercial kit (Cwbio, Beijing, China). Real-time quantitative polymerase chain reaction (qPCR) was conducted using the Light-Cycler 480 (Roche, USA) instrument with the

Light Cycler 480 SYBR Green I Master kit (Roche) according to the manufacturer's instructions. Gene expression was normalized to β -actin mRNA levels and the $2^{-\Delta\Delta CT}$ method was used to determine the relative levels of mRNA. qPCR was performed in duplicates. PCR primers used were as follows: TNF- α , forward 5'-GGTGATCGGTCCCAACAAGGA-3' and reverse 5'-CAGCTGGCTCAGCCACTC-3'; MCP-1, forward 5'-CTATGCAGGTCTCTGTCACGCTTC-3' and reverse 5'-CAGCCGACTCATTGGGATCA-3'; collagen IV, forward 5'-CCCCTGTAAGACACCATTAGC-3' and reverse 5'-AGCACCCAGTTTGTAGTTGTCC-3'; fibronectin, forward 5'-AACAAATCTCCTGCCTGGGACTGA-3' and reverse 5'-TGAGTTGGCGGTGACATCAGAAGA-3'; and β -actin, forward 5'-CTGAACCCTAAGGCCAACCG-3' and reverse 5'-GACCAGAGGCATACAGGGACAA-3'. Thermal cycling conditions were 95 °C for a 5 min initial denaturation step followed by 45 cycles of 94 °C for 10 sec, 58 °C for 20 sec, and 72 °C for 30 sec.

Histological examination

Formalin (10%)-fixed kidney tissues were embedded in paraffin, and serial sections (5 μ m) were cut using a microtome (Leica RM 2125; Leica, Germany). Sections were stained with hematoxylin and eosin (H&E), Periodic acid-Schiff stain (PAS), and Masson trichrome and at least ten fields per slide were observed under a light microscope (Nikon, Tokyo, Japan).

Western blot

For Western blot assays, renal proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat dried milk dissolved in TBS buffer containing 0.03% Tween 20 for 1 hr and then incubated with a primary antibody at 4 °C overnight. The membranes were washed with TBS buffer containing 0.3% Tween 20, and then incubated with Goat anti-rabbit IgG-HRP:2000 at 37 °C for 1 hr. The immunoreactions were visualized with enhanced chemiluminescent (ECL) detection reagent (Advansta,

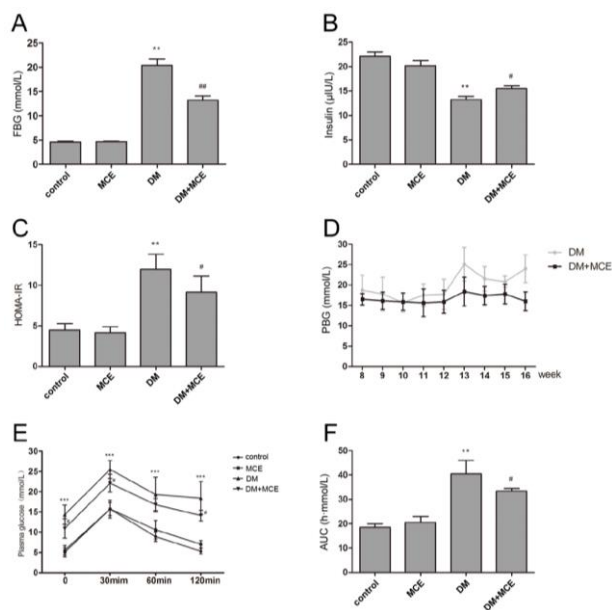


Figure 2. Preventive effect of *Mori cortex* extract on diabetic rats. FBG (A), plasma insulin (B), HOMA-IR (C), PBG (D), OGTT (E), and AUC (F) are shown. Control: Normal control; MCE: Normal control + *Mori cortex*; DM: type 2 diabetes mellitus (T2DM); DM+MCE: T2DM + *Mori cortex*. FBG: Fasting blood glucose; HOMA-IR: Homeostasis model assessment of IR; PBG: Postprandial blood glucose; OGTT: Oral glucose tolerance test; AUC: Area under the curve. Data are presented as mean±SEM (n = 6). * $P < 0.05$, ** $P < 0.01$ vs control; # $P < 0.05$, ## $P < 0.01$ vs DM

USA) and detected with a gel imaging and analysis system (Tanon, China). The density values of protein bands were quantified using the Image J program (NIH, MD, USA).

Immunohistochemistry

Immunohistochemistry for collagen IV, fibronectin, TNF- α , and MCP-1 was performed in rat kidney paraffin sections. These sections were dewaxed by dimethyl benzene and dehydrated with graded ethanol. Then the sections were blocked with 5% serum for 30 min at room temperature; antigen retrieval was performed by heating the sections in a 10 mM citrate buffer (pH 6.0). Samples were incubated with primary antibody (1:200 in PBS+1%BSA) for 1 hr at 37 °C. Thereafter, goat anti-rabbit IgG-HRP antibody was added and incubated for 1 hr at room temperature. The color reaction was developed by incubating the sections with diaminobenzidine (DAB, Boster, China) and the sections were counterstained with hematoxylin. Quantitative analysis of the brown positive staining in the glomerulus and tubules was performed using Image-Pro plus 6.0 (Media Cybernetics, USA).

Statistical analysis

Data are expressed as mean±SEM. Analyses were performed using SPSS 13.0. Group comparisons were conducted with one-way ANOVA followed by the Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant.

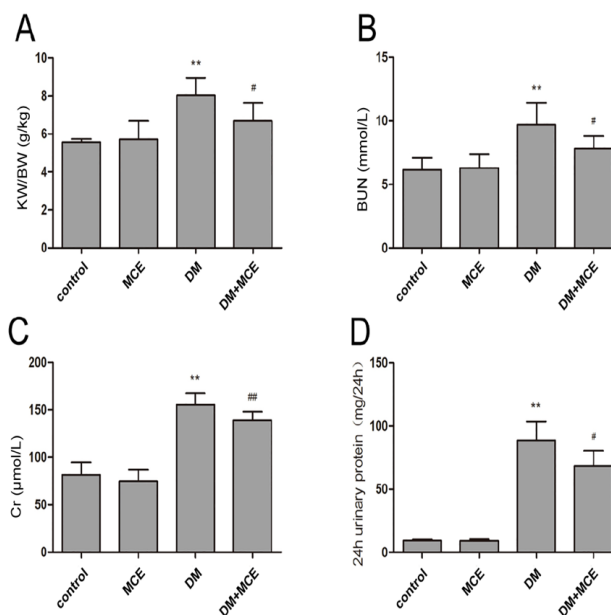


Figure 3. Renal function in different groups. KW/BW (A) and the levels of BUN (B), Cr (C), and 24-hr urinary protein (D) are shown. Control: Normal control; MCE: Normal control + *Mori cortex*; DM: Type 2 diabetes mellitus (T2DM); DM+MCE: T2DM + *Mori cortex*. KW/BW: kidney weight to body weight ratio; BUN: blood urea nitrogen; Cr: creatinine. Data are presented as mean ± SEM (n = 6). * $P < 0.05$, ** $P < 0.01$ vs control; # $P < 0.05$, ## $P < 0.01$ vs DM

Results

Mori cortex extract has a preventive effect on diabetes

HOMA-IR and OGTT were used to monitor the development of diabetes. Prevention with MCE in diabetic rats significantly decreased FBG ($P < 0.01$) and HOMA-IR ($P < 0.05$) and increased insulin levels compared with saline-treated diabetic rats (Figure 2A-C). MCE treatment also stabilized blood glucose (Figure 2D). In the OGTT, the blood glucose level was higher ($P < 0.05$) at all the time points after glucose administration in the diabetic rats compared with normal rats (Figure 2E), indicating that diabetic control rats developed glucose intolerance and IR. Compared with the DM group, the DM+MCE group had significantly ($P < 0.05$) reduced AUC for OGTT (Figure 2F), suggesting that MCE can be beneficial against diabetes.

Mori cortex extract improves renal function in rats

BUN, Cr, and 24-hr urinary protein were used to measure renal function. Kidney weight to body weight ratio (KW/BW), BUN, Cr, and 24-hr urinary protein in the DM group were higher ($P < 0.05$) than in the control group. However, MCE treatment significantly decreased KW/BW ($P < 0.05$), BUN ($P < 0.05$), Cr ($P < 0.01$), and 24-hr urinary protein ($P < 0.05$) in diabetic rats (Figure 3A-D).

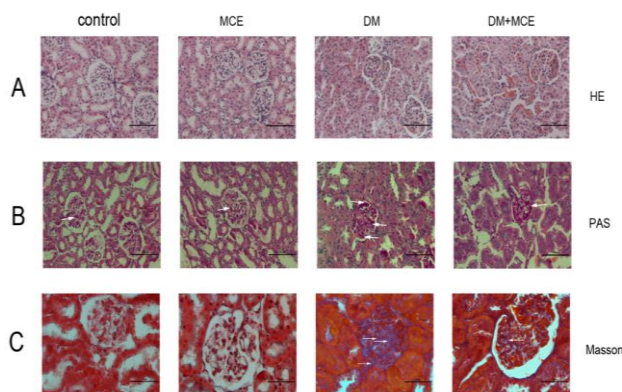


Figure 4. Histological assessment of the experiment rat kidneys H&E (A, 20 \times , scale bar 50 μ m); PAS (B, 20 \times , scale bar 50 μ m); Masson (C, 40 \times , scale bar 100 μ m). Control: Normal control; MCE: Normal control + *Mori cortex*; DM: Type 2 diabetes mellitus (T2DM); DM+MCE: T2DM + *Mori cortex*. Data are presented as mean \pm SEM (n=6). * P <0.05, ** P <0.01 vs control; # P <0.05, ## P <0.01 vs DM, white \rightarrow glycogen; black \rightarrow collagen

Mori cortex extract protects the kidneys from fibrosis

Renal morphologies of rats in different treatment groups are shown in Figure 4. Rats treated with MCE alone showed normal kidney structure, similar to that of normal rats. Significantly thickened glomerular basement membranes, tubular epithelial cell hypertrophy, and glomerular capillary dilation were observed in diabetic rat kidneys. In addition, bowman's space and renal tubular lumen disappeared while collagen deposition was evident in diabetic rats. MCE preventive treatment reversed these changes to some extent. Compared with those in the DM group, the bowman's capsule space and tubular lumen in the DM+MCE group were wider, and glomerular capillaries and tubular hypertrophy were decreased (Figure 4A).

Glycogen was assessed by PAS staining of rat kidney sections. PAS staining showed that basement membrane glycogen was increased; in other words, basement membrane was thicker in diabetic rat kidneys. In contrast, the kidney morphology of MCE-treated diabetic rats was greatly improved (Figure 4B). Masson trichrome staining showed substantial collagen deposition in diabetic rats, whereas MCE treatment in diabetic rats reduced the amount of collagen (Figure 4C).

Mori cortex extract reduces inflammation

The expression of MCP-1 and TNF- α in rat kidneys was measured by RT-PCR and immunohistochemistry. The expression of MCP-1 and TNF- α was significantly increased (P <0.05) in the diabetic rats in comparison to normal rats, at both the mRNA and protein levels (Figure 5). MCE treatment reduced MCP-1 and TNF- α expression (Figure 5). Thus MCE reversed the increase in the expression of inflammatory cytokines in DN.

Mori cortex extract prevents kidney fibrosis

Expression of fibrosis-related proteins in the kidney of all groups was measured using immunohistochemistry and RT-PCR. The levels of collagen IV and

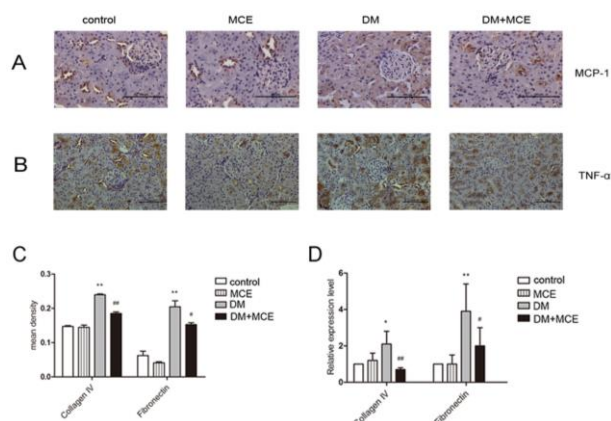


Figure 5. Suppressive effect of *Mori cortex* extract on MCP-1 and TNF- α expression. Immunohistochemical staining of MCP-1 and TNF- α (A-B, 20 \times , scale bar 50 μ m); Panel C represents mean density of MCP-1 and TNF- α . Panel D represents mRNA levels of MCP-1 and TNF- α . Control: Normal control; MCE: Normal control + *Mori Cortex*; DM: type 2 diabetes mellitus (T2DM); DM+MCE: T2DM + *Mori cortex*. Data are presented as mean \pm SEM (n=6). * P <0.05, ** P <0.01 vs control; # P <0.05, ## P <0.01 vs DM

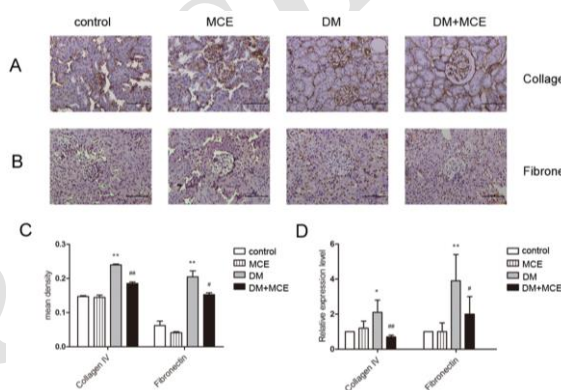


Figure 6. *Mori cortex* extract inhibited collagen IV and fibronectin expression in renal tissues. Immunohistochemical staining of collagen IV and fibronectin (A-B, 20 \times , scale bar 50 μ m); Panel C represents mean density of collagen IV and fibronectin. Panel D represents mRNA levels of collagen IV and fibronectin. Control: Normal control; MCE: Normal control + *Mori cortex*; DM: Type 2 diabetes mellitus (T2DM); DM+MCE: T2DM + *Mori cortex*. Data are presented as mean \pm SEM (n=6). * P <0.05, ** P <0.01 vs control; # P <0.05, ## P <0.01 vs DM

fibronectin were higher in diabetic rats as compared with normal rats (Figure 6). MCE treatment significantly reduced the expression of collagen IV (P <0.01) and fibronectin (P <0.05) in diabetic rats (Figure 6).

Discussion

In the present study, we demonstrate that MCE prevented the development of DN in HFD/STZ-induced type 2 diabetic rats. Many researchers have used HFD/STZ-induced type 2 diabetic rats as a model of DN because they show characteristics of the human DN (21, 22). In this study, we successfully replicated DN using this model.

At the end of the experiment, KW/BW, BUN, Cr, and 24-hr urinary protein were significantly higher in diabetic group rats than in normal rats, indicating

impaired kidney function. Compared with the untreated diabetic rats, MCE-treated diabetic rats had significantly reduced KW/BW, BUN, serum Cr, and 24-hr urine protein, indicating that MCE can improve renal function in diabetic rats.

In addition, MCE improved glomerular and tubular structures in this diabetic kidney damage model. Moreover, MCE had no adverse effects on normal rat kidneys. The effect of MCE against DN is likely a collective result of multiple factors. Here we further show that MCE can reduce the increase in the expression of inflammatory cytokines MCP-1 and TNF- α and extracellular matrix components fibronectin and collagen IV.

MCP-1 is a chemokine that plays a key role in the progression of diabetic renal injury. MCP-1 induces monocyte to migrate to sites with the highest expression of MCP-1 and differentiate into macrophages (23). MCP-1 augments extracellular matrix production and tubule-interstitial fibrosis (23). Researchers have found that patients with diabetes produce more MCP-1 in the kidney than healthy subjects and that urine MCP-1 levels can be used to assess renal inflammation in diabetic patients (24, 25). In addition, rodent studies have identified MCP-1 as an important therapeutic target for treating DN (26). Experiments using animal models have shown that kidney MCP-1 is increased during the early stages of DN development and is most abundant in the tubules (27). MCP-1-deficient diabetic kidneys had reduced accumulation of interstitial myofibroblasts and deposition of glomerular and interstitial collagen IV in a mouse model (27, 28). MCP-1 deficiency also reduces glomerular fibronectin protein production as well as mRNA and protein levels of fibronectin in diabetic mice (29). TNF- α levels rise in the glomeruli of diabetic rats shortly after STZ injection (30). Many studies have proven that TNF- α plays a pivotal role in experimental diabetic kidney disease and suggested that TNF- α may contribute to proteinuria and glomerular sclerosis in DN (31, 32). In the present study we found that MCE suppressed the expression of MCP-1 and TNF- α . Thus MCE might protect kidney function by reducing renal inflammation.

Another main finding of our study is that MCE inhibited collagen IV and fibronectin expression. Collagen IV and fibronectin are important components of the mesangial matrix and are often used as an indicator of accumulation of extracellular matrix. Collagen IV and fibronectin were significantly higher in human mesangial cells grown under a high glucose condition than those grown under a normal glucose condition, suggesting that high glucose can stimulate human mesangial cells to secrete extracellular matrix (33). The present study showed that collagen IV and fibronectin mRNA expression, as well as their protein accumulation, were significantly increased in the renal tissues of diabetic rats compared with normal rats.

After MCE preventive treatment, the expression of collagen IV and fibronectin decreased in glomerular mesangial cells, and the accumulation of extracellular matrix was reduced, suggesting that MCE might prevent or delay the process of glomerular sclerosis.

Our results showed that MCE treatment inhibited MCP-1 and TNF- α expression and significantly reduced collagen IV and fibronectin expression in a rat model. MCE may prevent DN through these mechanisms. To the best of our knowledge, this is the first study to demonstrate the renoprotective effect of MCE in experimental diabetic rats.

Conclusion

In a rat model of type 2 diabetes, MCE treatment prevented the development of DN, possibly via inhibiting the expression of MCP-1, TNF- α , collagen IV, and fibronectin.

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