The Effect of Moringa Isothiocyanate-1 on Renal Damage in Diabetic Nephropathy

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Introduction. Diabetic nephropathy (DN) is the most common clinical complication of diabetes mellitus. Moringa isothiocyanate-1 (MIC-1) is effective in the treatment of diabetes mellitus, but its mechanism of action in DN remains obscure. This research specifically probed the role of MIC-1 in modulating renal injury in DN.

Methods. Six db/m mice were assigned to control group and twelve db/db mice were randomly allocated to the db/db and db/db + MIC-1 groups. The body and kidney weights of the mice were monitored. Renal function indicators and oxidative stress-related markers were assessed by automatic biochemical analyzer and ELISA method. The pathological changes, apoptosis of renal tissues, extracellular regulated protein kinases (ERK) 1/2/ Nuclear factor erythroid2-related factor 2 (Nrf2) pathway-related markers, and the positive expressions of podocalyxin (Pod) and synaptopodin (Syn) were measured by H&E, PAS, and TUNEL staining, Western blot, and IHC assay.

Results. MIC-1 reduced the body and kidney weights, and increased the kidney organ index (calculated as 100*kidney weight/ body weight) in db/db mice. In addition, MIC-1 improved renal function, kidney tissue injury, and apoptosis of db/db mice. MIC-1 noticeably repressed the contents of reactive oxygen species (ROS) and malondialdehyde (MDA) and enhanced the contents of (glutathione) GSH, superoxide dismutase (SOD), and catalase (CAT) in db/db mice. At molecular level, db/db mice showed a decrease in p-ERK/ERK, Nrf2, SOD-1, heme oxygenase 1 (HO-1), and CAT and an increase in p- inhibitor kappa B alpha (IKBα) and p-Nuclear factor-kappa B (P65/P65), which were reversed when MIC-1 was administered. Furthermore, MIC-1 facilitated the positive expressions of Pod and Syn of the kidney tissues in db/db mice.

Conclusion. MIC-1 reduces oxidative stress and renal injury by activating the ERK/Nrf2/HO-1 signaling and repressing the NF-κB signaling in db/db mice.

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Original Paper

INTRODUCTION

Diabetic nephropathy (DN) is the most common complication of diabetes mellitus, in which kidney damage is caused by the disorders of glucose and lipid metabolism due to persistent hyperglycemia.[1] DN is a significant contributor to renal failure, causing approximately 40% of new end-stage kidney disease (ESKD).[2] In China, the incidence and

prevalence of DN have dramatically increased over the past decade.[3] By 2040, it is expected that the number of people with diabetes will increase to 642 million worldwide, of whom 30 to 40% will develop DN.[4] DN is characterized by glomerular sclerosis, renal vascular degeneration, and tubulointerstitial fibrosis, resulting from microvascular lesions due to chronic hyperglycemia.[5] The clinical manifestations of DN include edema, proteinuria, and hypertension, which are the key factors in the progression of the patients' disease to ESKD and renal failure.[6] Currently, therapeutic strategies for DN concentrate on the control of blood glucose and lipids, blood pressure, and suppression of the renin-angiotensin system; however, these approaches have not thoroughly reduced the prevalence of DN.[7, 8] Therefore, the development of medications with low toxicity and high efficiency for prevention or treatment of DN is becoming innovative.

Increasing evidence suggests that numerous types of traditional Chinese medicine (TCM) were used to treat DN, including berberine, *Astragali Radix*, and *Moringa oleifera Lam*.[9-11] *Moringa oleifera Lam.* is a cruciferous plant that belongs to the Moringaceae family.[12] Moringa Oleifera seeds (MOS) are rich in a variety of active ingredients, which are very useful for human health. MOS isothiocyanate derivatives have biological activities, such as anti-inflammatory, anti-oxidative, anti-bacterial and anti-diabetic effects.[12-14] Moringa isothiocyanate-1 (MIC-1) is the major isothiocyanate extract of MOS.[15] Some researchers have pointed out that MOS extract could restrain oxidative stress and renal fibrosis via the activation of the GSK-3β and Nrf2/HO-1 pathways, thereby protecting kidney function.[16] Waterman *et al.* reported that MIC was the primary moringa concentrate with anti-obesity and anti-diabetic biological activity.[17] According to the findings of Cheng *et al.*, MIC-1 could activate the anti-oxidant response system *in vivo* by activating the NRF2-ARE pathway, leading to the improvement of DN.[18] However, the mechanism of action of MIC-1 on DN is still unclear and needs to be explored.

In this study, C57BLKS/J db/m mice and C57BLKS/J db/db mice were chosen as the research objects. This research intended to check the effect and mechanism of MIC-1 on DN, in order to provide a scientific basis for the use of MIC-1 in the treatment of DN.

MATERIALS AND METHODS

Ethics Statement

All procedures involving animal study was conducted in accordance with the Institutional Animal Care and Use Committee. Animals were maintained as granted by the Ethics Committee of Zhejiang Eyong Pharmaceutical Research and Development Center.

Animals and Treatment

Male C57BLKS/J db/m mice (n = 6) and C57BLKS/J db/db mice (n = 12), aged 8-weeks, were procured form Shanghai SLAC Laboratory Animal Co., Ltd. All mice were fed in standard conditions (temperature at 25 ± 2°C, humidness at 55 ± 5%, and 12 hours (h) day-night cycle) with free standard food and tap water.

Six db/m mice were assigned to control group and twelve db/db mice were randomly allocated to two groups of db/db (n = 6) and db/db + MIC-1 (n = 6). In the db/db + MIC-1 group, the mice were subjected to receiving MIC-1 (100 mg/kg/d, oral) in 10% dimethyl sulfoxide (DMSO, D8372, Solarbio, China) for 21 days. In the db/m group and db/db group, the mice were subjected to receiving excipient solution in the same manner. Fasting blood glucose (FBG) was measured by using a blood glucose meter (580, Yuwell, China). After 21 days, we used a metabolic cage to collect 24 hours urine sample from each mouse. The 24 hours urine albumin was obtained with the use of a liquid protein extraction reagent (P1255) supplied from Applygen (China).

Sample Collection

After 21 days, blood samples were collected from the right ventricle of mice by cardiac puncture under anesthesia. The serum was obtained after centrifuging the blood at 4 °C, 1000 g, for 20 minutes. In addition, one of the kidneys was resected and weighed after euthanasia. The kidney organ index was calculated by the formula: kidney organ index = 100*kidney weight/body weight. The kidney tissues were fixed in 4% paraformaldehyde (PFA, M002, GEFAN, China) for histopathological assay, TUNEL assay, and immunohistochemistry (IHC) assay. After being dehydrated and paraffin-wax-embedded, the renal tissues were sectioned (4 μm) and were placed at -80°C for ELISA and western blot tests.

Assessment of Renal Function

Serum creatinine (Scr), blood urea nitrogen

(BUN), insulin, and uric acid were evaluated by using an automatic biochemical analyzer (AU680), acquired from Beckman (USA). Insulin resistance (IR) was calculated according to homeostatic model assessment (HOMA): HOMA-IR = fasting insulin (FINS) × fasting plasma glucose (FBG)/22.5.

Kidney Histopathological Analysis

Servicebio (China) supplied H&E (G1003) and Periodic Acid-Schiff (PAS) Kits (G1008). The renal tissue slices were dewaxed by xylene (37 ℃, 10 min., 3 times) and then dehydrated in gradient alcohols (70 to 100%). Then, the slices were stained with the use of H&E and PAS Kits, respectively. After being dehydrated, the slices were cover-slipped with Glycerol Jelly Mounting Medium (M085, GEFAN, China). Finally, the slices were examined and captured with the help of an optical microscope (Eclipse E100, Nikon, Japan).

Evaluation of Oxidative Stress-associated Factors

MDA assay kit (MM-0388M1), ROS assay kit (MM-043700M1), GSH assay kit (MM-0758M1), SOD assay kit (MM-0389M1), and CAT assay kit (MM-44125M1) were provided by Meimian (China). The renal tissues were subjected to lysate (R0010, Solarbio, China) and homogenized, followed by centrifugation. The supernatant was separated. The contents of ROS, MDA, GSH, SOD, and CAT were determined with the use of the corresponding kits.

Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick-End Labeling (TUNEL) Assay

TUNEL Kit (G1501) was provided by Servicebio (China). The dewaxed and hydrated renal tissue slices were exposed to proteinase K (G1205, Servicebio, China) for 20 min. (37 ℃), which were then reacted with the prepared TUNEL solution for 60 min. (37 ℃). DAPI solution (G1012, Servicebio, China) was added afterwards. After being dehydrated, the dehydrated slices were cover-slipped with Glycerol Jelly Mounting Medium. Finally, apoptosis in the renal tissues was examined and a photograph was taken with the use of an optical microscope.

Western Blot Test

Tissue lysis was performed with the use of a Radio immunoprecipitation assay (RIPA) buffer (P0013D, Beyotime, China) to obtain the total protein. Furthermore, a BCA kit (pc0020, Solarbio, China) was used for quantification of protein. Subsequently, the quantified proteins were subjected to denaturation and electrophoresis. The protein in the gel was blotted onto nitrocellulose membranes, which was then sealed with 5% non-fat milk at 37 ℃ for 90 min. The blocked membranes were immersed in primary antibodies at 4 ℃ (overnight) followed by intervention with goat anti-rabbit secondary antibody (sc-2004, SCB, USA) (37 ℃, 60 min.). Electrochemiluminescence (ECL) luminescence reagent (E266188, Aladdin, China) was added for protein visualization. Later, the results were analyzed with the help of a gel imaging system (610020-9Q, Clinx, China). The primary antibodies of p-ERK (1:1000, ab47310), ERK (1:1000, ab32537), Nrf2 (1:1000, ab92946), SOD-1 (1:50000, ab51254), HO-1 (1:50000, ab68477), CAT (1:2000, ab209211), P65 (1:10000, ab16502), IKBα (1:80000, ab32518), and GAPDH (1:10000, ab181603) were acquired from Abcam (UK). Affinity (USA) provided p-IKBα antibody (1:2000, AF2002) and p-P65 antibody (1:1000, AF2006).

IHC Assay

The dewaxed and hydrated renal tissue slices underwent antigen repair with citric acid antigen repair solution (G1202, servicebio, China). Next, the slices were immersed in 3% H_2O_2 to block endogenous peroxidase activity, which were then blocked with BSA (G5001, Servicebio, China). The blocked slices were probed with podocalyxin (Pod) antibody (1:200, abx129536, abbexa, USA) and synaptopodin (Syn) antibody (1:200, abx005849, abbexa, USA) at 4 ℃, overnight. A further one-hour reaction was performed after the addition of HRP-labeled secondary antibody (S0001, Affinity, USA). The slices were then developed with the use of DAB (G1211, Servicebio, China). Hematoxylin staining solution was applied for staining the nucleus and nuclear material. The stained slices were placed in the optical microscope after sealing and photographed.

Statistical Analysis

All experiments were performed at least three times and data were analyzed by SPSS software (version 16.0, IBM, USA). The contrast among multiple groups was done by a one-way ANOVA. Comparison between groups was performed by

Student-Newman-Keuls (SNK) test. Kruskal-Wallis H test was applied for heterogeneity of variance. Data were expressed as mean ± standard deviation. $P < .05$ would suggest that the difference was statistically significant.

RESULTS

MIC-1 Reduced the Body Weight and Kidney Weight and Increased Kidney Organ Index in db/db Mice

We measured the body and kidney weights and calculated the kidney index of db/db mice. The results showed that the body and kidney weights were higher and the kidney index was lower in the db/db group compared to the db/m group (Figure 1A to 1C, $P < .01$). MIC-1 administration effectively decreased the body and kidney weights and enhanced the kidney index of db/db mice (Figure 1A to 1C, $P < .05$).

MIC-1 Ameliorated Renal Dysfunction in db/db Mice

Biochemical indicators of each group are presented in Figure 2A to 2F. As it is seen, the levels of Scr, BUN, insulin, uric acid, 24 hours

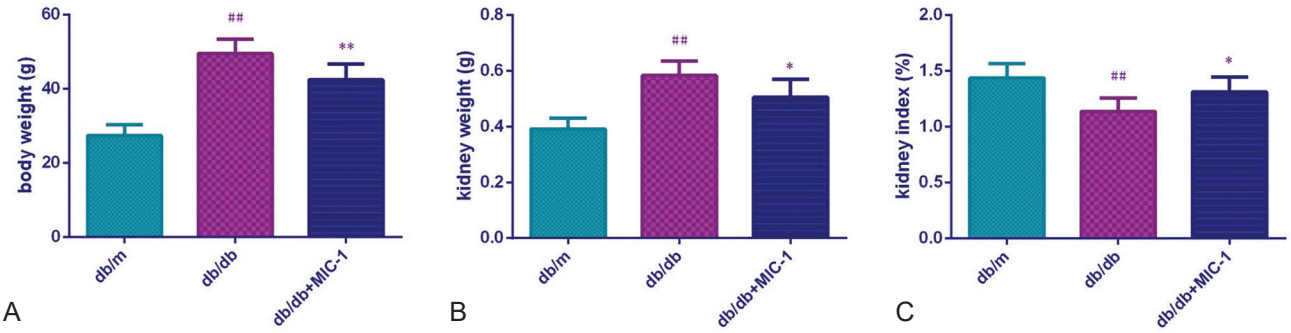

Figure 1. MIC-1 weakened the body weight and kidney weight and increased kidney organ index of db/db mice [The body weight (A), kidney weight (B), and kidney/body weight ratio of mice (C) in each group were assessed]. Data were described as mean ± SD (n = 6) (##P < .01 vs. db/m group; *P < .05, **P < .01 vs. db/db group).

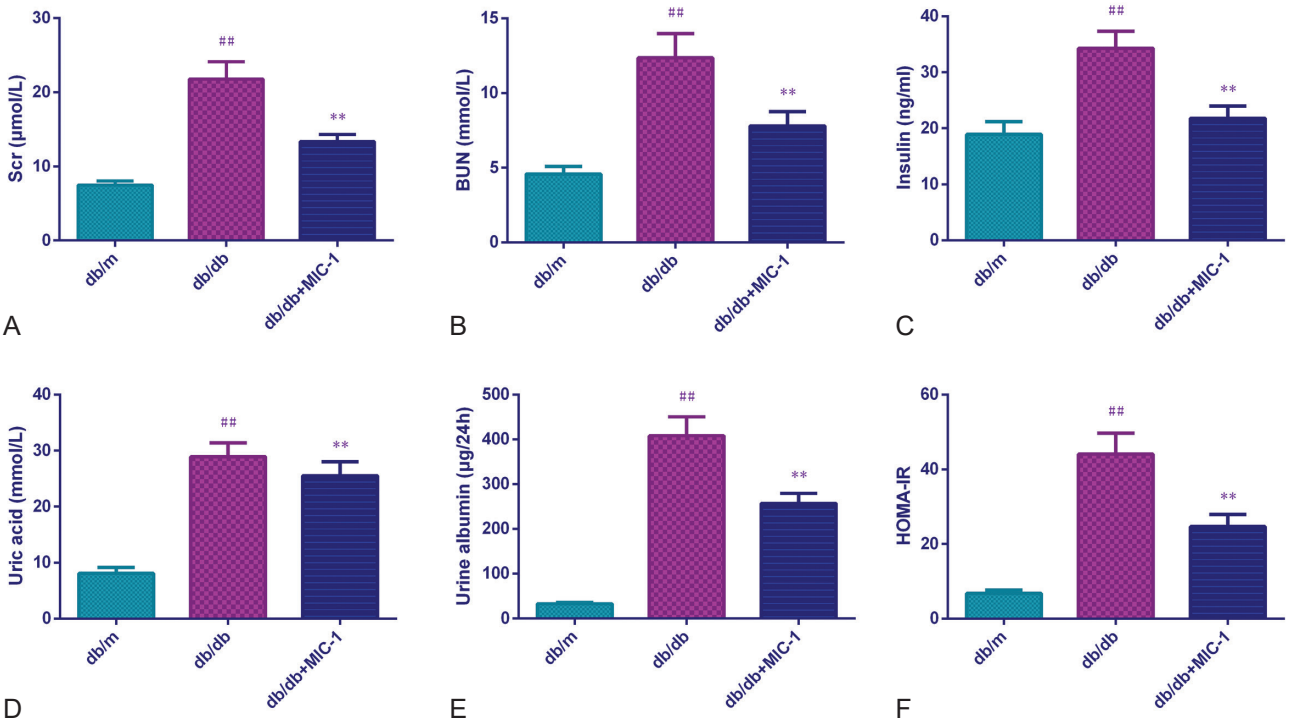

Figure 2. MIC-1 decreased renal dysfunction of db/db mice [The content of serum creatinine (Scr) (A), blood urea nitrogen (BUN) (B), insulin (C), uric acid (D), urine albumin (E), and HOMA-IR (F) were measured in db/m mice and db/db mice treated with or without moringa isothiocyanate-1 (MIC-1) were measured]. Quantitative data were described as mean ± SD (n = 6) (##P < .01 vs. db/m group; **P < .01 vs. db/db group).

urine albumin, and HOMA-IR in the db/db group are extremely elevated compared with the db/m group ($P < .01$). Notably, abnormalities of these biochemical indices tended to turn normal after treatment with MIC-1 (Figure 2A to 2F, $P < .01$).

MIC-1 Alleviated Kidney Histopathological Damage in db/db Mice

Histopathological staining was exploited to assess kidney damage (Figure 3). H&E staining showed that the glomerular structure of the db/m mice was intact, without obvious abnormality (Figure 3A). The db/db mice renal tissue displayed glomerular hypercellularity and hypertrophy, while MIC-1 administration mitigated the above-mentioned histopathological changes in db/db mice (Figure 3A). The result of PAS staining revealed that the mice in the db/db group exhibited glomerular basement membrane thickening, extracellular matrix accumulation, and mesangial hyperplasia, whereas MIC-1 treatment prominently alleviated the histopathological injury (Figure 3B).

MIC-1 Inhibited Oxidative Stress of db/db Mice

Oxidative stress-related markers in mice kidney tissues were determined by ELISA method. The elevated levels of ROS and MDA, and the reduced levels of GSH, SOD, and CAT were observed in the db/db group (Figure 4A to 4E, $P < .01$). MIC-1 treatment obviously decreased the levels of ROS and MDA and increased the levels of GSH, SOD, and CAT in db/db mice (Figure 4A to 4E, $P < .05$ or $P < .01$).

MIC-1 Restrained Apoptosis of db/db Mice

The apoptosis of the kidney tissues was evaluated

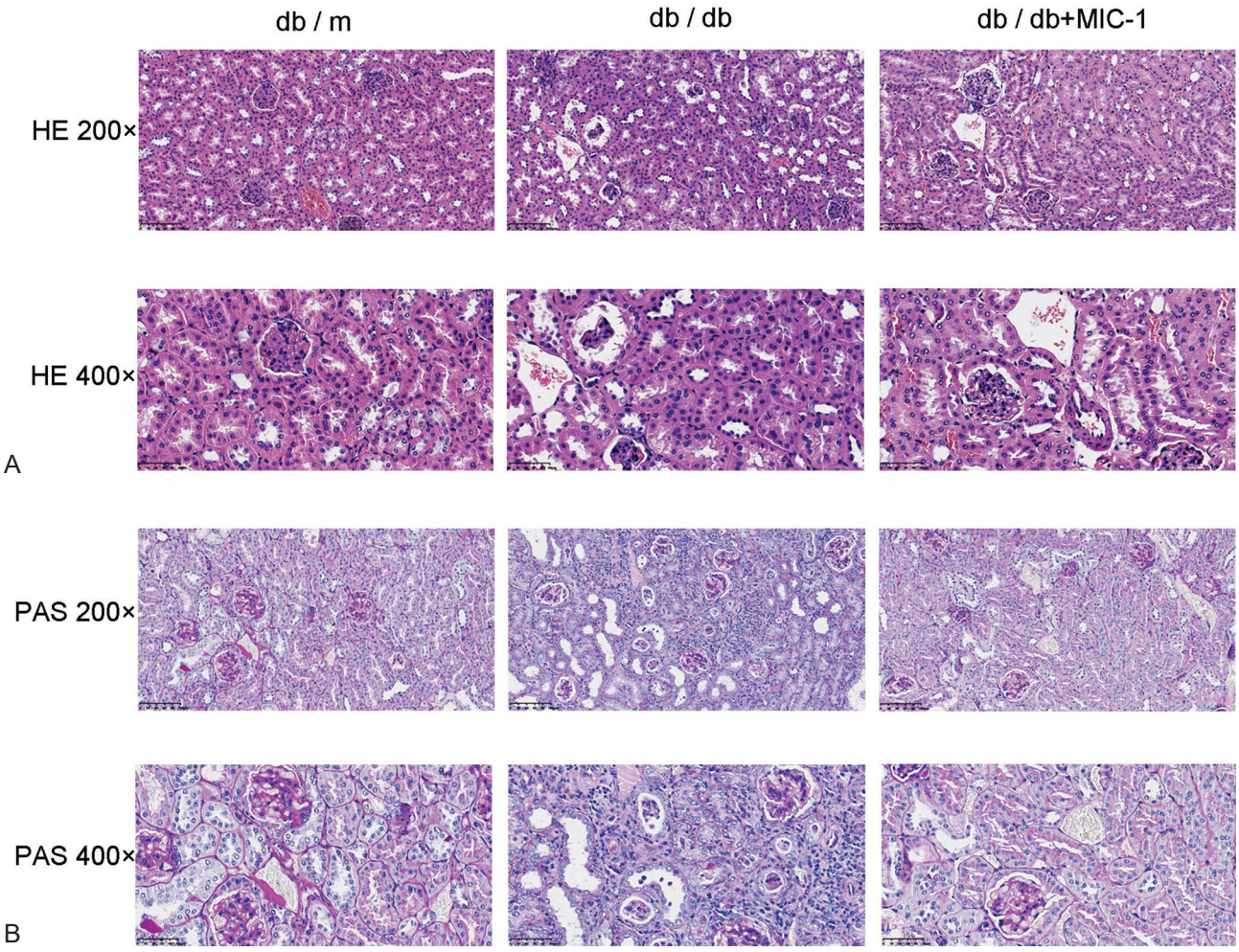

Figure 3. MIC-1 alleviated kidney damage in db/db mice [The kidney tissue injury of db/db mice was assessed by H&E staining (A) and Periodic Acid-Schiff (PAS) staining (B)].

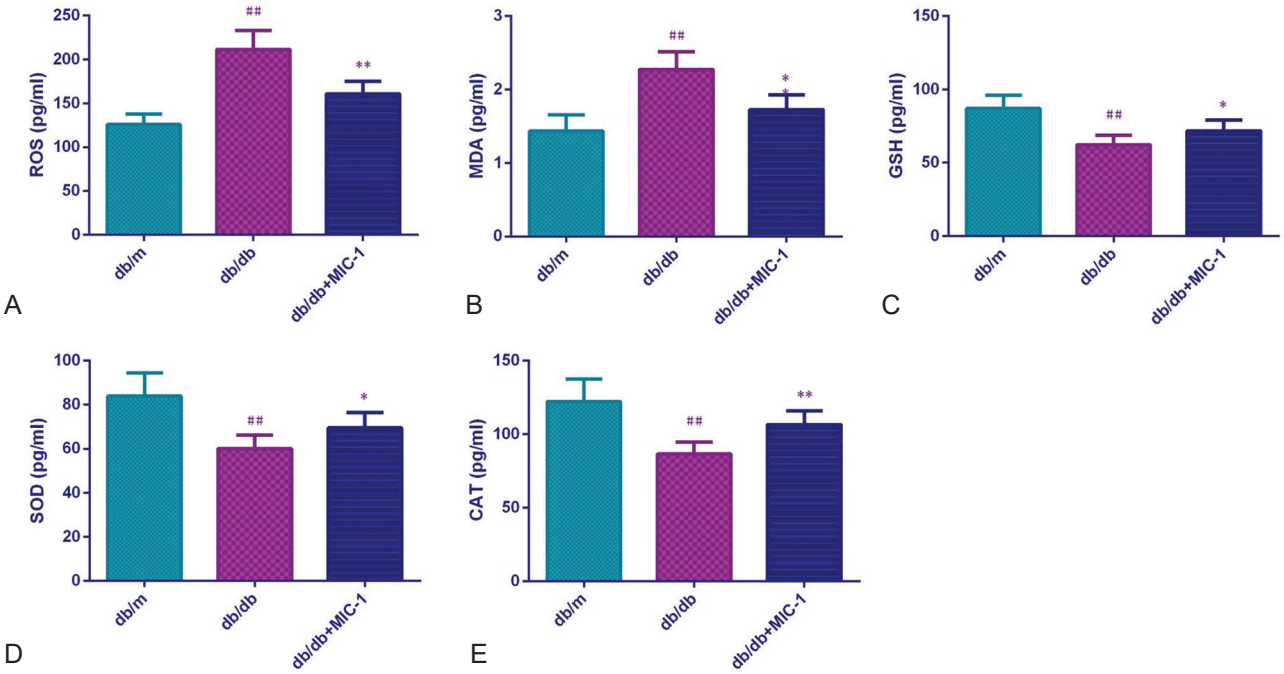

Figure 4. MIC-1 inhibited oxidative stress of db/db mice [The content of reactive oxygen species (ROS) (A), malondialdehyde (MDA) (B), glutathione (GSH) (C), superoxide dismutase (SOD) (D), and catalase (CAT) (E) in mouse kidney tissue homogenate was measured]. Quantitative data were described as mean ± SD (n = 6) (##P < .01 vs. db/m group; *P < .05, **P < .01 vs. db/db group).

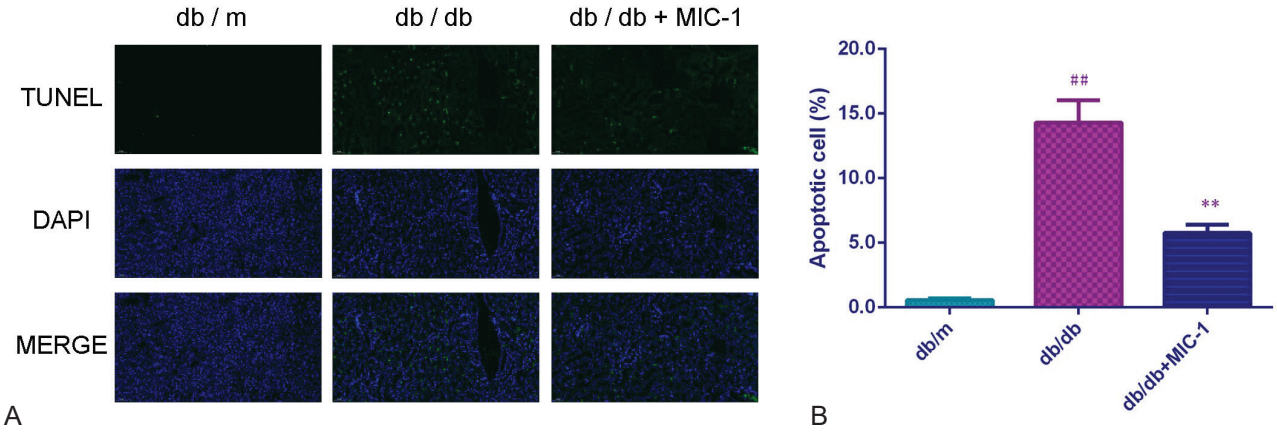

Figure 5. MIC-1 restrained apoptosis of db/db mice [(A-B) TUNEL staining was employed to assess apoptosis of the kidney tissues]. Quantitative data were described as mean ± SD (n = 3) (##P < .01 vs. db/m group; **P < .01 vs. db/db group).

by TUNEL staining. Compared with the db/m group, the apoptotic cell rate in the db/db group was extremely high (Figure 5A to 5B, $P < .01$), while the apoptotic cells in the db/db + MIC-1 group decreased markedly compared with the db/db group (Figure 5A to 5B, $P < .01$).

MIC-1 Activated the ERK/Nrf2 Pathway of db/db Mice

The western blot test revealed that db/db mice had lower levels of p-ERK/ERK, Nrf2, SOD-1, HO-1, and CAT and higher levels of p-IKBα and p-P65/P65, whereas these aberrant ERK/Nrf2 pathway-related markers returned to normal after administration of MIC-1 (Figure 6A to 6B, $P < .05$).

MIC-1 Facilitated the Positive Expressions of Pod and Syn of the Kidney Tissues in db/db Mice

The IHC staining illustrated that the positive

expressions of Pod and Syn in the renal tissues in db/db mice were lower than that in the db/m mice (Figure 7A to 7B, $P < .01$). Interestingly, MIC-1 prominently increased the positive expressions of Pod and Syn in the renal tissues in db/db mice (Figure 7A to 7B, $P < .05$).

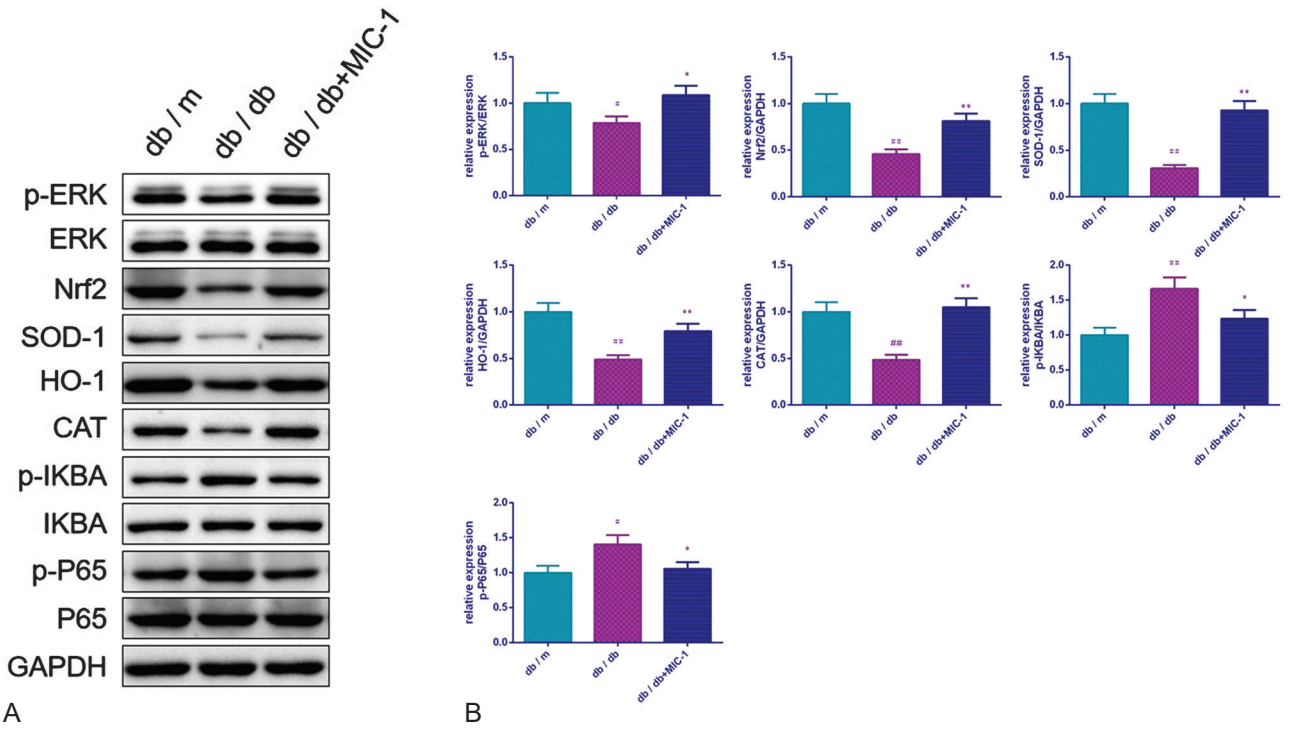

Figure 6. MIC-1 activated the ERK/Nrf2 pathway in db/db mice [(A-B) Western blot test was applied to examine the ERK/Nrf2 pathway-related markers]. Quantitative data were presented as mean ± SD (n = 3) (#P < .05, ##P < .01 vs. db/m group; *P < .05, **P < .01 vs. db/db group).

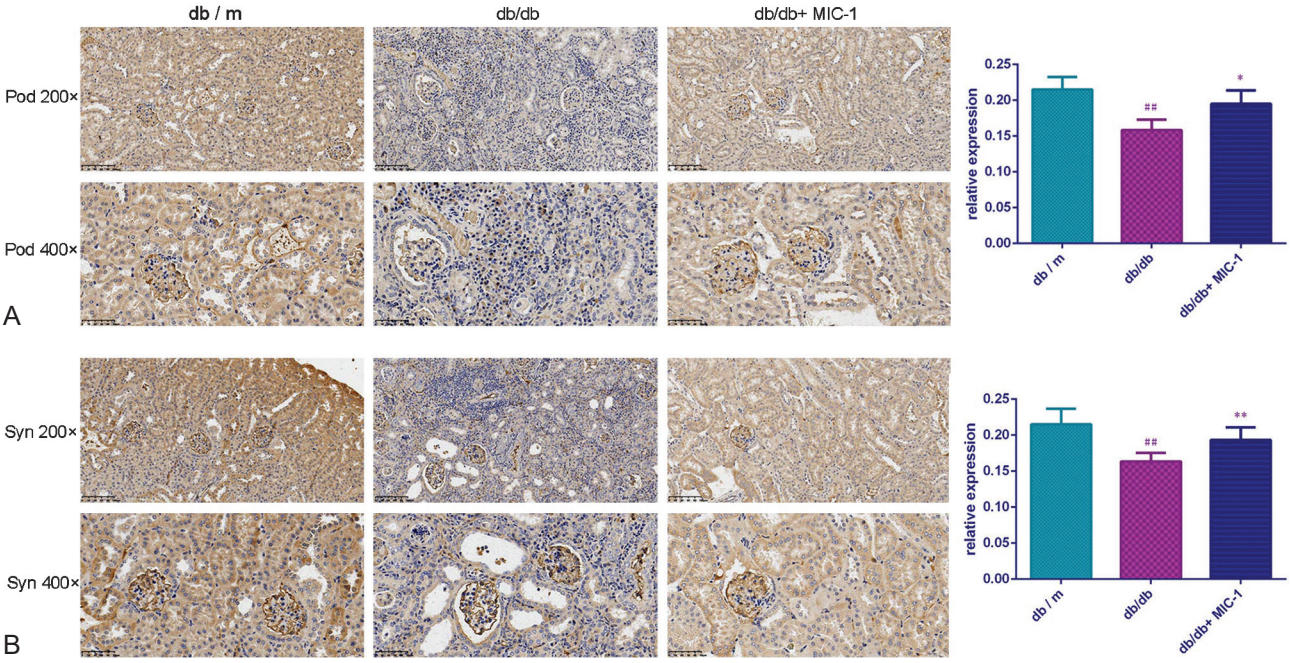

Figure 7. MIC-1 facilitated the positive expressions of Pod and Syn of the kidney tissues in db/db mice (IHC assay was exploited to examine the positive expressions of Pod (A) and Syn (B) of the kidney tissues in db/db mice). Quantitative data were presented as mean ± SD (n = 3) (##P < .01 vs. db/m group, **P < .01 vs. db/db group).

DISCUSSION

MOS and MIC-1 have been used in the treatment of multiple chronic diseases, including diabetes mellitus. According to several studies, MIC-1 could counteract the changes of epigenome and transcriptome in high glucose-induced mouse renal mesangial cells.[19] In streptozotocin-induced diabetic rat model, methanolic extract of MOS protected the kidney against diabetic kidney injury through limiting the effect of high blood glucose on inflammation, and oxidative stress.[20] Wen *et al.* demonstrate that MOS extract can serve as an antioxidant and prevent renal fibrosis to slow the progression of DN.[16] In the present study, we performed *in vivo* experiments to assess the impact of MIC-1 on DN which demonstrated its protective role against renal injury in db/db mice.

In this study, we firstly found that the treatment with MIC-1 effectively decreased the body and kidney weights and enhanced the kidney index in db/db mice. Next, we analyzed the impact of MIC-1 on renal function in db/db mice by evaluating the renal function markers (Scr, BUN, uric acid, and urine albumin), as they could significantly indicate the severity of DN.[21] Damage to the kidneys or a reduction in glomerular filtration rate lead to diminished excretion of BUN and Scr, leading to elevated the levels of both substances. As DN advances, urine albumin can be excreted in the urine of patients, so the content of urine albumin is closely related to the progression of DN.[22] Based on our findings, MIC-1 has a renal protective role that is latent and manifests itself by preventing the abnormal accumulation of Scr, BUN, uric acid, and urine albumin. The fatty acids from the ethanolic extract of MOS reduced the increased Scr and BUN in gentamicin-induced AKI.[23] Moreover, the insulin level and HOMA-IR index are generally considered to be the major indicators of DN in clinical practice.[24] As insulin plays a ctitical role in the development of diabetes.[25] Our results verified that MIC-1 decreases the level of insulin and HOMA-IR index in DN mice. These findings suggest that MIC-1 may be an effective promising treatment for DN.

The histopathological features of DN includes glomerular hypertrophy, edema and dilation of renal tubules, thickening of glomerular basement membrane, and inflammatory cell infiltration of renal interstitial.[26] It was reported that MOS extract could alleviate renal tissue damage.[16] In the current study, histopathological staining showed that the kidney injury of db/db mice improved after MIC-1 administration, suggesting that MIC-1 might be able to repair damaged renal tissue, verifying its protective effect on DN renal function at the pathological level.

Oxidative stress can stimulate cell proliferation and lead to proliferation of glomerular mesangial cells and matrix, which is strongly correlated with the initiation and development of DN.[27] The efficient anti-oxidant properties of MIC-1 have been fully demonstrated.[18] For instance, Sailaja *et al.* explained that MIC-1 strongly alleviated the level of ROS in macrophages induced by lipopolysaccharides (LPS).[13] In this study, we found that MIC-1 could reduce oxidative stress in DN mice, as evident by the reduction of ROS and MDA levels, and the increase of GSH, SOD, and CAT, all of which contributing to its protective effect against DN. Furthermore, apoptosis is one of the considerable pathological features of DN.[28] Several studies also proved that MIC-1 was effective in a variety of human diseases by modulating apoptosis pathway.[29, 30] Our results demonstrated that MIC-1 restrained apoptosis of the kidney tissues in db/db mice.

ERK is a NF-κB transcription factor involved in diabetes mellitus.[31] NF-κB signaling is a major signaling mechanism participating in the transcriptional regulation of inflammation and inflammatory mediators. NF-κBp65 subunit phosphorylation and acetylation are central to NF-κB activation.[32] ERK/Nrf2/HO-1 signaling is a major anti-oxidant and anti-inflammatory signal in the body and is involved in the pathogenesis of DN.[33] Enhancing the phosphorylation level of ERK can up-regulate the expression of Nrf2, exert anti-oxidative and anti-inflammatory effects, protect neural function, and alleviate DN kidney injury.[34, 35] An *in vitro* experiment manifested that MIC-1 attenuated oxidative stress response in high glucose-mediated renal cells by activating Nrf2 signaling.[18] The protective effect of DN by MOS was also reported to exhibit through the activating of the GSK-3β and Nrf2/HO-1 pathways.[16] Notably, this study found that MIC-1 administration exerted a protective role on DN by up-regulating anti-oxidant genes (p-ERK, Nrf2, SOD-1, HO-1, and CAT) and down-regulating NF-κB signaling (p-IKBα and p-P65)-mediated inflammation and

oxidative stress.

A growing number of studies suggested that Pod and Syn proteins are the most commonly used markers that reflect renal injury in patients with early DN.[36] By conducting IHC assay, we discovered that MIC-1 prominently elevated the positive expressions of Pod and Syn of the kidney tissues, indicating that MIC-1 could alleviate the loss of Pod and Syn proteins on the kidney tissues in db/db mice.

CONCLUSION

The study found that MIC-1 reduced the oxidative stress and renal injury by activating the ERK/Nrf2/HO-1 signaling and repressing the NF-κB signaling in db/db mice, offering a new method for the treatment of DN.

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