

Moringa isothiocyanate-1 mitigates the damage of oxidative stress and apoptosis in diabetic nephropathy mice

Zhou Hua¹, JiuHong Deng² and Guiying Wang³

¹Department of Nephrology, The People's Hospital of Suichang County, Lishui City, ²Department of Endocrinology, Second People's Hospital of Pingyang County, Wenzhou City and ³Department of Nephrology, Shangyu People's Hospital of Shaoxing, Shaoxing City, Zhejiang Province, China

Summary. Objective. Diabetic nephropathy (DN) is a prevalent cause of end-stage kidney disease worldwide. Moringa isothiocyanate-1 (MIC-1) has shown potential for DN management, however, the exact mechanisms remain unclear. This research intended to evaluate the impact and mechanism of MIC-1 on DN.

Methods. Six C57BLKS/J-db/m mice served as controls. Eighteen C57BLKS/J-db/db mice were randomly separated into three groups: db/db, db/db + irbesartan (IBS), and db/db + MIC-1. Three weeks post-drug administration, the body weight and kidney weight of mice in each group were measured. Concurrently, serum creatinine (Scr), urine albumin, insulin, glycosylated hemoglobin (GHb), oxidative stress-, and inflammatory-related factors were determined. Additionally, the pathological injury, apoptosis, apoptosis-related markers, NLRP3, and ASC levels in the kidney tissues were examined utilizing H&E, Masson, PAS, TUNEL staining, and Western blot.

Results. MIC-1 decreased the body weight, kidney weight, the levels of Glu, Scr, and urine albumin in db/db mice. Moreover, MIC-1 significantly suppressed the levels of MDA, insulin, GHb, TNF- α , IL-1 β , and IL-6, while increased the activities of SOD, CAT, and GPX in the serum of db/db mice. MIC-1 also mitigated the kidney tissue injury in db/db mice. Western blot assay showed that MIC-1 enhanced the Bcl-2 level and suppressed the Bax, cleaved caspase-3, cleaved caspase-9, NLRP3, ASC, and caspase-1 levels of the kidney tissues in db/db mice.

Conclusions. MIC-1 ameliorated the kidney injury in DN mice, and its mechanism may be associated with the suppression of renal cell apoptosis, oxidative stress, and inflammatory responses.

Key words: Diabetic nephropathy, Moringa isothiocyanate-1, Oxidative stress, Apoptosis

Introduction

Diabetic nephropathy (DN) is a disease with distinctive pathological structure and function changes engendered by diabetes, and it has grown to be the foremost cause of end-stage kidney disease (ESKD) (Vasanth Rao et al., 2019). In China, the incidence and prevalence of DN have increased dramatically over the past decade (Samsu, 2021). By 2040, the global population of diabetes is estimated to elevate to 642 million, of which 30-40% will develop to DN (Tang and Yiu, 2020). The primary clinical characteristics of DN are renal lesions, such as edema, proteinuria, hypertension, and renal insufficiency, which are the major reasons responsible for ESKD and renal failure (Ioannou, 2017). At present, conventional therapies for DN mainly focus on blood glucose control, blood pressure moderation, repression of the renin-angiotensin system, and renal transplantation (Yamazaki et al., 2021). However, these interventions do not prevent the progression of chronic kidney disease (CKD) in the majority of patients with DN. Hence, discovering anti-DN medicaments with low toxicity and profound efficacy has emerging as a novel hot spot in the management of DN.

In recent years, the theoretical and clinical exploration of traditional Chinese medicine (TCM) in treating DN has made continuous progress, exhibiting unique advantages in alleviating oxidative stress, reducing inflammatory response, and ameliorating podocyte injury (Ma et al., 2021; Ou et al., 2021). *Moringa Oleifera* Lam., a perennial tropical tree of the Moringaceae, has high economic and medicinal value. *Moringa Oleifera* seeds (MOS) are rich in diverse bioactive ingredients, which are beneficial for human

Corresponding Author: Guiying Wang, Department of Nephrology, Shangyu People's Hospital of Shaoxing, Shaoxing City 312300, Zhejiang Province, PR China. e-mail: wgy8320@163.com
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health (Fakurazi et al., 2008; Araújo et al., 2013; Wen et al., 2022). MOS contained isothiocyanate derivatives with biological activities such as anti-inflammatory, anti-oxidative, anti-bacterial, and anti-diabetic (Padla et al., 2012; Kou et al., 2018; Sailaja et al., 2021). Of these, Moringa isothiocyanate-1 (MIC-1) is the main isothiocyanate (Sailaja et al., 2021). A study has clarified that MOS extracts alleviated oxidative stress injury and renal fibrosis by activating GSK-3 β and Nrf2/HO-1 pathways, thereby protecting renal function (Wen et al., 2022). Waterman et al. reported that MIC-enriched seed extract postponed the onset of diabetes in type-2 diabetes rats (Waterman et al., 2020). Some scholars have illustrated that MIC-1 reversed epigenome and transcriptome changes in mouse renal mesangial cells mediated by high glucose (Li et al., 2019). In addition, numerous studies have demonstrated that MIC-1 could alleviate inflammation, apoptosis, and oxidative processes closely related to human diseases, including DN (Giacoppo et al., 2017; Kim et al., 2017; Xie et al., 2021). Nonetheless, it remains unclear whether MIC-1 can retard DN progression by restraining apoptosis, inflammation, and oxidative stress.

Therefore, in this study, db/db mice were selected as the research subjects to explore the effect of MIC-1 on renal function and pathological damage, apoptosis, inflammation, and oxidative stress in db/db mice, to offer a scientific foundation for the TCM therapy of DN.

Materials and methods

Experimental animals

Shanghai SLAC Laboratory Animal Co., Ltd offered male C57BLKS/J db/m mice (n=6, 8-week-old) and C57BLKS/J db/db mice (n=18, 8-week-old). These animals underwent a seven-day acclimatization, aligning with standard norms encompassing temperature (25 $\pm$ 2°C), relative humidity (55 $\pm$ 5%), and illumination (12-hour segregation between day and night cycles).

Experimental design

Irbesartan (IBS, I129263) was bought from Aladdin (China). MIC-1 was extracted from *Moringa Oleifera* seeds referring previous report (Xie et al., 2021). Six db/m mice served as controls. Eighteen db/db mice were randomized into the db/db, db/db + IBS, and db/db + MIC-1 groups, with six mice in each group. The mice in the db/db + IBS group were subjected to IBS by gastric lavage at a dose of 50 mg/kg/d in 10% DMSO for 21 days (Deng et al., 2016). The mice in the db/db + MIC-1 group were treated with MIC-1 by gastric lavage at a dose of 100 mg/kg/d in 10% DMSO for 21 days (Kim et al., 2018). The dosage was adjusted weekly to accommodate changes in body weight during the study period. The mice in the db/m and db/db groups were given excipient solution in the same way. The design of the study is presented in Figure 1A. Blood glucose (Glu)

levels were assessed by a blood glucose meter (580, Yuwell, China) every two weeks. After 21 consecutive days of drug treatment, each mouse was placed in a metabolic cage to harvest a 24-hour urine for analysis. The body weight of the mice in each group was measured. A liquid protein extraction reagent was applied to obtain urine albumin (P1255, Applygen, China). Next, a BCA kit (P1513, Applygen, China) was exploited to quantify the extracted urine albumin.

Sample collection

Upon completion of the 21-day treatment, mice were anesthetized with 2% isoflurane (R510-22, RWD Life Science Co., China), and blood samples were collected from the eyes. All blood samples were centrifuged (4°C, 1000 g, 20 min) and serum was obtained. After that, mice were euthanized by CO₂, and kidneys were quickly harvested and weighed. Then the kidney-to-body weight ratio was calculated according to the formula: 100*kidney weight/body weight. Part of the kidney samples was fixed via 4% paraformaldehyde (PFA, G1101, Servicebio, China), dehydrated, paraffin-embedded, sliced (4 μ m), and the remainder stored at -80°C for future analysis.

Measurement of renal function

Serum creatinine (Scr) was estimated utilizing an automatic biochemical analyzer (AU680, Beckman, USA), and glycosylated hemoglobin (GHb) content was estimated utilizing another automated GHb analyzer (Variant II, BIO-RAD, USA). Serum insulin was assessed by the ELISA method utilizing the Mouse Ins1 / Insulin-1 ELISA Kit (RAB0817, Sigma-Aldrich, USA).

Determination of oxidative stress-associated biomarkers

In brief, lysate (P0013, Beyotime, China) was added to the kidney samples and then homogenized. The proportion of tissue weight to lysate was 10%. After centrifugation, the supernatant was harvested. Then, the contents of MDA, SOD, CAT, and GPX were examined utilizing the MDA (S0131M, Beyotime, China), SOD (S0109, Beyotime, China), CAT (S0051, Beyotime, China), and GPX assay kits (S0056, Beyotime, China), separately.

Kidney histopathological analysis

Servicebio (China) offered the Hematoxylin and Eosin (H&E) (G1003), Masson (G1006), and Periodic Acid-Schiff (PAS) kits (G1008). After being routinely dewaxed utilizing xylene and then hydrated utilizing gradient ethanol, the kidney slices were stained with the H&E, Masson, and PAS kits, respectively. After rinsing, slices were dehydrated and permeabilized. Thereafter, neutral resin (abs9177, Absin, China) was exploited to block the slices. Lastly, the pathological damage and

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degree of fibrosis of kidney tissues were captured using an optical microscope (BX53M, Olympus, Japan).

TUNEL assay

The TUNEL kit (C1090, Beyotime, China) was employed to assess the apoptosis of kidney tissues. The proteinase K solution (P1120, Solarbio, China) was added to the dewaxed and hydrated slices at room temperature for 20 min. After rinsing, the prepared TUNEL solution was applied to treat the slices at RT for 60 min. After that, a DAPI solution (C1005, Beyotime, China) was added. After being dehydrated and transparentized, the slices were subjected to neutral resin. Apoptosis of the kidney tissues was observed utilizing an optical microscope (Nikon Eclipse Ci-L, Nikon, Japan).

Detection of inflammation-related factors

Meimian (China) provided the TNF- α (MM-

0132M1), IL-1 β (MM-0040M1), and IL-6 kits (MM-0163M1). The contents of TNF- α , IL-1 β , and IL-6 in serum samples were estimated in strict accordance with the corresponding instructions of the TNF- α , IL-1 β , and IL-6 kits by ELISA assay.

Western blot analysis

Total protein from the kidney tissues was lysed with lysis buffer (abs9229, Absin, China). After being quantified with the help of a BCA kit (G2026, Servicebio, China), protein samples were subjected to denaturation and electrophoresis and then blotted onto nitrocellulose membranes. They were subsequently sealed with 5% non-fat milk at 37°C for 90 min. After the blocked membranes were hybridized with primary antibodies at 4°C overnight, the bound antibodies were then exposed to Goat Anti-Rabbit IgG (H+L) HRP (1:5000, S0001, Affinity) at 37°C for 60 min. Thereafter, the membranes were examined with a gel imaging system (A44114, Invitrogen, USA) after being

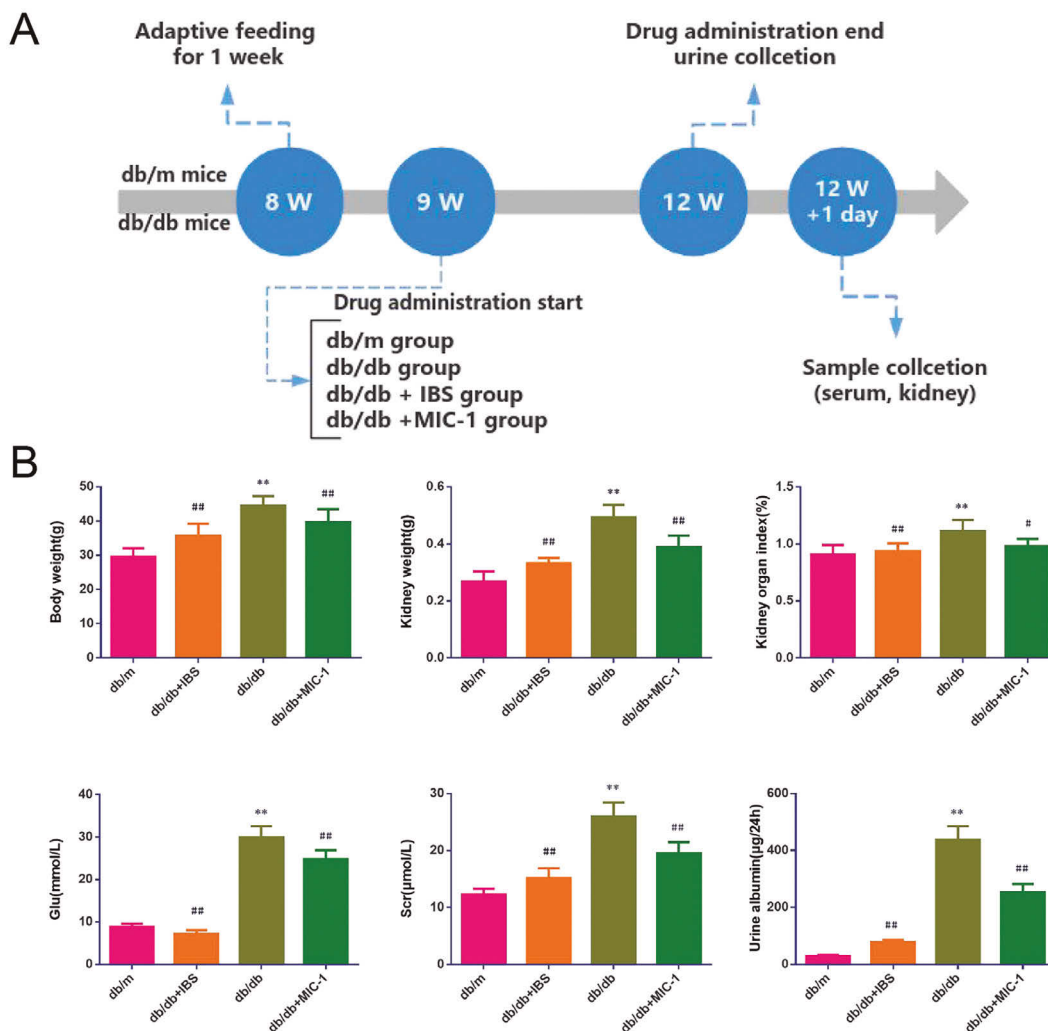


Fig. 1. MIC-1 attenuated the renal impairment of db/db mice. **A.** The schematic representation of the animal model. **B.** The body weight, kidney weight, kidney/body weight ratio of mice, fasting blood Glu, serum creatinine, and urine albumin in each group were assessed ($n=6$). Quantitative data were described as mean $\pm$ standard deviation. ** $P<0.01$ vs. db/m; # $P<0.05$, ## $P<0.01$ vs. db/db. In the db/m group, C57BLKS/J-db/m mice were used as a control. The C57BLKS/J-db/db mice in the db/db + irbesartan (IBS) group were subjected to IBS at a dose of 50 mg/kg/d for 21 days. The db/db mice in the db/db + moringa isothiocyanate-1 (MIC-1) group were treated with MIC-1 at a dose of 100 mg/kg/d for 21 days.

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developed with a color reagent (E266188, Aladdin, China). The primary antibodies of Bcl-2 (1:2000, AF6139), Bax (1:8000, AF0120), cleaved caspase-3 (1:5000, AF7022), caspase-3 (1:2000, AF6311), ASC1 (1:5000, DF13176), NLRP3 (1:1000, DF7438), caspase-1 (1:1000, AF5418), pro-caspase-1 (1:1000, Ab179515), cleaved caspase-9 (1:2000, AF5240), caspase-9 (1:2000, AF6348) and β -actin (1:5000, AF7018) were bought from Affinity (USA).

Statistical analysis

Quantitative data were expressed as mean $\pm$ standard deviation. All experiments were conducted at least three times and SPSS software (16.0, IBM, USA) was utilized to process data. Statistical analyses were conducted with the use of a one-way ANOVA for comparing differences among multiple groups. Comparison between groups was evaluated by the LSD test. The Kruskal-Wallis H test was deployed for heterogeneity of variance. $P < 0.05$ represented statistical significance.

Results

MIC-1 attenuated the renal dysfunction of db/db mice

Assessing the general physical signs of the mice in each group, as exhibited in Figure 1B, the body weight, kidney weight, and kidney organ index in the db/db group were higher relative to the db/m group ($P < 0.01$). Interestingly, IBS and MIC-1 clearly alleviated these basic indicators in db/db mice (Fig. 1B, $P < 0.05$). Moreover, we also noted that the levels of Glu, Scr, and urine albumin in db/db mice were upregulated, while

were greatly decreased by IBS and MIC-1 intervention (Fig. 1B, $P < 0.01$).

MIC-1 suppressed the oxidative stress of db/db mice

Oxidative stress has been acknowledged as a considerable stimulator in DN development. In Figure 2A-D, when compared with the db/m group, the level of MDA was elevated and the activities of SOD, CAT, and GPX were decreased in the db/db group ($P < 0.01$). Importantly, IBS and MIC-1 notably decreased the MDA level and increased the activities of SOD, CAT, and GPX in serum of DN mice (Fig. 2A-D, $P < 0.01$).

MIC-1 mitigated the kidney tissue injury of db/db mice

We utilized histological staining to observe the renal pathological changes in db/db mice. H&E staining results manifested that the kidney structure of db/db mice was severely damaged relative to db/m mice, characterized by severe cellular defects and blurred vascular striae (Fig. 3). MIC-1 and IBS intervention alleviated the above renal histopathological injuries in db/db mice (Fig. 3). Injury to the kidney tissue of db/db mice was also observed by Masson staining results, with a large number of fat vacuoles in the cytoplasm of cells and distinct blue fibrous tissue hyperplasia (Fig. 3). Compared with the db/db group, the db/db + MIC-1 and db/db + IBS groups present less cell rupture and notably decreased blue fibrous tissue (Fig. 3). PAS staining indicated a marked increase in the mucus secretion of kidney tissue in DN mice and the proliferation of bronchial goblet cells, while MIC-1 and IBS intervention lessened these

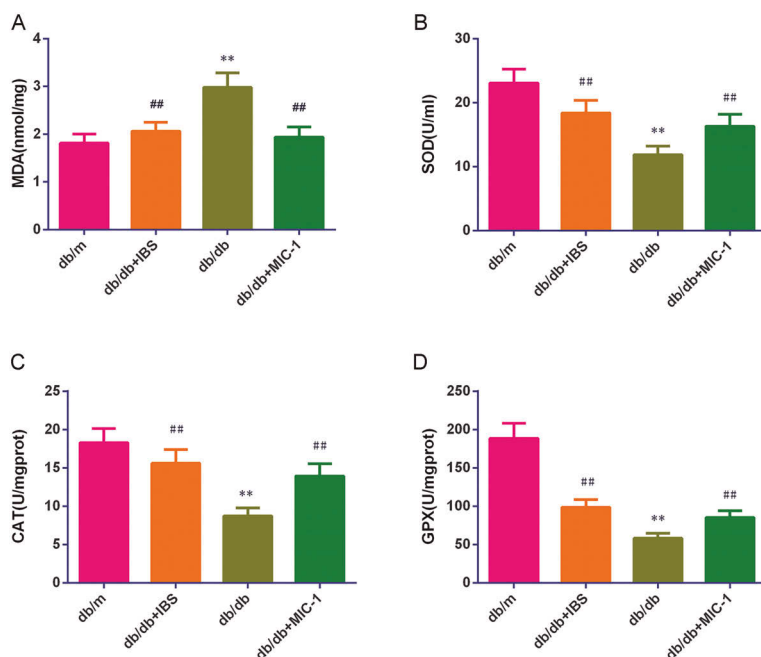


Fig. 2. MIC-1 suppressed oxidative stress of db/db mice. Corresponding kits were utilized to examine the levels of malondialdehyde (MDA) (A), activities of superoxide dismutase (SOD) (B), catalase (CAT) (C), and glutathione peroxidase (GPX) (D) in the serum of db/db and db/m mice ($n=6$). Quantitative data were expressed as mean $\pm$ standard deviation. ** $P < 0.01$ vs. db/m; ## $P < 0.01$ vs. db/db.

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abnormal pathological injuries (Fig. 3).

The contents of insulin and GHb in db/db mice were decreased by MIC-1

As illustrated in Figure 4A,B, the levels of insulin and GHb were highly increased in db/db mice ($P<0.01$). After intervened with MIC-1 and IBS, the levels of insulin and GHb were downregulated ($P<0.01$).

MIC-1 inhibited the cell apoptosis of kidney tissues of db/db mice

The apoptosis of kidney tissues was observed by the TUNEL assay. Compared with the db/m group, the apoptotic cell rate of the db/db group was significantly

higher; compared with the db/db + MIC-1 and db/db + IBS mice decreased notably (Fig. 5A, $P<0.01$). In addition, at the molecular level, significant downregulation of Bcl-2 protein and upregulation of Bax, cleaved caspase-3, and cleaved caspase-9 were observed in DN kidneys compared to db/m (Fig. 5B, $P<0.01$). Treatment with MIC-1 or IBS restored the Bcl-2 protein level and consequently restrained the expressions of Bax, cleaved caspase-3, and cleaved caspase-9 proteins within db/db+IBS and db/db+MIC-1 groups (Fig. 5B, $P<0.05$).

MIC-1 alleviated the inflammatory response of db/db mice

The increased levels of TNF- α , IL-1 β , and IL-6 in

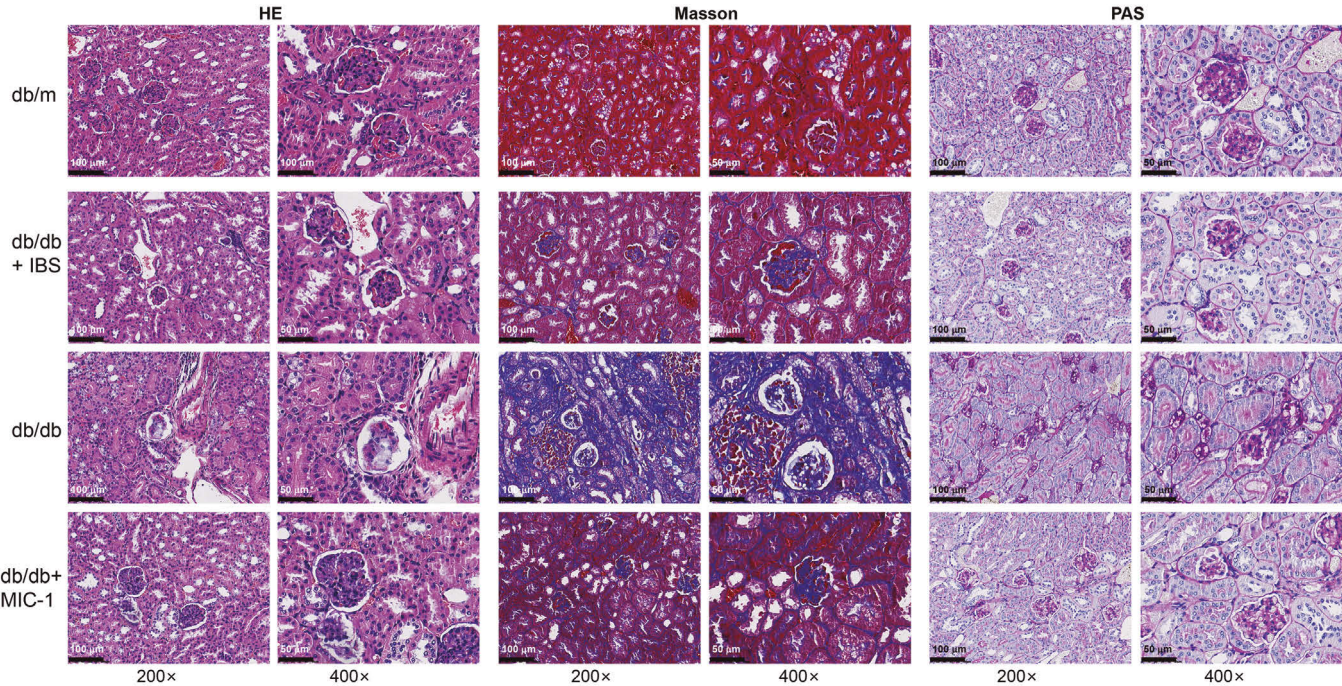


Fig. 3. MIC-1 mitigated the kidney tissue injury of db/db mice The kidney tissue injury of db/db mice was assessed by Hematoxylin & Eosin (H&E), Masson, and Periodic Acid-Schiff (PAS) staining.

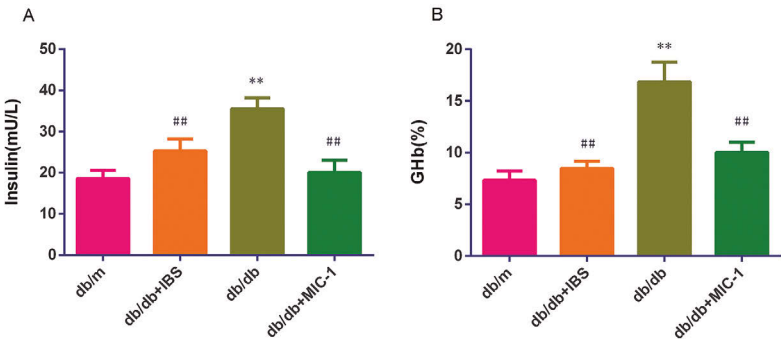


Fig. 4. The insulin and GHb contents in db/db mice were decreased by MIC-1 treatment in db/db mice. **A.** Serum insulin was assessed utilizing the Mouse Ins1 / Insulin-1 ELISA Kit (n=6). **B.** The level of glycosylated hemoglobin (GHb) was estimated utilizing an automated GHb analyzer (n=6). Quantitative data were described as mean $\pm$ standard deviation. ** $P<0.01$ vs. db/m; ## $P<0.01$ vs. db/db.

DN mouse serum of the db/db group were detected than those in the db/m group (Fig. 6A-C, $P<0.01$). In contrast, after the intervention of IBS and MIC-1, the above inflammatory factors were significantly decreased (Fig. 6A-C, $P<0.01$).

MIC-1 inhibited the NLRP3, ASC, and caspase-1 levels of the kidney tissues in db/db mice

Western blot showed that the protein levels of NLRP3, ASC, and caspase-1 in the kidney of the db/db group were obviously higher than those of the db/m group (Fig. 7A,B, $P<0.01$). Remarkably, MIC-1 or IBS treatment decreased the expression of these proteins (Fig. 7A,B, $P<0.05$).

Discussion

MIC-1 has been exploited to treat various chronic

diseases associated with inflammation, including diabetes. Waterman et al. elucidated that MIC-1 intervention effectively delayed the onset of type 2 diabetes in a rat model (Waterman et al., 2020). In this study, we conducted animal experiments to evaluate the effect of MIC-1 on DN. Our results demonstrated that, after MIC-1 intervention, the body weight, kidney weight, and kidney organ index of db/db mice decreased notably. Then we assessed renal function indicators, as they can reflect the severity of DN to a large extent, especially Scr, a crucial indicator of renal function (Jin et al., 2019). Our study demonstrated that MIC-1 exhibits renal protective potential, evident as the reduction of Scr and urine albumin levels. In addition, the levels of blood glucose, insulin, and GHb are generally considered to be the primary indicators of DN testing in clinical practice (Tang et al., 2021). One study reported that both plasma insulin and GHb were remarkably upregulated in db/db mice (Gould et al.,

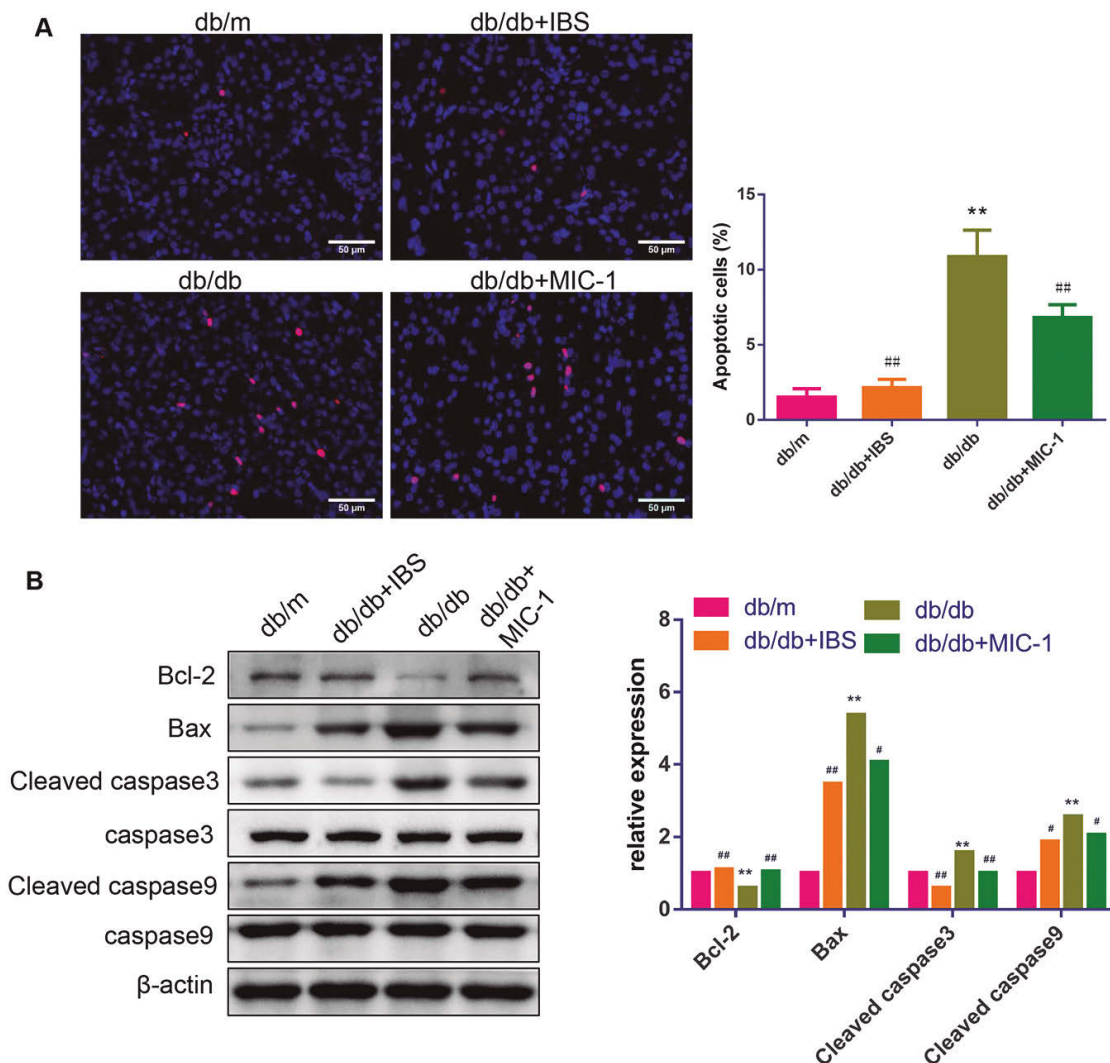


Fig. 5. MIC-1 inhibited the apoptosis of db/db mice. **A.** TUNEL staining was used to assess the apoptosis of the kidney tissues (n=6). **B.** The protein levels of Bcl-2, Bax, cleaved caspase-3, caspase-3, caspase-9, and cleaved caspase-9 in the kidney tissues of each group were assessed by Western blot (n=3). Quantitative data were manifested as mean $\pm$ standard deviation. ** $P<0.01$ vs. db/m; # $P<0.05$, ## $P<0.01$ vs. db/db.

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1986). Our results illustrated that MIC-1 could enhance insulin resistance and decrease the level of GHb in DN mice. Collectively, these findings suggested that MIC-1 can act as an effective therapeutic agent for DN.

The detection of renal pathological morphology can directly indicate the degree of pathological damage of the disease and is a primary indicator for evaluating the therapeutic effect. The histopathological features of DN kidney mainly include glomerular enlargement, mesangial and basement membrane thickening, renal tubular epithelial edema, and inflammatory cell infiltration (Chen et al., 2018). MOS extract has been reported to impede renal tissue damage (Wen et al., 2022). Based on the results of H&E, Masson, and PAS staining of renal tissues, our study proved that MIC-1 could significantly ameliorate the histological damage of glomerulus and renal tubules in DN model rats, indicating that MIC-1 can repair damaged renal tissue and confirm its protective potential on DN renal function at the pathological level.

The pathogenesis of DN is attributed to a multitude of factors such as glucose and lipid metabolism disorders, genetic factors, apoptosis, oxidative stress,

and abnormal expression of inflammatory factors, ultimately leading to renal fibrosis (Warren et al., 2019; Sagoo and Gnudi 2020). Among them, apoptosis, inflammatory injury, and oxidative injury are the common pathways involved in and promoting the progression of DN (Turkmen, 2017). Epithelial cell apoptosis is the primary feature of early DN, and inhibition of renal tubular epithelial cell apoptosis is particularly considerable for the treatment of DN (Zhang et al., 2014). Bax is a pro-apoptotic molecule, forming a homologous dimer altering mitochondrial membrane permeability, resulting in increased release of cytochrome C, which further activates the caspase cascade reaction and induces apoptosis (Fu et al., 2020). Conversely, Bcl-2 is an anti-apoptotic molecule, which forms a heterodimer with Bax when its expression increases, weakening the pro-apoptotic effect of Bax (Czabotar et al., 2014). Our study determined apoptosis-related markers in the kidney tissue of DN mice. MIC-1 effectively suppressed apoptosis, as evidenced by increasing Bcl-2 levels and suppressing Bax, cleaved caspase-3, and cleaved caspase-9 levels of kidney tissues in DN mice, unveiling that apoptosis inhibition was one

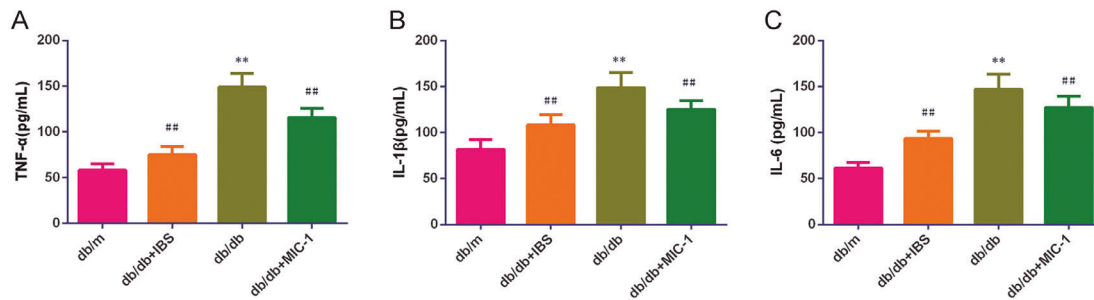


Fig. 6. MIC-1 alleviated the inflammatory response of db/db mice. The tumor necrosis factor- α (TNF- α) (A), interleukin-1 β (IL-1 β) (B), and IL-6 (C) levels of the serum in each group were assessed by the corresponding kits (n=6). Quantitative data were described as mean $\pm$ standard deviation. ** P <0.01 vs. db/m; ## P <0.01 vs. db/db.

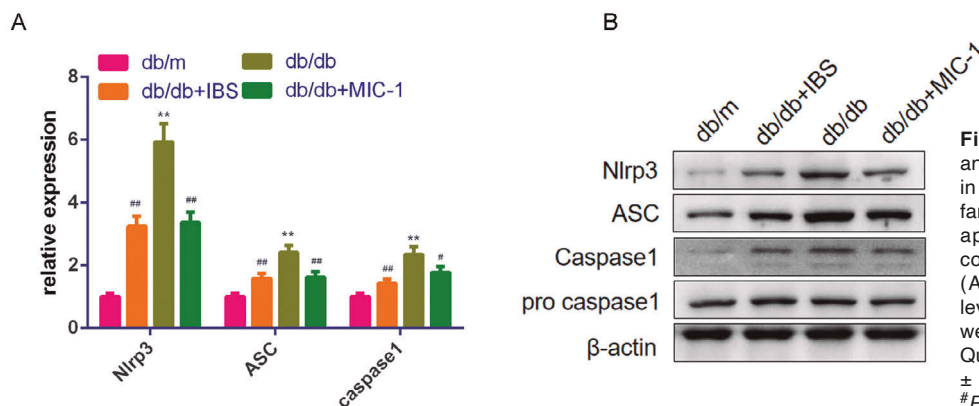


Fig. 7. MIC-1 suppressed NLRP3, ASC, and caspase-1 levels of the kidney tissues in db/db mice. A, B. The NOD-like receptor family pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), pro-caspase-1, and caspase-1 levels of the kidney tissues in db/db mice were examined utilizing Western blot (n=3). Quantitative data were presented as mean $\pm$ standard deviation. ** P <0.01 vs. db/m; # P <0.05, ## P <0.01 vs. db/db.

of the mechanisms by which MIC-1 produced anti-DN effects.

Furthermore, the renal inflammatory response and oxidative stress response can stimulate cell proliferation, leading to hyperplasia of mesangial cells and stroma, which is closely associated with the initiation and development of DN (Aghadavod et al., 2016). By detecting the level of MDA, the activities of SOD, CAT, and GPX in the serum of db/db mice, we found that MIC-1 could inhibit oxidative stress in DN mice, thus contributing to its protective function against DN. Moreover, oxidative stress frequently accompanies the inflammatory response, and NLRP3 is an inflammasome widely existing in cells, mainly composed of NLRP3 protein, ASC, and caspase-1 (Jo et al., 2016; Brydges et al., 2013). Activated NLRP3 inflammasome can accelerate the maturation and secretion of various inflammatory factors, and participating in the inflammatory cascade (Kelley et al., 2019). Gao et al. demonstrated that suppression of NLRP3 inflammasome reduced podocyte injury and evidently improved renal tissue injury in DN (Gao et al., 2014). An investigation has reported that MIC-1 inhibited inflammatory factors (IL-6, IL-1 β , and TNF- α) in high glucose-mediated human renal proximal tubule cells by activating the Nrf2-ARE pathway (Cheng et al., 2019). This study unveiled that TNF- α , IL-1 β , IL-6, NLRP3, ASC, and Caspase-1 expressions were clearly decreased in the kidney tissue homogenate of DN mice by MIC-1 intervention. The above evidence indicated that MIC-1 can weaken the renal inflammatory response and improve the peroxidation state *in vivo*, thereby mitigating the severity of renal tissue injury and playing a protective role in the kidney.

In conclusion, MIC-1 notably improve the kidney injury of DN mice, and its mechanism might be correlated with the decrease in renal cell apoptosis, oxidative stress, and inflammatory responses. This finding provides a clue for studying the specific molecular mechanism of DN, which will be verified in primary renal tubular epithelial cells in further research.

Acknowledgements. Not applicable.

Ethics statement. All animal protocols were performed in compliance with the Institutional Animal Care and Use Committee. Ethical approval for animal manipulations was granted by the Ethics Committee of Zhejiang Eyong Biotechnological Co., Ltd. We spared no effort to lighten the suffering of animals during the experiments.

Conflict of Interests. The authors report there are no competing interests to declare.

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