

Isomangiferin Attenuates Renal Injury in Diabetic Mice via Inhibiting Inflammation

This article was published in the following Dove Press journal:
Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy

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Aim: Renal injury induced by diabetes is reported to be associated with inflammation. Isomangiferin (ISO), a xanthone C-glucoside from the Cyclopia subfamily, exhibits many pharmacological properties. This study aimed to evaluate the protection of ISO against renal damage in diabetic mice.

Methods: Serum glucose, insulin, uric acid, creatinine, total cholesterol (TC), triglyceride (TG), and inflammatory cytokines in serum and the kidney of db/db diabetes model mice were detected. The components of high mobility group protein B1 (HMGB1)/NACHT leucine-rich repeat- and PYD-containing 3 (NLRP3)/nuclear factor kappa-B (NF-κB) pathway in the kidney were detected by Western blot and immunohistochemical analysis.

Results: ISO improved lipid profile and glucose tolerance, and inhibited the production of inflammatory cytokines in a db/db model mice. Moreover, ISO decreased biochemical indexes in the serum and inhibited the activation of HMGB1/NLRP3/NF-κB signaling in the kidney of db/db model mice.

Conclusion: ISO provides protection against renal injury via inhibiting HMGB1/NLRP3/NF-κB signaling in a diabetic mouse model.

Keywords: isomangiferin, renal injury, inflammation, HMGB1/NLRP3/NF-κB, diabetics

Introduction

Diabetes mellitus (DM) is characterized by hyperglycemia because of abnormal insulin secretion, and causes a variety of complications including renal failure, atherosclerosis, liver injury, and blindness.¹⁻⁴ Diabetic nephropathy (DN) is a common microvascular complication of diabetes, and is one main cause of death of diabetes patients.^{5,6} At present, the main treatment strategy for DN is to reduce renal injury by controlling blood glucose, blood fat, and anti-hypertension.⁷ Recent studies have revealed novel mechanisms underlying the pathogenesis of DN and suggested that targeting inflammation is a promising approach for DN treatment.⁸⁻¹⁰ The inflammatory pathways involved in DN mainly include mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF-κB).¹¹⁻¹³ High mobility group protein B1 (HMGB1) can combine with receptor for advanced glycation end products (RAGE) to induce inflammation.¹⁴⁻¹⁶

Isomangiferin (ISO) is a xanthone C-glucoside found in plants of the Cyclopia family with anti-inflammation and anti-cancer activities.¹⁷ In particular, a recent study reported that ISO acted as a potent inhibitor of vascular endothelial growth factor receptor 2 kinase to suppress breast metastasis and angiogenesis.¹⁸ In addition, many Chinese herbal extracts, in particular those containing ISO, can improve diabetes and renal function.¹⁹⁻²⁴ However, whether ISO is beneficial to DN remains unclear. Therefore,

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in this study we aimed to evaluate the efficacy of ISO on DN and explore possible mechanisms.

Methods

Reagents

ISO was purchased from YuanYe Biotech (Shanghai, China), glucose was from Sigma (St. Louis, USA), enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-6, IL-1 β , and tumor necrosis factor (TNF)- α were from Elabscience (Wuhan, China), biochemical kits to measure triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL); low-density lipoprotein (LDL); uric acid (UA), urea nitrogen, and creatinine (Cr) were from Jiancheng Biotech (Nanjing, China). Primary antibodies were purchased from Cell Signaling Technology.

Animals

Type 2 diabetes mellitus (T2DM) model mice (C57BL/KsJ db/db mice, male) and male db/m mice were provided by Animal Model Center of Nanjing University, and kept in specific pathogen-free (SPF) condition at room temperature of 21 \pm 1 $^{\circ}$ C and humidity of 55 \pm 5%. The mice were divided randomly into six groups (n=10): db/m group, db/db group, db/db+ISO (ISO, 10 mg/kg) group, db/db+ISO (ISO, 20 mg/kg) group, db/db+metformin (met, 400 mg/kg) group, and db/db+saline group. ISO (10, 20 mg/kg) was administered orally every day for 12 weeks. The protocols were approved by Animal Care and Use Committee of China Pharmaceutical University and followed Chinese National Guidelines for Experimental Animal Welfare, and animals were euthanized by cervical dislocation.

Oral Glucose Tolerance Test (OGTT)

The mice were fasted for 18 hours and then received glucose (2 g/kg) orally. Blood samples collected at 0, 30, 60, 90, and 120 minutes were centrifuged at 4 $^{\circ}$ C (4,000 g) for 10 minutes to separate serum. Glucose level was determined by automatic blood glucose instruments.

Elisa

The levels of IL-1 β , IL-6, and TNF- α in the serum and renal tissues were measured using ELISA kits following the manufacturer's protocols.

Immunohistochemistry

Renal tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μ m sections. The sections were

blocked by incubation with 5% sheep serum for 20 minutes, then incubated with antibodies to HMGB1 (1:1,000) and p-NF- κ Bp65 (1:200) at 4 $^{\circ}$ C overnight. The sections were then washed and incubated with secondary antibody. The section were also stained with hematoxylin and eosin (H&E), and observed under an optical microscope (Nikon, Tokyo, Japan).

Western Blotting

Proteins were extracted from renal tissues using RIPA buffer (Beyonce, Nanjing, China), resolved on 10% polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies (Cell Signaling Technology) at 4 $^{\circ}$ C overnight and then incubated with secondary antibody conjugated with horseradish peroxidase at room temperature for 1 hour. Protein bands were detected using an enhanced chemiluminescence kit (Tanon Tech., China).

Statistical Analysis

Data were presented as means \pm SD and analyzed by ANOVA. P <0.05 was considered significant.

Results

ISO Reduced UA and Cr Levels in Db/Db Mice

UA, Cr, urea nitrogen, and albuminuria levels were significantly higher in db/db mice than in db/m mice, while ISO (10, 20 mg/kg) significantly reduced high UA, Cr, urea nitrogen, and albuminuria levels in db/db mice (Figure 1). As the control, saline could not reduce high UA, Cr, urea nitrogen, and albuminuria levels in db/db mice.

ISO Reduced Serum Insulin Levels and Improved Lipid Profiles in Db/Db Mice

Blood glucose level was significantly higher in db/db mice than in db/m mice, while ISO (10, 20 mg/kg) significantly reduced blood glucose level (Figure 2A). In addition, ISO (10, 20 mg/kg) treatment reduced the high serum insulin level in db/db mice (Figure 2B).

Compared to db/m mice, plasma TC, TG, and LDL levels were significantly higher while serum HDL levels were significantly lower in db/db mice, and ISO (10, 20 mg/kg) significantly reduced serum TC, TG, and LDL levels while it increased serum HDL levels in db/db mice (Figure 2C–F).

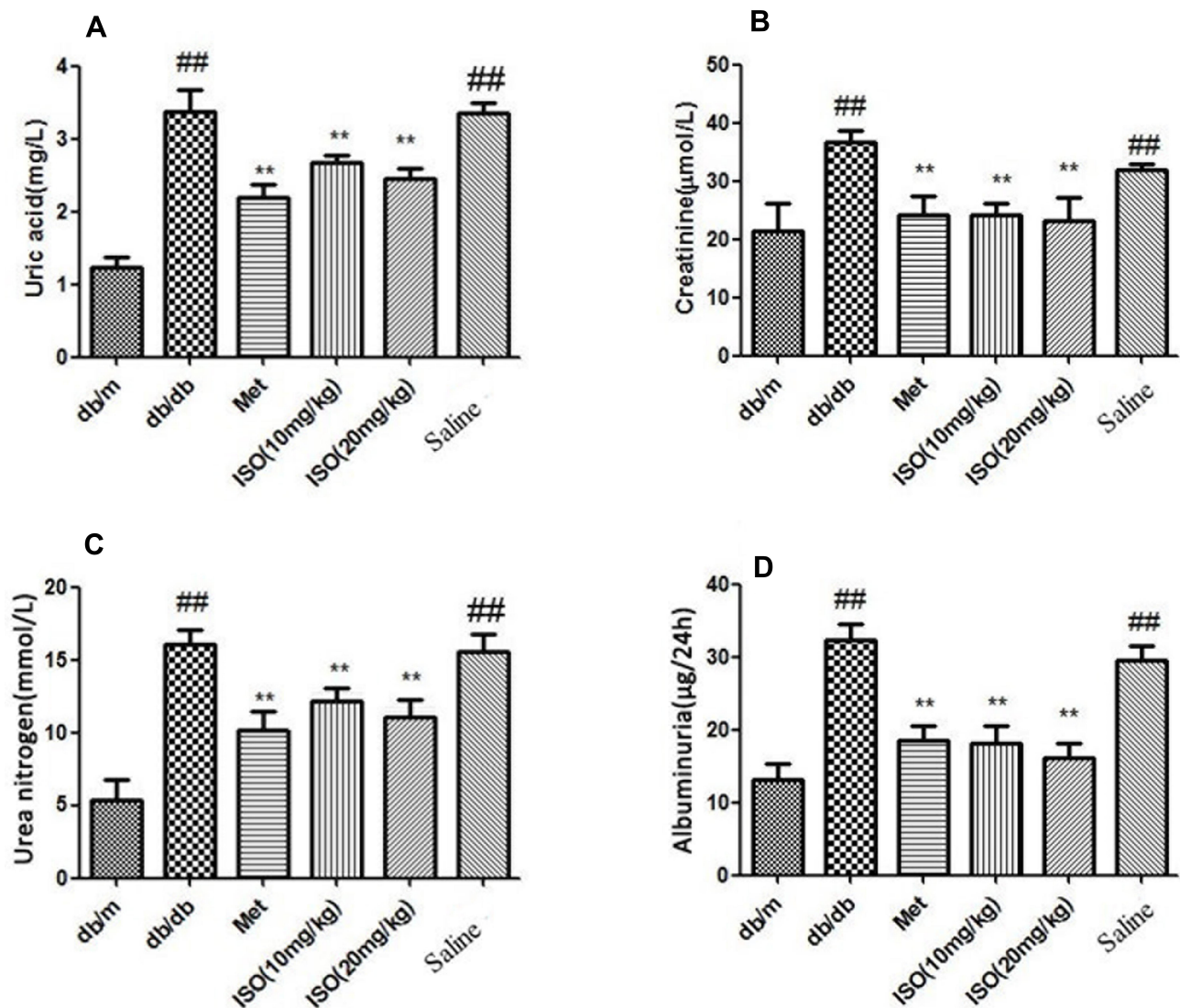


Figure 1 ISO decreased serum levels of UA and Cr in db/db mice. (A) UA level in mice serum. (B) Cr level in mice serum. (C) urea nitrogen and (D) albuminuria. All data presented as mean±SD (n=10). Compared with db/m: ^{##}P<0.01. Compared with db/db: ^{**}P<0.01.

ISO Attenuated Kidney Damage

Histopathological examination demonstrated significant lipid accumulation and infiltration of monocytes and neutrophils in db/db mice compared to db/m mice. Mice in the saline group showed significant tubular injuries, such as vacuolar degeneration. However, the animals treated with ISO (10, 20 mg/kg) showed less inflammation and tubular injuries (Figure 3).

ISO Reduced Inflammatory Cytokine Levels in Serum and the Kidney

IL-1 β , IL-6, and TNF- α levels were significantly higher in serum and kidney tissues of db/db mice compared to db/m mice, while ISO (10, 20 mg/kg) significantly

decreased their levels in serum and kidney tissues (Figure 4A and B).

ISO Inhibited HMGB1/NACHT Leucine-Rich Repeat- and PYD-Containing 3 (NLRP3)/NF- κ B Pathway in Db/Db Mice

Western blot analysis showed that protein levels of HMGB1, NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), interleukin-1 β converting enzyme (Caspase-1), p-NF- κ B, and IL-1 β increased in db/db mice compared to db/m mice, while ISO (10, 20 mg/kg) significantly decreased their levels in db/db mice (Figure 5A and B). Furthermore, immunohistochemistry showed higher HMGB1 and p-NF- κ Bp65

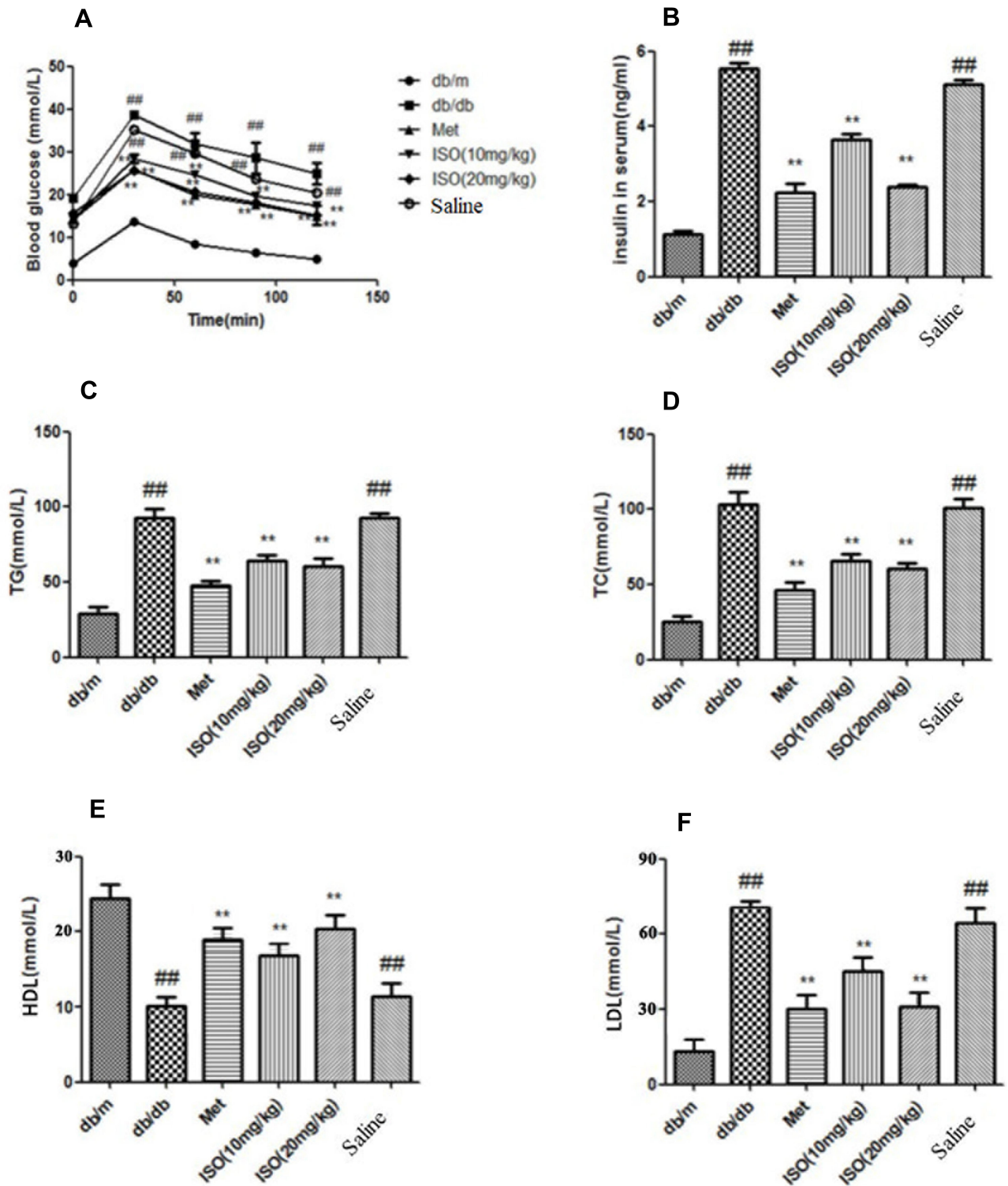


Figure 2 ISO decreased the levels of blood glucose, insulin, TG, and TC in db/db mice. (A) Glucose level in mice blood. (B) Insulin level in serum. (C) TG level in serum. (D) TC level in serum. (E) HDL level in serum. (F) LDL level in serum. All data presented as mean±SD (n=10). Compared with db/m: ###p<0.01. Compared with db/db: **p<0.01.

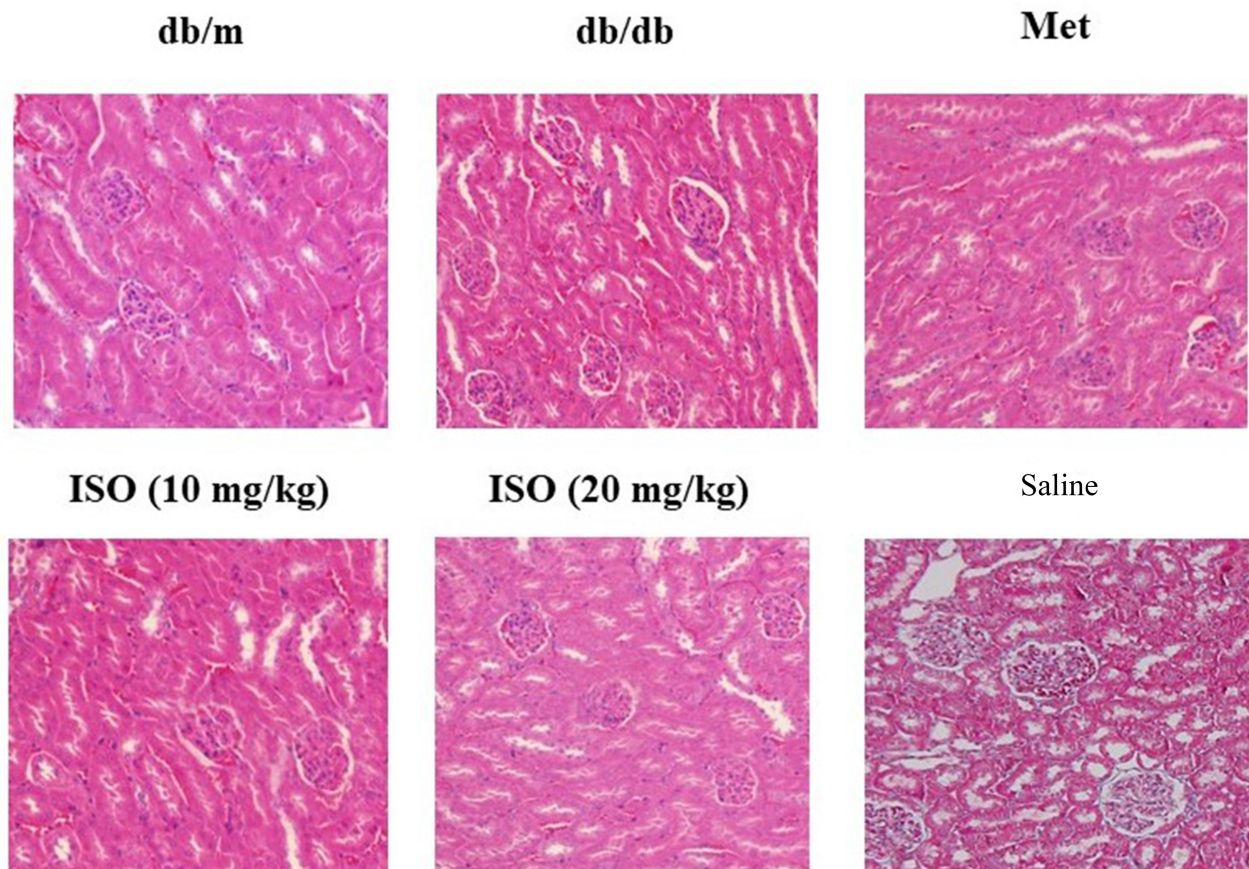


Figure 3 Effects of ISO on kidney pathology. Shown were H&E staining of the kidney tissues. Magnification: 20x.

levels in the kidney tissues of db/db mice compared to db/m mice, while ISO significantly reduced their levels in db/db mice (Figure 6).

Discussion

Currently several methods have been developed for DN therapy, such as controlling blood pressure and blood sugar, but their efficacy is not satisfactory. In this study, we demonstrated that ISO had an anti-diabetes effect and attenuated kidney damage by targeting the HMGB1/NLRP3/NF- κ B pathway.

DN is characterized by chronic hyperglycemia and proteinuria.²⁵ It is important to diminish the risk of diabetes and diabetes complications to prevent DN caused by diabetes.²⁶ In this study we showed that ISO significantly decreased serum TG and TC levels, reduced insulin resistance in db/db mice, reduced serum levels of uric acid and creatinine, and reduced kidney injury caused by hyperglycemia.

Kidney inflammation indicates the activation of immune response and the production of inflammatory

cytokines such as IL-6 and TNF- α .²⁷ The production of IL-1 β inhibits insulin signaling, leading to insulin resistance.²⁸ Additionally, increased serum IL-6 level indicates the progression of T2DM.²⁹ In this study we found increased levels of IL-1 β , TNF- α , and IL-6 in serum and kidney tissues of db/db mice, but ISO markedly reduced their levels in serum and kidney tissues.

It is acknowledged that HMGB1 plays a crucial role in inflammatory cascade. HMGB1 induces the upregulation of IL-6 to regulate inflammatory progression in macrophages.^{30–32} ISO reduced TC and TG levels and improved lipid metabolism by inhibiting HMGB1 in db/db mice. In particular, we found that ISO inhibited the production of pro-inflammatory factors IL-1 β , IL-6, and TNF- α . Moreover, our results showed that ISO reduced the levels of HMGB1, ASC, NLRP3, Caspase-1, p-NF- κ B, and IL-1 β in db/db mice.

To our knowledge, this is the first study to demonstrate beneficial effects of ISO on DN. More importantly, our results provided a clue that beneficial effects of ISO on DN

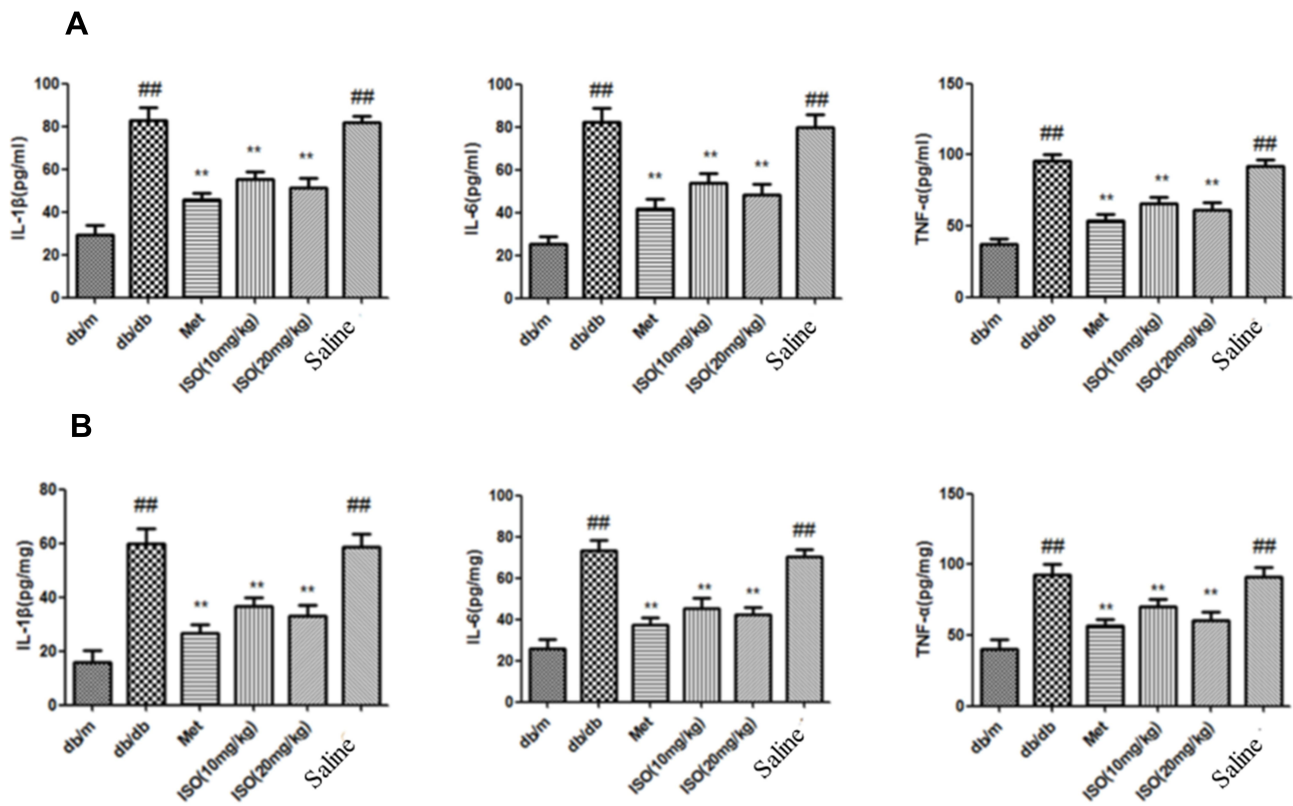


Figure 4 ISO mitigated the increase of inflammatory cytokines in serum and kidney of db/db mice. **(A)** Levels of IL-1β, IL-6 and TNF-α in mice serum. **(B)** Levels of IL-1β, IL-6, and TNF-α in mice kidney. All data presented as mean±SD (n=10). Compared with control: ###P<0.01. Compared with model: **P<0.01.

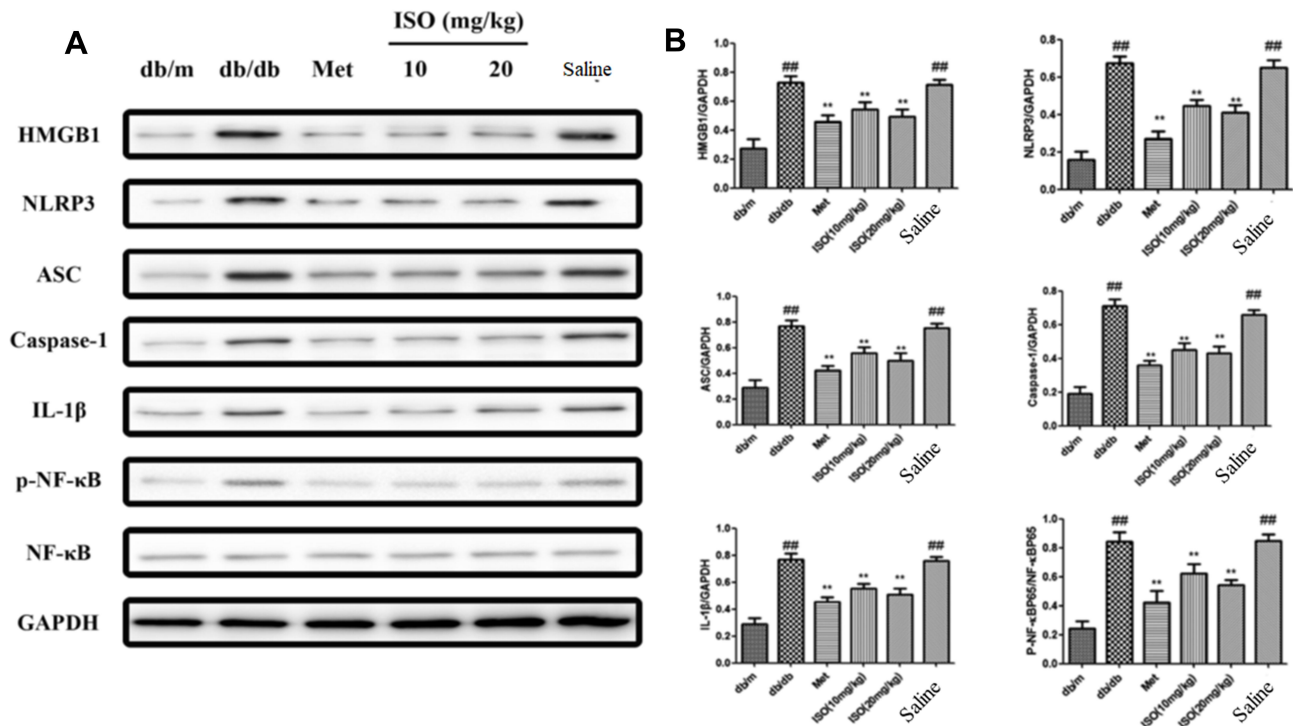


Figure 5 ISO inhibited HMGB1/NLRP3/NF-κB pathway in db/db mice. **(A)** Levels of HMGB1, NLRP3, ASC, Caspase-1, IL-1β, p-NF-κB, NF-κB, and GAPDH were detected by Western blot analysis. **(B)** Quantitation by densitometry analysis. All data presented as mean±SD (n=3). Compared with control: ###P<0.01. Compared with model: **P<0.01.

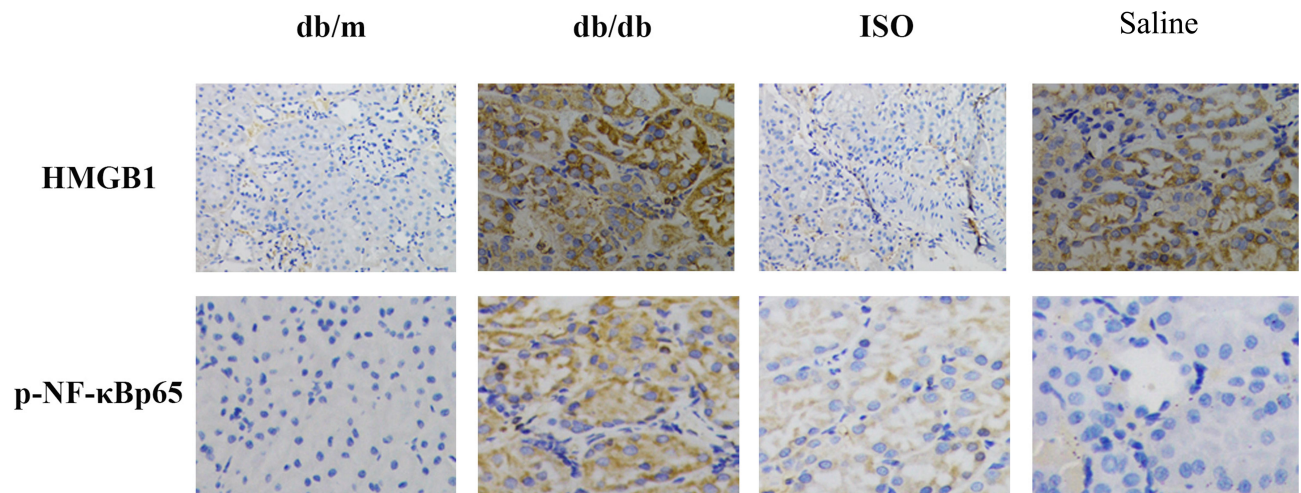


Figure 6 Effects of ISO on inflammatory factors in the kidney. Immunohistochemical staining of HMGB1 and p-NF-κB in the kidney tissues. Magnification: 20x.

is related to anti-inflammation activity of ISO, consistent with the recent opinion that targeting inflammation is a promising therapeutic approach for DN.⁸ However, this study has some limitations. First, we could not perform quantitative analysis of the effects of ISO on pathological changes in the kidney of diabetic mice. Second, we could not determine the signaling pathway that mediates beneficial effects of ISO on DN by using in vitro cellular models.

In conclusion, ISO improved hyperglycemia, insulin resistance, and kidney inflammation in diabetes model mice, and these beneficial effects may be at least partially mediated by the inhibition of HMGB1/NLRP3/NF-κB signaling in the kidney. ISO may be a new agent to treat DN.

Acknowledgment

This study was supported by Natural Science Foundation of Jiangsu Province [BK20180574, BK20171064], which supported the animals, medicine, and page charges in the study design; the Technology Incubator Scheme (Social Programs) of Jiangning District [2018Ca12], the Research Foundation of Jiangsu Provincial Medical Youth Talent, the Project of Invigorating Health Care through Science, Technology and Education [QNRC2016858], the Nanjing Medical University Science Research and Development Foundation [2016NJMU013], the Natural Science Research Fund (General Program) for the Higher Education of Jiangsu Province of China [18KJB350005], and the Zhenjiang City 2017 Science and Technology Innovation Fund [SH2017043].

Disclosure

The authors declare no competing interests.

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