Original Article
Protective effect of Shenkang injection against renal ischemia-reperfusion injury via inflammation inhibition in type 2 diabetic rats

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Abstract: *Aim:* To investigate the therapeutic effects of Shenkang Injection (SKI) on renal ischemia-reperfusion injury (IRI) and its possible mechanism in type 2 diabetes mellitus (T2DM) rats. *Materials and methods:* The T2DM rat model was induced by a high-sugar, high-fat diet and intraperitoneal injection of low-dose streptozotocin. Insulin resistance of the diabetic rats was detected using the hyperinsulinemic-euglycemic clamp test. Renal IRI in the T2DM rats (DMIR) were induced by clamping the left renal artery for 45 minutes with surgical removal of the right kidney, and then reperfusion for 24 hours. Four experimental groups were set in this study: The rats were treated with SKI (6.0 g/kg/d) for eight weeks after the T2DM model was established and then the renal IRI operation were performed as the experimental group (DMIR+SKI group), T2DM rats with IRI (DMIR), T2DM rats with sham operation (DMS), and the normal rats with sham operation (NCS) as the controls in this study. The level of serum creatinine (SCr), blood urea nitrogen (BUN), and the level of urine N-acetyl-D-glucosaminidase (NAG), kidney injury molecule-1 (KIM-1) were detected. The renal pathological changes were evaluated. Furthermore, expression of toll like receptor (TLR) 2, TLR4, myeloid differentiation factor 88 (MyD88), nuclear factor kappaBp56 (NF-κBp56), and high-mobility-group box 1 (HMGB1) in the renal tissue was detected using immunohistochemical staining, Western blot and real-time polymerase chain reaction, respectively. *Results:* The level of SCr, BUN, urine NAG, and KIM-1 were markedly increased after induction of renal IRI in the T2DM rats. The renal histologic results showed severe tubular injury in the DMIR rats. Expression of HMGB1, TLR2, TLR4, MyD88, and NF-κB in the renal tissue was significantly increased both at the gene and protein level after induction of renal IRI. With eight weeks pre-treatment of SKI before induction of renal IRI, all the above changes were significantly improved in the T2DM rats. *Conclusion:* Our results suggest that the TLR2/4 pathway activation plays an important role on initiating and accelerating renal IRI in T2DM rats. SKI pre-treatment could significantly decrease the level of SCr, BUN, urine NAG, and KIM-1, and ameliorate renal histologic injury after induction of renal IRI in T2DM rats. These effects are probably related to TLR2/4 pathway inhibition.

Keywords: Diabetes mellitus, ischemia-reperfusion injury, toll-like receptor 2, toll-like receptor 4, shenkang injection

Introduction

Ischemia-reperfusion injury (IRI) occurs in many tissues and organs of the human body, such as the heart, brain, liver, kidney, lung, and gastro-intestinal tract [1]. Kidney is a high-perfusion organ which is prone to sustain ischemia, ischemia-reperfusion (IR), and eventually acute kidney injury (AKI). This condition of IRI is worsened in the case of diabetes mellitus (DM). Chronic hyperglycemia lesions of DM compromise the renal tolerance to IR, and the kidney is more prone to AKI [2]. It has been reported that

DM is a risk factor for increasing susceptibility of AKI in a variety of clinical settings, such as severe sepsis episodes, contrast nephropathy, following cardiopulmonary bypass operations, and kidney transplantation [3-6]. Furthermore, Oliveira et al. has also showed that DM is an independent risk factor for development of AKI according to logistic regression analysis [7]. The pathogenesis of renal IRI is mediated by multiple mechanisms including inflammatory, oxidative stress, endothelial dysfunction, apoptosis, and necrosis [2, 8, 9]. Among the above mechanisms, inflammatory responses play an

important role on initiating and accelerating renal IRI and toll-like receptors (TLRs) activation and may be the beginning of this episode [10, 11].

TLRs are pattern recognition receptors (PRRs) which play a key role in the innate immune system by triggering pro-inflammatory signaling pathways. These receptors recognize both exogenous ligand such as microbial pathogens and endogenous agonists of non-microbial origin such as heat shock protein 70 (HSP70) and high-mobility-group box 1 (HMGB1). TLRs are involved in non-infectious inflammatory conditions in response to endogenous ligands [12, 13]. Among the eleven kinds of human TLRs, TLR2, and TLR4 are closely associated with renal IRI [10, 14]. During the IRI, expression of HMGB1 in the renal tissue was significantly elevated, which may subsequently bind to TLR2/4 as endogenous ligands and through the activation of myeloid differentiation primary response 88 (Myd88) and NF-κB, leading to a pro-inflammatory response [14, 15].

Pharmacological conditioning is one of the most commonly preferred methods to reduce the serious effects of IRI. Shenkang injection (SKI), a traditional Chinese herbal formula, consists of extracts from four herbs: *Rheum palmatum* L. (Da Huang), *Salvia miltiorrhiza* Bunge (Dan Shen), *Astragalus membranaceus* Bunge (Huang Qi), *Carthamus tinctorius* L. (Hong Hua). SKI is widely used and has shown good clinical effects in chronic kidney disease and diabetic nephropathy (DN) in China. Our previous study showed that SKI could improve early renal injury and inhibit tubulointerstitial inflammation and macrophages infiltration in type 2 diabetic rats [16]. There are limited experimental studies of the protective effects of SKI on renal IRI in diabetic kidneys. The aim of this study was to evaluate the effects of SKI administration on the lesions of renal IRI and their mechanism in the rat renal IRI model with diabetes.

Materials and methods

Animals and drugs

Sprague-Dawley (SD) rats were purchased from Beijing Vital River Laboratory Animal Technology Co, Ltd. (Beijing, China). All rats used in this study were female, four weeks of age, and weighed between 80 and 100 grams. All the rats were maintained in a specific pathogen-free, laminar-flow housing apparatus under controlled temperature (23±2°C), humidity (65-70%) and a strict 12 hour light/dark cycle, and were given ad libitum access to food and water in the experimental animal center of Chinese PLA General Hospital. This project was approved by the Animal Care and Use Committee of the Chinese PLA General Hospital (2013-XC-12). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

SKI was prepared and provided by Xi'an Shijishengkang Pharmaceutical Industry Co., Ltd. (Xi'an, China). It is composed of four crude plant medicines (*Rheum palmatum* L., *Salvia miltiorrhiza* Bunge, *Astragalus membranaceus* Bunge, *Carthamus tinctorius* L.) at a ratio of 1:1:3:1. The concentration of SKI is 1.0 g/ml for animal administration in this study.

Establishment of type 2 diabetes mellitus (T2DM) rat model

A total of thirty rats were randomly divided into two groups: a group of normal control with sham operation (NCS, n=6) and a group of diabetes mellitus (DM, n=24) after the rats were fed adaptively for one week. The rats of the DM group were fed with a high sugar and high fat diet (conventional feed + 10% sucrose + 10% lard + 10% egg yolk powder) for six weeks. Then, the rats were administered with streptozotocin (STZ, 35 mg/kg) by a single intraperitoneal injection after being deprived of food but not water for 12 hours. STZ (Sigma, St. Louis, USA) was dissolved in 0.1 M ice-cold sodium citrate buffer (pH=4.6). The rats of DM group received 5% sucrose solution orally for the first 48 hours after STZ injection to prevent those rats death from hypoglycemia. The level of blood glucose was measured using gluco-check apparatus (Accu-Check Active, Penzberg, Germany) at the first week after STZ injection. If the level of blood glucose was higher than 16.67 mm and maintained for at least one week, the T2DM rat model were thought established successfully. The rats of the NCS group received an equal amount of citrate buffer without STZ.

Table 1. Primers for rat renal cortex mRNA detection

Primer name	Primer sequence
TLR2: forward	5'-CCCTGCTCTTTCTCACAGCA-3'
TLR2: reverse	5'-TGACGGCCTGTATCCCTGTA-3'
TLR4: forward	5'-TCCACAAGAGCCGGAAAGTT-3'
TLR4: reverse	5'-CCAGAGCGGCTACTCAGAAA-3'
MyD88: forward	5'-TTTCGACGCCTTCATCTGCT-3'
MyD88: reverse	5'-TGACGGCCTGTATCCCTGTA-3'
NF-κBp56: forward	5'-CGACGTATTGCTGTGCCTTC-3'
NF-κBp56: reverse	5'-AACAGGTGCAGACAGGATTG-3'
β-actin: forward	5'-CCCATCTATGAGGGTTACGC-3'
β-actin: reverse	5'-TTTAATGTCACGCACGATTTC-3'

TLR2: Toll like receptor 2; TLR4: Toll like receptor 4; Myd88: Myeloid differentiation factor 88; NF-κBp65: nuclear factor-kappa B p65.

Hyperinsulinemic-euglycemic clamp test

The hyperinsulinemic-euglycemic clamp test was performed at the time of the T2DM rat model established for assessment of insulin resistance. Three rats were randomly selected from the two groups for the clamp test. The procedure was performed as previously described [16]. Briefly, local anesthesia of rat tail root with 2% lidocaine. The rat tail vein was intubated with trocar for infusion of insulin or glucose. The blood samples were taken from the end of the rat tail artery. The human insulin (40 mU/ml) was infused at the rate of 12 mU/kg/ml with injectomat agilia (Fresenius Kabi, Bad Homburg, Germany) to decrease the fasting blood glucose levels by 0.5 mmol/L in 10 minutes, then the insulin infusion rate was decreased at 4 mU/kg/ml in the following 110 minutes. The blood samples were collected every 5 minutes to measure the level of blood glucose. The blood glucose level was maintained at 4.4~5.5 mmol/L (normal fasting blood glucose) via adjusting 10% glucose injection rate (GIR) according to the level of blood glucose measured. The total time of the clamp test was about two hours (generally up to steady state). The mean GIR of 60~120 minutes, reflecting the sensitivity of insulin, was calculated. The blood glucose coefficient of variation (CV_{BG}) reflects the accuracy of the clamp technique and the GIR coefficient of variation (CV_{GIR}) reflects the reliability of the clamp

technique. At the end of the test, 2 ml of 10% glucose were slowly injected thought the tail vein to prevent the rat from hypoglycemia.

Experimental grouping and drug administration

The T2DM rats (n=21, among the twenty-four DM rats, three of them failed to develop hyperglycemia) were randomly divided into three groups: DMIR (n=7) only group, DMIR treated with SKI (DMIR+SKI, n=7), and DM with sham operation (DMS, n=7). The DMIR+SKI rats were treated with SKI (6.0 g/kg/d) by intraperitoneal injection for eight weeks after T2DM model established. Meanwhile, the rats of the DMIR, DMS, and NCS groups were injected with equal amount of saline as the controls.

Induction of renal ischemia-reperfusion

After eight weeks of SKI or saline administration in each group, the renal IR rat model were induced by clamping the left renal artery for 45 minutes plus a right kidney removed as previously described [17]. Briefly, after being fasted for 12 hours, the rats were anesthetized with 1% pentobarbital (30 mg/kg, Sigma, St. Louis, USA) via intraperitoneal injection. The rat was placed on a heating pad to maintain its body temperature at 37-37.5°C during surgery. The abdomen was opened along the linea alba incision, and the right kidney was removed rapidly. Then, the left renal artery was isolated carefully and clamped with non-traumatic microvascular clip for 45 minutes. To minimize dehydration, the abdomen was covered with a moist sterile pad during the waiting. The clamp was released at an eligible time to initiate reperfusion and the blood flow to the kidney was re-established with visual verification of blood supply. Subsequently, the abdomen was sutured and the animals were returned to their cages for 24 hours. The rats of the DMIR and DMIR+SKI groups were strictly in accordance with the above surgical procedures. The rats of the NCS and DMS groups were attached to the exact same surgical procedure without clamping the left renal artery. The random urine samples were collected during reperfusion. The rats were sacrificed after 24 hours of reperfusion. Blood samples and kidney tissues were collected for the followed examination.

Table 2. Results of hyperinsulinemic-euglycemic clamp test

Group	Body Weight (g)	CV_{BG} (%)	CV_{GIR} (%)	BG (mmol/L)	GIR (mg·kg^{-1}·min^{-1})
NCS (n=3)	222.00±11.06	2.86	2.59	5.09±0.22	27.26±3.34
DM (n=3)	298.06±23.43[a]	3.81	8.87	4.95±0.21	8.11±1.09[a]

NC: normal control group; DM: diabetes mellitus group; GIR: glucose injection rate; CV_{BG}: the blood glucose coefficient of variation, reflects the accuracy of the clamp technique; CV_{GIR}: the GIR coefficient of variation, reflects the reliability of the clamp technique; BG: blood glucose. Compared with NC, [a]$P<0.01$.
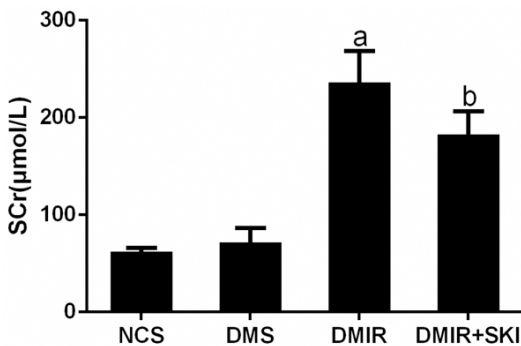

Figure 1. Changes of serum creatinine. NCS: normal control sham group; DMS: DM ischemia-reperfusion sham group; DMIR: DM ischemia-reperfusion group; DMIR+SKI: DMIR treated with SKI group; SCr: Compared with DMS, [a]$P<0.01$; Compared with DMIR, [b]$P<0.01$.

Urinary detection and blood biochemistry

The level of urine N-acetyl-D-glucoseaminidase (NAG) and kidney injury molecule-1 (KIM-1) were detected using commercial ELISA kits purchased from Jiancheng Bioengineering Institute (Nanjing, China). Blood samples were collected via abdominal aorta puncture. Serum creatinine (SCr) and blood urea nitrogen (BUN) were detected using 7150 automatic biochemical analyzer (Hitachi, Tokyo, Japan). The serum HMGB1 was measured using commercial ELISA kits purchased from Jiancheng Bioengineering Institute (Nanjing, China).

Renal pathologic analysis

The left kidney was harvested and part of the renal tissue was fixed with 4% paraformaldehyde/PBS buffer at 4°C overnight, and then routine dehydration, paraffin embedding. Two μm-thick renal tissue sections were stained with Periodic Acid-Schiff (PAS). Renal histopathologic changes were observed under a light microscopy (Olympus, Tokyo, Japan). Changes of renal tubular injury were recorded according to the Paller's renal tubular injury score (TIS)

system [18]. Briefly, 100 cortical tubules from at least 10 different fields were scored for each kidney. Points were given for the presence and extent of tubular epithelial cell flattening (1 point), brush border loss (1 point), cell membrane bleb formation (1 or 2 points), interstitial edema (1 point), cytoplasmic vacuolization (1 point), cell necrosis (1 or 2 points), and tubular lumen obstruction (1 or 2 points).

Immunohistochemistry analysis

Immunohistochemistry analysis was performed as previously described [19]. Four μm-thick paraffin sections of rat renal tissue were incubated with rabbit anti-rat HMGB1 antibody (1:400, Boster, Wuhan, China), rabbit anti-rat TLR2 antibody (1:100; Abcam, Massachusetts, USA), rabbit anti-rat TLR4 antibody (1:100, Boster, Wuhan, China) at 4°C overnight respectively. After washing with PBS buffer (pH 7.4), sections were incubated with polymer helper (Zsbio, Beijing, China) for 30 minutes. Sections were then washed with PBS buffer and incubated with polyperoxidase-anti-rabbit IgG (Zsbio, Beijing, China) for 30 minutes. The positive staining area of the cortical tubular was quantitated using Image Pro Plus Software 5.0 (Media Cybernetics, Maryland, USA) and expressed as Integrated Optical Density (IOD).

Western blot analysis

Kidney tissue was homogenized and suspended on ice with RIPA lysis buffer containing proteinase inhibitors for 20 minutes, then centrifuged at 13,000 rpm for 20 minutes at 4°C. Equal aliquots of proteins were separated by SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% BSA-TBST for 1 hour at room temperature, the membranes were incubated overnight at 4°C with primary antibodies included anti-TLR2 (1:500, Abcam, Massachusetts, USA) and anti-TLR4 (1:500, Boster, Wuhan, China), anti-MyD88 (1:500, Boster, Wuhan, China), anti-NF-κBp56 (1:500, Boster, Wuhan, China) and anti-β-actin (1:1000, Epitomics, USA). After thorough washing, the membranes were incubated for 1 hour at room temperature with peroxidase conjugated secondary antibodies (Jackson, USA). Immuno-
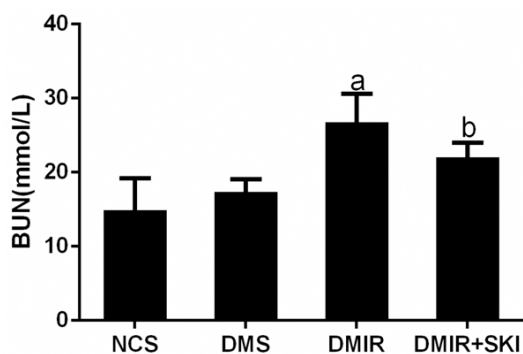
Figure 2. Changes of BUN. NCS: normal control sham group; DMS: DM ischemia-reperfusion sham group; DMIR: DM ischemia-reperfusion group; DMIR+SKI: DMIR treated with SKI group; BUN: blood urea nitrogen. Compared with DMS, [a]$P<0.01$; Compared with DMIR, [b]$P<0.01$.
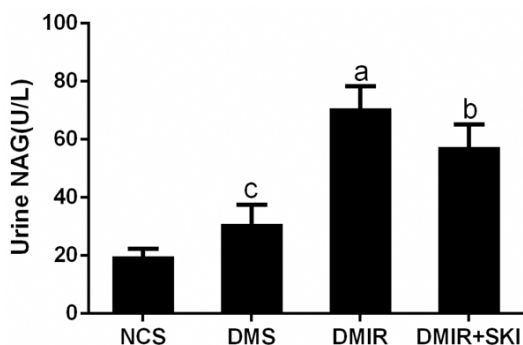

Figure 3. Changes of urine NAG. NCS: normal control sham group; DMS: DM ischemia-reperfusion sham group; DMIR: DM ischemia-reperfusion group; DMIR-SKI: DMIR treated with SKI group; NAG: N-acetyl-D-glucoseaminidase. Compared with DMS, [a]$P<0.01$; Compared with DMIR, [b]$P<0.01$; Compared with NCS, [c]$P<0.01$.
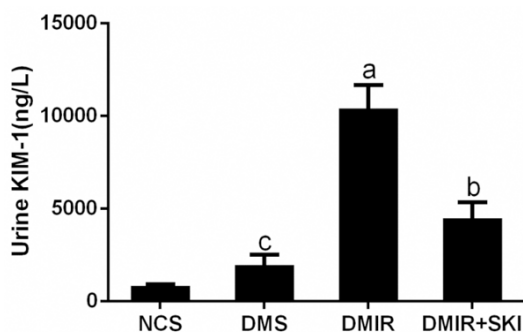

Figure 4. Changes of urine KIM-1. NCS: normal control sham group; DMS: DM ischemia-reperfusion sham group; DMIR: DM ischemia-reperfusion group; DMIR+SKI: DMIR treated with SKI group; KIM-1: kidney injury molecule-1. Compared with DMS, [a]$P<0.01$; Compared with DMIR, [b]$P<0.01$; Compared with NCS, [c]$P<0.01$.

reactive bands were visualized using an ECL system (Millipore, USA). The results were quantified by a densitometric analysis using a Bio-Image Analysis System (Bio-Rad). Each band was normalized relative to the actin band in the same sample.

Quantitative real-time polymerase chain reaction (rt-PCR)

Total RNA was extracted from rat kidney with TRI[zol] reagent (Invitrogen, Shanghai, China) and was reverse transcribed to cDNA using TIANScript RT Kit (Invitrogen, Shanghai, China). Gene expression was analyzed by real-time PCR in an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The primers were used for rat renal cortex mRNA detection were showed in **Table 1**. Expression levels were normalized relative to those of actin in the same samples using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was conducted using computer program SPSS (version 13.0, IBM, NY, USA). All quantitative data were subjected to normality tests and the data were analyzed by one-way ANOVA or Rank-sum test. Differences were considered statistically significant at $p<0.05$.

Results

Obvious insulin resistance of T2DM rats

After six weeks of high sugar and high fat diet, the body weight of DM rats increased obviously compared with the NCS rats ($p<0.01$, Showed as the **Table 2**). The CV_{BG} and CV_{GIR} values of hyperinsulinemic-euglycemic clamp test were within the normal range which suggested that the clamp technology was accurate and reliable. The GIR values in the rats with DM were significantly lower than that of the NCS rats ($p<0.01$, Showed as the **Table 2**). This data implied that the diabetic rats in this study showed significant insulin resistance.

SKI prevented renal function decline after induction of renal IRI

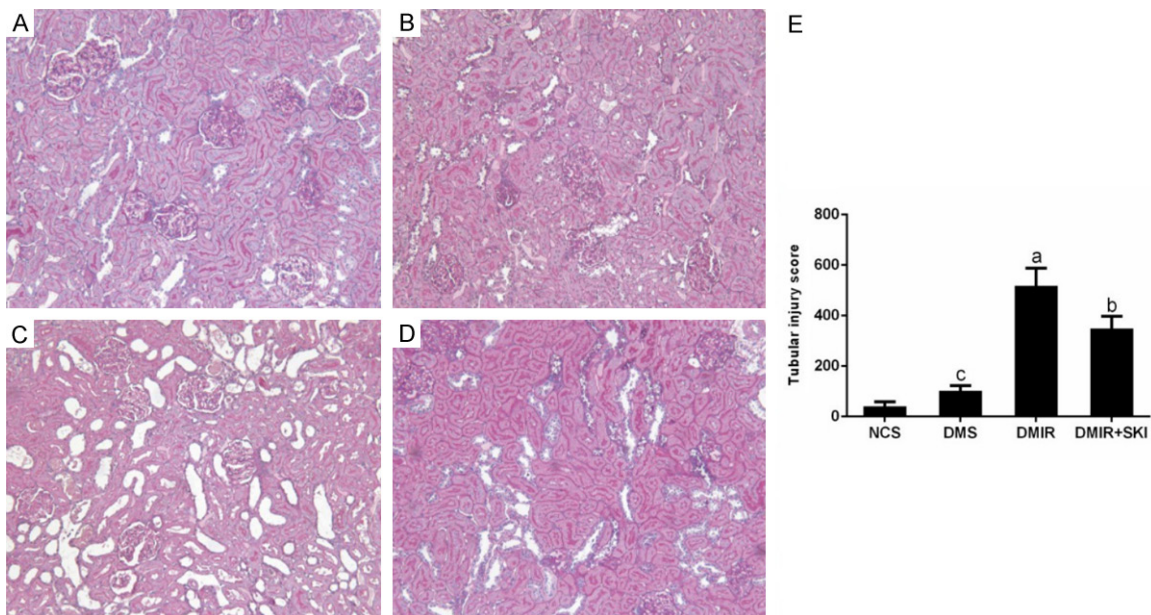Compared with the DMS group, the levels of the SCr and BUN in the rats of DMIR group were

Figure 5. Lesions of the renal tubulointerstitium. NCS: normal control sham group; DMS: DM ischemia-reperfusion sham group; DMIR: DM ischemia-reperfusion group; DMIR+SKI: DMIR treated with SKI group. A: Renal tubular changes of NCS. B: Renal tubular changes of DMS. C: Renal tubular changes of DMIR. D: Renal tubular changes of DMIR-SKI. E: Tubular injury score. Compared with DMS, [a]$P<0.01$; Compared with DMIR, [b]$P<0.01$; Compared with NCS, [c]$P<0.01$. Periodic Acid-Schiff staining ×200.

significantly increased after induction of renal IRI (**Figures 1**, **2**, $p<0.01$). With SKI treatment, the levels of the SCr and BUN were markedly decreased in the rats with DMIR+SKI (**Figures 1**, **2**, $p<0.01$).

SKI improved renal IRI-induced renal tubular injury

The levels of urine NAG and KIM-1, biomarkers of renal tubular injury, were significantly increased in the rats with DMIR group compared with the DMS group after induction of renal IRI (**Figures 3**, **4**, $p<0.01$). Treatment with SKI markedly decreased the level of urine NAG and KIM-1 in the rats with DMIR+SKI (**Figures 3**, **4**, $p<0.01$).

Histopathological light microscopic findings of the rat kidney tissue are shown in **Figure 6**. Blind review of specimens from DMIR rats revealed greater tubular injury than that of DMS rats (**Figure 5B** and **5C**). TIS in the DMIR rats was significantly higher than that in the DMS rats (**Figure 5E**, $p<0.01$). Although all the rats with DMIR and DMIR+SKI showed heterogeneous loss of the brush border, bleb formation, cytoplasmic vacuolization, cellular necrosis,

tubular luminal debris and obstruction, the lesions of renal tubulointerstitum in DMIR+SKI group was alleviated markedly (**Figure 5C** and **5D**). TIS in the rats with DMIR+SKI was also decreased significantly than that in the DMIR rats (**Figure 5E**, $p<0.01$). The differences of renal histologic changes between DMIR and DMIR+SKI rats were mostly due to the greater extent of cellular necrosis and tubular obstruction. There were no differences between DMIR and DMIR+SKI rats with regard to the pattern or distribution of lesions.

SKI reduces tubulointerstitial inflammation via TLR2/4 pathway inhibition

Expression of HMGB1 was mainly detected in nuclear of proximal and distal tubular cells in all rats (**Figure 6A-D**). Compared with the DMS rats, strong staining of HMGB1 was detected in the DMIR rats after induction of renal IRI (**Figure 6B** and **6C**). Meanwhile, with SKI treatment, weak staining of HMGB1 was detected on the tubular cells in the DMIR+SKI rats (**Figure 6C** and **6D**). Expression of TLR2 and TLR4 were detected predominantly in the proximal tubules, the distal tubules, and the peritubular capillaries in all rats (**Figure 6E-K**). The variation ten-
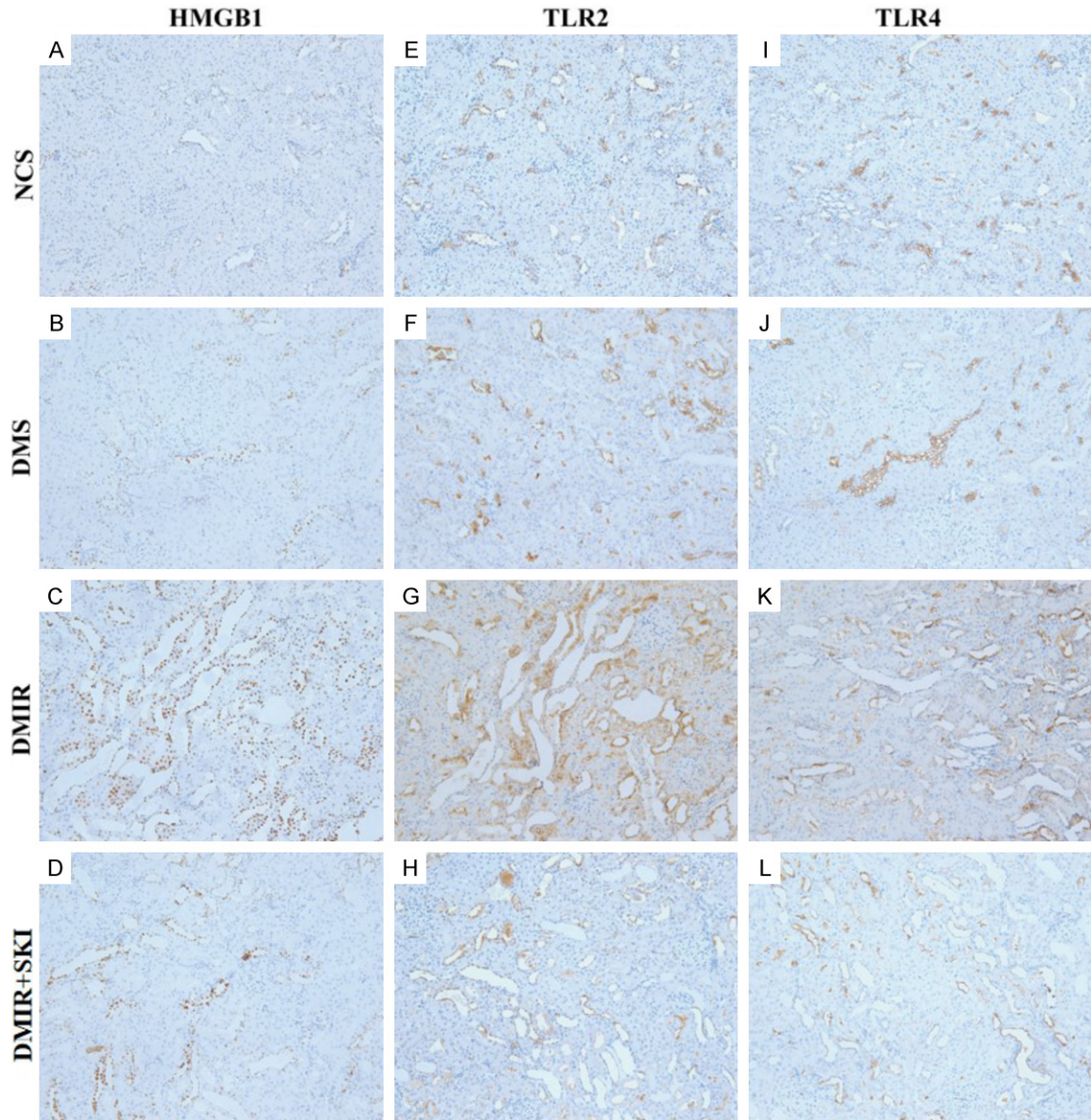
Figure 6. Immunohistochemical staining of HMGB1, TLR2 and TLR4. NCS: normal control sham group; DMS: DM ischemia-reperfusion sham group; DMIR: DM ischemia-reperfusion group; DMIR+SKI: DMIR treated with SKI group. HMGB1: High-mobility-group box 1; TLR2: Toll like receptor 2; TLR4: Toll like receptor 4. Immunohistochemistry ×200.

dencies of TLR2 and TLR4 expression were similar to HMGB1, which means SKI treatment also inhibited the expression of TLR2 and TLR4 (**Figure 6G**, **6H**, **6K** and **6L**). The semi-quantitative analyses of the HMGB1, TLR2 and TLR4 in the renal tubules were showed as **Figure 7A-C**.

To confirm differential expression of TLR2 and TLR4 in each group, we also performed Western blot and RT-PCR (**Figures 7**, **8**). High expression of TLR2 and TLR4 protein was ob-

served in DMIR rats by Western blot, and their downstream proteins MyD88 and NF-κBp-56 were also activated. With SKI treatment, expression of TLR2, TLR4, MyD88, and NF-κBp56 protein in the DMIR+SKI rats were obviously decreased than that in the DMIR rats (**Figure 8**, $p<0.01$). The results of gene detection are similar to what was observed by the Western blot. The fold changes of TLR2, TLR4, MyD88, and NF-κBp56 mRNA are shown in **Figure 7D-G**.
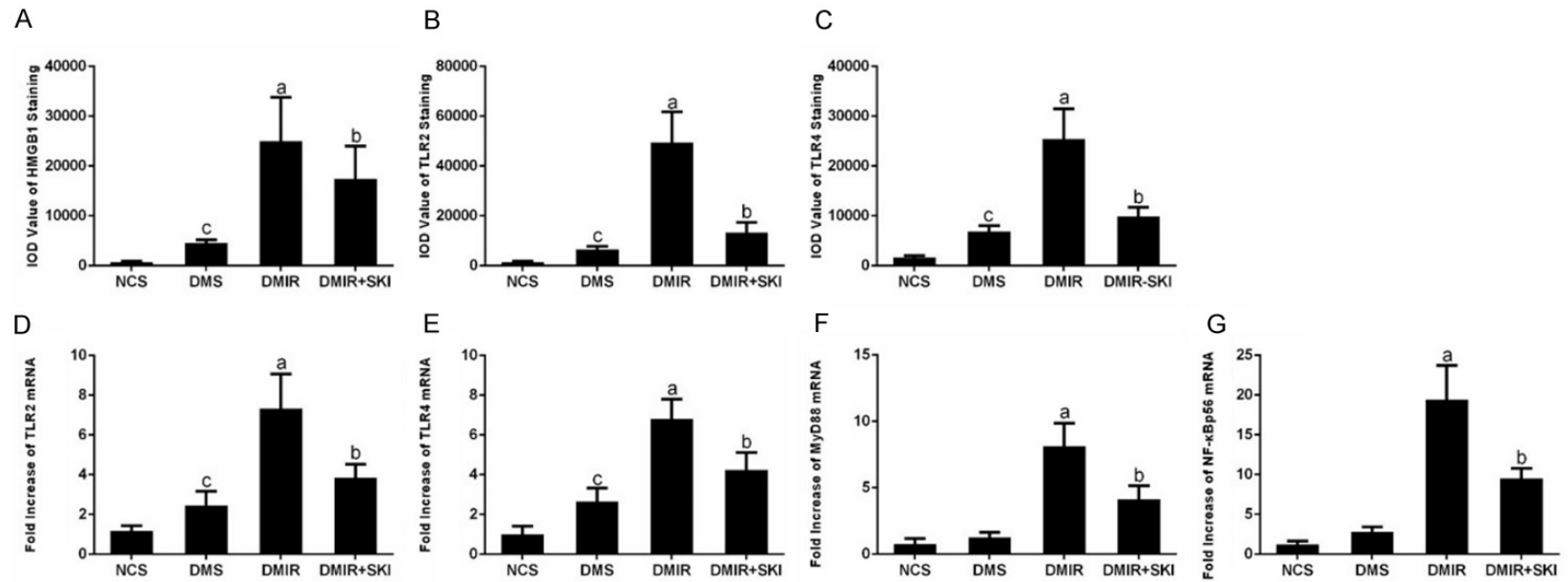
Figure 7. Quantitative results of immunohistochemistry and real-time polymerase chain reaction. NCS: normal control sham group; DMS: DM ischemia-reperfusion sham group; DMIR: DM ischemia-reperfusion group; DMIR+SKI: DMIR treated with SKI group. HMGB1: High-mobility-group box 1; TLR2: Toll like receptor 2; TLR4: Toll like receptor 4; Myd88: Myeloid differentiation factor 88; NF-κBp65: nuclear factor-kappa B p65. IOD: Integrated Optical Density. Compared with DMS, [a]$P<0.01$; Compared with DMIR, [b]$P<0.01$; Compared with NCS, [c]$P<0.01$.
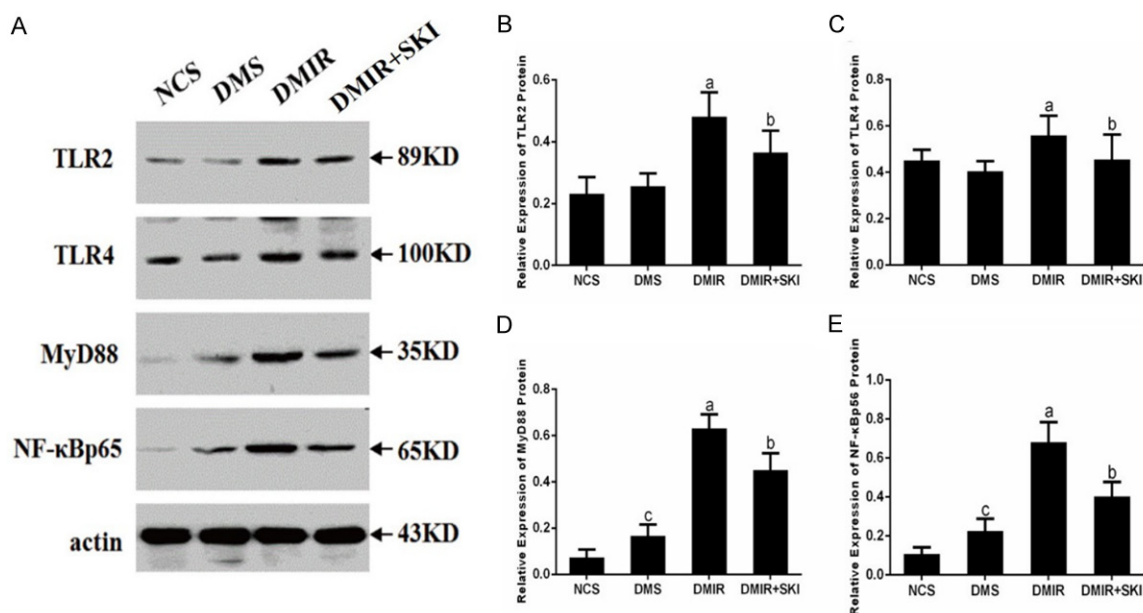
Figure 8. The results of Western blot. NCS: normal control with sham operation group; DMS: DM ischemia-reperfusion with sham operation group; DMIR: DM ischemia-reperfusion group; DMIR+SKI: DMIR treated with SKI group. TLR2: Toll like receptor 2; TLR4: Toll like receptor 4; Myd88: Myeloid differentiation factor 88; NF-κBp65: nuclear factor-kappa B p65. Compared with DMS, [a]$P<0.01$; Compared with DMIR, [b]$P<0.01$; Compared with NCS, [c]$P<0.01$.

Discussion

Insulin resistance is the basis of T2DM and should be proven in T2DM rat model. Hyper-insulinemic-euglycemic clamp test is the gold standard for detecting the existence of insulin resistance. Our results show that the GIR value of the DM rats was significantly lower than that of the NCS rats which means the obvious insulin resistance was existed in the diabetic rats in this study. Taken together with the increased body weight and higher blood glucose of DM rats, we conclude that the T2DM rat model was established successfully in our study.

Acute kidney ischemia was induced by clamping the left renal artery for 45 minutes plus the right kidney removed. After 24 hours reperfusion, the level of SCr and BUN, as well as the renal tubular injury biomarker urine NAG and KIM-1, were markedly increased in the DMIR rats. The renal histologic changes also showed severe tubular injury, such as loss of the brush border, bleb formation, cytoplasmic vacuolization, cellular necrosis, tubular luminal debris and obstruction in the DMIR rats. These results showed that the renal IRI rat model was induced successfully.

Toll-like receptors have been increasingly recognized as playing the initiating and critical role in the pathogenesis of renal IRI. During the renal IRI, TLR2, and TLR4 were up-regulated and activation by binding with endogenous HMGB1 which are released by stressed and necrotic cells, as well as degraded products of endogenous macromolecules, then induced inflammatory responses through MyD88 and NF-κB activation, leading to acute tubulointer-stitial injury [10, 20, 21]. A cross sectional study found that the circulating HMGB1 were elevated and related to inflammatory parameters release in the patients with AKI [22]. It has been reported that the released HMGB1 played a pro-inflammatory role to initiate innate immune responses and involved in renal IRI [23]. Wu et al. also reported that neutralizing anti-HMGB1 antibody treating could protect the wild-type mice against the renal IRI [24]. Furthermore, Lin et al. found that the renal tubular expression of TLR2 and TLR4 were obviously increased after renal IRI, and consistent with the high expression of NF-κB [14]. Additionally, TLR4 deficient mice were protected against renal IRI [24]. These results imply that TLR2/4 activation is involved in renal IRI. In our study, we found that the tubular expression of HMGB1 was elevated after induction of renal

IRI in T2DM rats. Strong staining of TLR2 and TLR4 were directly observed on the proximal tubules, the distal tubules, and the peritubular capillaries with immunohistochemistry analysis after renal IRI in T2DM rats. Moreover, we confirm activation of TLR2 and TLR4 both at the gene and protein level and their downstream pathway MyD88 and NF-κBp56 were also significantly up-regulated after renal IRI in T2DM rats. Taken together, we deduce that renal IRI induced cell damage and increased HMGB1 release, and then the released HMGB1 bound to up-regulated TLR2/4 to transduce inflammatory response through MyD88 and NF-κB activation and contribute to initiation and development of tubulointerstitial injury in the T2DM rats.

The studies of SKI were mostly focus on the renal protection in CKD and DN. To our knowledge, this study is the first to report the potential therapeutic value of SKI on the renal IRI in the rats with T2DM. In our study, we found that the level of SCr and BUN in the rats with DMIR were decreased when treatment with SKI. Urine NAG and KIM-1, the renal tubular injury biomarkers, were also markedly decreased with treatment of SKI. Furthermore, the renal tubular histologic changes also improved in the rats with DMIR+SKI. Moreover, expression of HMGB1, TLR2, TLR4, MyD88, NF-κB were all significantly decreased both at the gene and protein level in kidney tissue with treatment of SKI. Therefore, the protective effects of SKI against renal IRI may be associated with TLR2/4 pathway Inhibition in T2DM rats. The therapeutic effects of SKI are inseparable from its composition. Emodin, Rhein, Tanshinone IIA, danshensu and Astragaloside IV are the main active components of SKI [25]. Guan et al. had reported that the rhubarb aglycone (mainly composed of aloe-emodin, rhein and emodin) had protective effects on the rats of cerebral IRI, such as improved neurological deficits, cerebral infarction, and neuronal apoptosis [26]. It was also found that intraperitoneal injection of emodin (5 mg/kg) significantly reduced the levels of SCr and the renal pathological damage, decreased the renal tubular score and necrotic area and inhibited the NF-κB expression in mice with renal ischemia for 25 minutes and reperfusion for 24 hours [27]. The Tanshinone IIA (10, 20, and 40 mg/kg/d) were given for 15 days before induction of rat myo-cardial ischemia (Rami anterior descending branch of coronary artery was ligated for 30 minutes and then re-perfused for 120 minutes to establish a reperfusion model). The study found that Tanshinone IIA could effectively improve the oxidative stress, attenuate the expression of HMGB1, and inhibit the inflammation [28]. In addition, Lu et al. also reported the cardioprotective effects of Astragaloside IV on myocardial IRI via regulation of TLR4-NF-κB signaling pathway [29]. Therefore, according to the above results, the renoprotective effects of SKI with renal IRI may probably associated with TLR2/4 pathway inhibition in the T2DM rats.

Conclusion

In summary, TLR2/4 pathway activation plays an important role on initiating and accelerating renal IRI in T2DM rats. Our study found that SKI treatment can significantly decrease the level of SCr, BUN, urine NAG, and KIM-1, ameliorate renal histologic injury after induction of renal IRI in T2DM rats. These effects probably relate to TLR2/4 pathway inhibition. Nevertheless, the exact mechanisms still warrant further research.

Acknowledgements

Disclosure of conflict of interest

None.

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