Toll-like receptor 7 involves the injury in acute kidney ischemia/reperfusion of STZ-induced diabetic rats[1]

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ABSTRACT

PURPOSE: To determine whether Toll-like receptor 7 (TLR7) is the potential targets of prevention or progression in the renal ischemia/reperfusion (I/R) injury of STZ-induced diabetic rats.

METHODS: Thirty six Sprague-Dawley rats were randomly arranged to the nondiabetic (ND) or diabetic group (DM), with each group further divided into sham (no I/R injury), I/R (ischemia-reperfusion) and CD (given by Chloroquine) group. Preoperatively, Chloroquine (40 mg/kg, intraperitoneal injection.) was administrated 6 days for treatment group. I/R animals were subjected to 25 min of bilateral renal ischemia. Renal function, histology, apoptosis, cytokines, expression of TLR7, MyD88 and NF-κB were detected.

RESULTS: The serum levels of blood urea nitrogen, creatinine, IL-6 and TNF-α, apoptotic tubular epithelial cells, expression of TLR7, MyD88 and NF-κB were significantly increased in DM+I/R group, compared with ND+I/R group ($p<0.05$). All these changes were further improved by TLR7 inhibition Chloroquine except Paller scores ($p<0.05$).

CONCLUSION: Toll-like receptor 7 inhibition attenuates the acute renal ischemia/reperfusion injury of STZ-induced diabetic in SD rats.

Key words: Toll-Like Receptor 7. Diabetes Mellitus. Ischemia. Reperfusion. Kidney. Rats.

Introducion

An increased susceptibility of the diabetic kidney to ischemic injury has been reported in diabetics[1-3]. The pathomechanisms of acute kidney ischemia/reperfusion (I/R) are not completely elucidated. It is mainly caused by complex inflammatory processes that affect the vascular as well as the tubular system in the kidney[4]. The mechanisms that induce the inflammatory response in the kidney may share a common pathway: pro-inflammatory response upon toll-like receptors (TLRs) activation[5,6]. They suggest that different TLRs play a varying role in renal I/R of diabetes mellitus[5,7,8]. However, the role of TLR7 in renal I/R of diabetic has not been examined.

In the present time, the research of TLR7 has been at least partly successful in terms of liver, lungs, coronary artery and related disorder[9-11]. In hepatitis viruses, TLR7 stimulation mediates an endogenous type I interferon response, that may lead to development of protective immunity and eradication of hepatitis B. However, TLR7 agonists result in the generation of proinflammatory cytokines, such as IL-2, IL-8, and TNF along with interferon α/β, which may result in development of undesirable adverse events[11]. TLR7 was overexpressed in lung cancer patients and have been implicated in contributing to inflammation, tumor growth, cell survival, and metastasis[12,13]. In addition, TLR7 was identified as a macrophage-specific target to selectively induce autophagy in macrophages from atherosclerotic plaques. TLR7 stimulation accelerates the spontaneous onset of autoimmune diabetes in NOD mice[14].

The TLR7 is activated by the myeloid differentiation primary response gene 88 (MyD88)-dependent (all TLRs except TLR3) pathways. The association of TLR7 and MyD88 leads to activation of IL-1-receptor-associated kinase (IRAK) by phosphorylation which in turn leads to activation of TNF receptor-associated factor 6 (TRAF6), TGF-β-activated kinase 1 (TAK1), inhibitor of κB kinase (IKK) and transcription factors nuclear factor (NF-κB)[15]. Activation of NF-κB triggers the production of pro-inflammatory cytokines and chemokines such as IFN-α, TNF-α, IL-1, IL-4, IL-6 and IL-12[16].

Chloroquine (CQ), the antimalarial drug that inhibits the function of endosomal TLRs, has been shown to inhibit the endosomal TLR7 signalling effectively[17,18]. Although most studies have been reported on the role of TLR7 in inflammation and cancer, little is known in AKI with T1DM. The purpose of this study was to determine whether TLR7 is the potential targets of prevention or progression in the renal ischemia/reperfusion (I/R) injury of STZ-induced diabetic rats.

Methods

This study was approved by the ethics committee of the Renmin Hospital of Wuhan University, Wuhan, China. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals.

Thirty six males Sprague-Dawley (SD) rats, six-week-old, with a body weight of 200–220 g were purchased from Huafukang Biotechnology Co. Ltd. (permission certificate no. SCXK (Beijing, China) 2015–0004). The animals were housed in a temperature-and humiditycontrolled room with a light/dark cycle of 12h, with standard diet and water ad libitum, in the animal center of Renmin Hospital of Wuhan University. The experiment started after a week of adaptation.

Groups, renal ischemia reperfusion of rat diabetes model and drug administration

All animals were randomly separated into the nondiabetic (ND) or diabetic group (DM), with each group further divided into sham , I/R and CD (given by Chloroquine) group. CD group was daily administered intraperitoneal by 0.5% Chloroquine diphosphate (40 mg/kg; Sigma-Aldrich Inc., St. Louis, MO, USA) which diluted in a vehicle (sterile 0.9% NaCl), for 6 days before surgery[18]. Other groups were daily administrated with the equal volume of vehicle. Each group has 6 samples.

After 1 week of taming, the rats were subjected to overnight fasting and Intravenous injection with a single dose of STZ (55 mg/kg body weight, Sigma-Aldrich, St Louis, MO, USA)[19]. Rats with blood glucose levels exceeding 16.7 mmol/L on 7 consecutive days were considered diabetic and used in this study. After 4 weeks, the rats were induced of kidney ischemia-reperfusion injury.

Four weeks after the initial STZ or control injection, SD rats were anesthetized by intraperitoneal injection of 7% chloral hydrate (350 mg/kg, i.p.)[20]. A midline incision (about 3cm) was made, both renal arteries and veins were clamped for 25 minutes with atraumatic microaneurysm clamps. After clamp removal kidneys were inspected for 1 min for restoration of blood flow and returning their original color then the incision was closed in 2 layers. Sham-operated rats received identical surgical procedures

except that the microaneurysm clamps was performed. During the entire experimental protocol, body temperature was maintained at 37°C by placing the animals on a heating pad. To maintain fluid balance and volume status, rats were supplemented with a few drops sterile 0.9% NaCl intra-peritoneal.

Histopathological evaluation

Rats were sacrificed 48h after reperfusion. Left kidney were divided up to be snap frozen in liquid nitrogen and stored in -80°C for different determinations, and right kidney were fixed in 4% paraformaldehyde overnight at 4°C and processed for paraffin embedding according to standard procedures. Sections were cut at 3-mm thickness for histology and immunohistochemistry[21]. Some tissue sections were stained with conventional H&E staining to observe histopathological changes under a light microscope. Morphologic damage was assessed by Paller scores[22].

For immunohistochemical analyses, some tissue sections were subjected to antigen retrieval by microwaving for 10 or 15 min in 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by a 10-min incubation in 3% hydrogen peroxide. Sections were washed with phosphatebuffered saline (PBS) and subsequently incubated with primary antibody (1:50 anti-TLR7 antibody) for 15 h incubation at 4°C. After three washes with PBS, the samples were incubated with biotin-conjugated anti-IgG for 30 min at room temperature. After a final wash in PBS, the sections were incubated with diaminobenzidine . Finally, the sections were incubated with haematoxylin and dehydrated through alcohol and xylene. The sections were examined by light microscopy.

In addition, Tunel assay was performed to detect apoptosis in situ cell death according to the manufacturer's instructions (TUNEL kit). The results of staining were analyzed and evaluated with American Image-Pro Plus software. The percentage of positive cells with TUNEL staining in five x400 sights served as apotosis index (AI).

Biochemical parameters

Blood samples collected via the ventricle were centrifuged (4.000×g for 10 min) to separate the serum and kept at - 20°C until analyses. Renal function was measured by serum blood urea nitrogen (BUN) and creatinine (Cr) levels after 48h reperfusion. Levels of Interleukin-6(IL-6) and Tumor Necrosis Factor-alpha (TNF-α) in the serum were assessed by commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' (Elabscience Biotechnology, China) protocols.

Preparation of protein

The upper pole of renal tissues used for western blotting were homogenized and sonicated in lysis buffer (containing 137 mM NaCl, 20 mM Tris/HCl, pH 8.0, 5 mM EDTA, 10% glycerol, 1% Triton X-100), centrifuged at 4°C at 12000 g for 15 min and supernatants were snap-frozen and stored at -80°C. Western blot was performed as previously described[23]. Protein concentrations were determined by densitometry values. Proteins extracted were separated by SDS-PAGE, transferred onto PVDF membranes, incubated with primary antibodies (TLR7: Novus USA, MyD88: Abcam, UK, NF-κB:CST, USA) and detected with peroxidase-conjugated secondary antibodies and chemiluminescence. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Statistical analysis

All results are expressed as the mean± standard error of the mean (SEM). Data were analyed by one-way analysis of variance (ANOVA) followed by LSD's post-hoc test. Values of $p<0.05$ were considered as statistically significant. All statistical analyses and figures were conducted using SPSS 19.0 and GraphPad Prism 3 software package (La Jolla, CA, USA).

Results

Injury of renal I/R in diabetic rats

The diabetic rats exhibited reduced body weight (Figure 1A) and increased blood glucose levels (Figure 1B) over 4 week period. The data shown the success of the model of diabetes.

In order to verify the success of the model of renal I/R injury, we detected BUN and Cr. We saw a rise in BUN and Creatinine levels in the kidney I/R groups (Figure1 C-D). Compared with ND Sham or DM Sham group, rats subjected to I/R injury showed significant increases in BUN and Cr levels. As shown in Figure 1, DM+I/R group injury caused a transient increase in BUN and Cr compared with ND+I/R group, demonstrating a severe renal dysfunction.

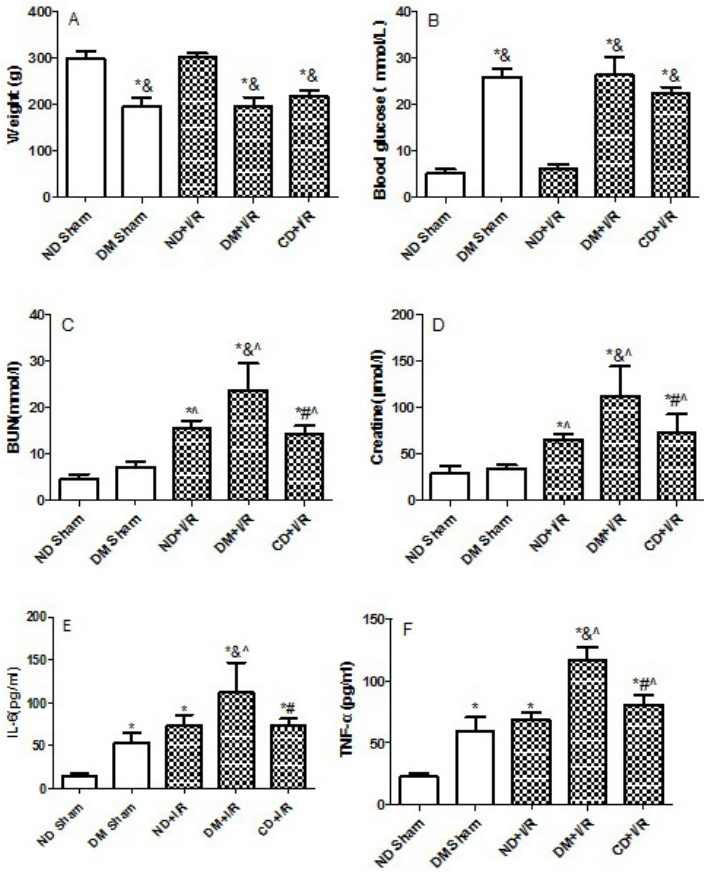

FIGURE 1 - Weight (**A**) and blood glucose (**B**) were measured before surgery. Serum BUN (**C**), Cr (**D**), IL-6 (**E**) and TNF-α (**F**) were measured after 48h reperfusion (n=6). *p<0.05 *vs*. ND Sham group; &p<0.05 *vs*. ND+I/R group; ^p<0.05 *vs*. DM Sham group. #p<0.05 *vs*. DM+I/R group.

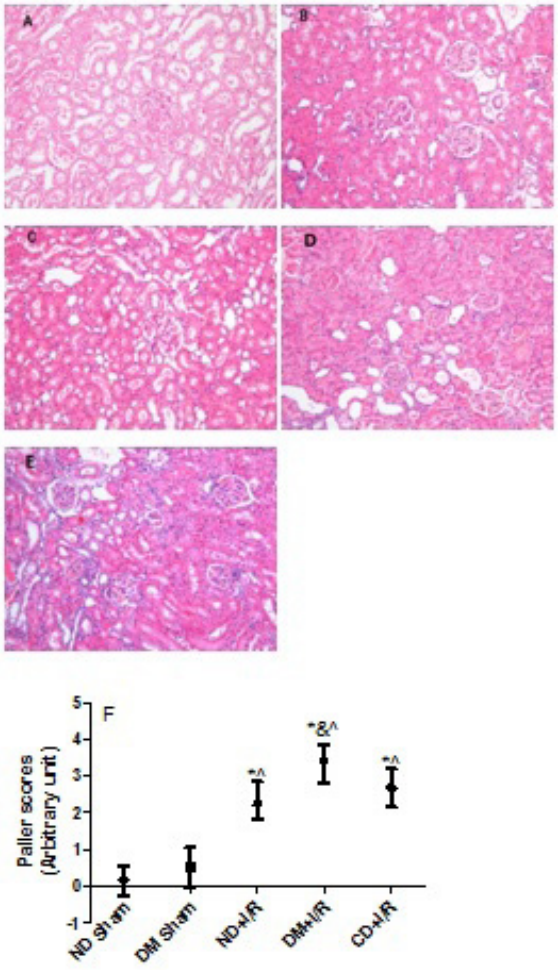

FIGURE 2 - Histological evaluation of renal tissue in five groups (×200). (**A**) ND Sham group; (**B**) DM Sham group; (**C**) ND+I/R group; (**D**) DM+I/R group; (**E**) CD+I/R group. Palle scores for renal tubular injury (**F**). Apoptosis was evaluated by TUNEL staining. Quantification of TUNEL positive cells was counted. Data are expressed as mean ± SD, n = 6. *p<0.05 *vs*. ND Sham group; &p<0.05 *vs*. ND+I/R group; ^p<0.05 *vs*. DM Sham group; #p<0.05 *vs*. DM+I/R group.

Histopathological changes were observed in renal tissue. Kidneys taken from ND Sham rats (Figure 2A) had normal tubular histology. In contrast, only mild damage was seen in the DM Sham group (Figure 2B). Animals experienced to I/R injury were showed dramatically more severe renal pathological injury compared to sham rats. These features included tubular cell swelling, widespread degeneration of tubular dilation, and nuclear condensation (Figure 2 C-D). Paller scores of rats in the I/R injury groups were significantly higher than Sham groups (Figure 2F, p<0.05). Moreover, the DM+I/R group was higher than ND+I/R group (p<0.05).

In addition, we determined the amount of apoptotic tubular epithelial cells. TUNEL staining assay can stains the positive apoptotic cells brown (Figure 3). TUNEL assay showed a significant increase in the number of positive cells after I/R compared with sham rats. Meanwhile, there was difference between ND Sham group and DM Sham group (Figure 3F, p<0.05). Moreover, the most pronounced increase was observed in DM+I/R group.

Pro-inflammatory cytokines (IL-6 and TNF-α) was detected in this study. There were significantly increase in DM+I/R animals when compared with ND+I/R group, detected by ELISA in the blood serum (Figure 1 E-F, p<0.05).

Whatever it is from histopathology, apoptosis or pro-inflammatory cytokines, all observations shown that the extent of acute renal damage after I/R injury is more serious in diabetic animals.

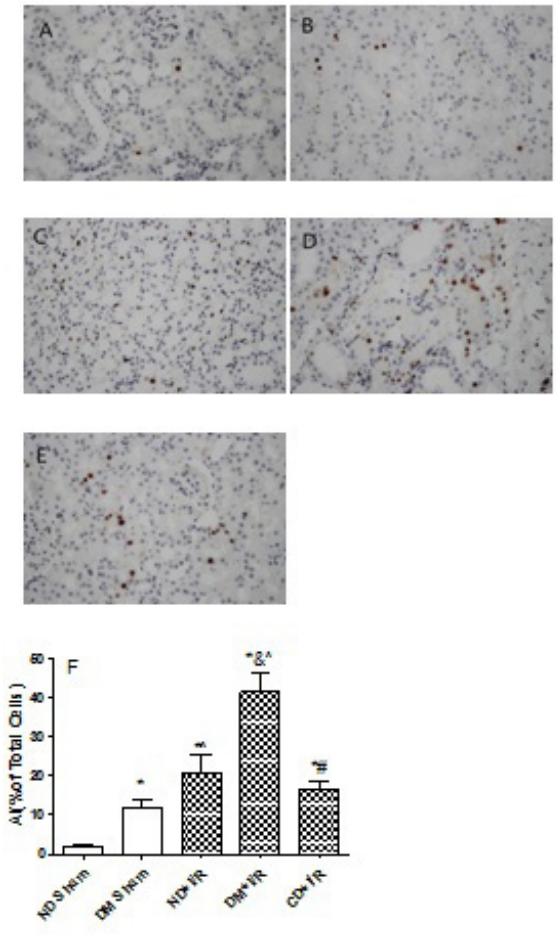

FIGURE 3 - Apoptosis of tubular epitheial cells of renal tissue in five groups(×400). (**A**) ND Sham group; (**B**) DM Sham group; (**C**) ND+I/R group; (**D**) DM+I/R group; (**E**) CD+I/R group. Apoptosis was evaluated by TUNEL staining. Quantification of TUNEL positive cells was counted. AI: apotosis index (**F**). Data are expressed as mean ± SD, n = 6. $^{*}p<0.05$ *vs*. ND Sham group; $^{\&}p<0.05$ *vs*. ND+I/R group; $^{\wedge}p<0.05$ *vs*. DM Sham group; $^{\#}p<0.05$ *vs*. DM+I/R group.

Expression of TLR7 in animals

We examined whether TLR7 expression was functionally important. Then we localized the expression of TLR7 with immunohistochemical staining assay (IHC). We found that TLR7 was expressed in each group. However, TLR7 was expressed predominantly in renal tubules, minimal in renal glomerulus as shown (Figure 4 A-D).

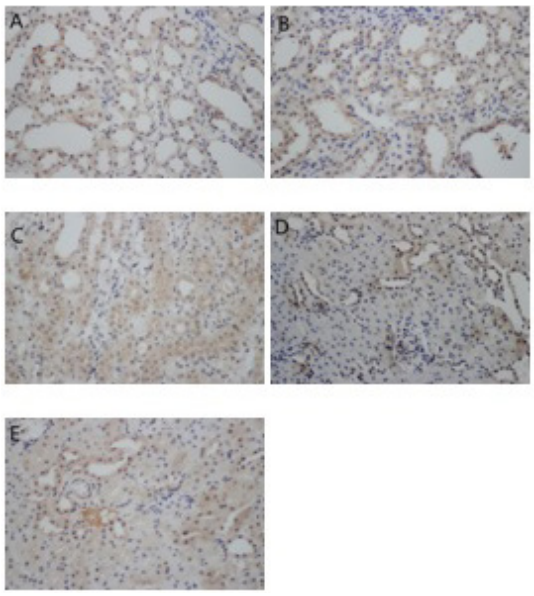

FIGURE 4 - Immunohistochemical detection of the TLR7 expression levels in renal tissue (×400). (**A**) ND Sham group; (**B**) DM Sham group; (**C**) ND+I/R group; (**D**) DM+I/R group; (**E**) CD+I/R group.

Then TLR7 was measured using western blotting (Figure 5A). Compared with ND Sham group, DM groups were higher. Compared with the DM Sham group, TLR7 was also substantially increased in diabetic rat kidneys after I/R injury. The most worthy of attention is that the expression of TLR7 in diabetic I/R group is higher than nondiabetic I/R group. The datas displayed that the changes of TLR7 were positively correlated with the degree of renal injury.

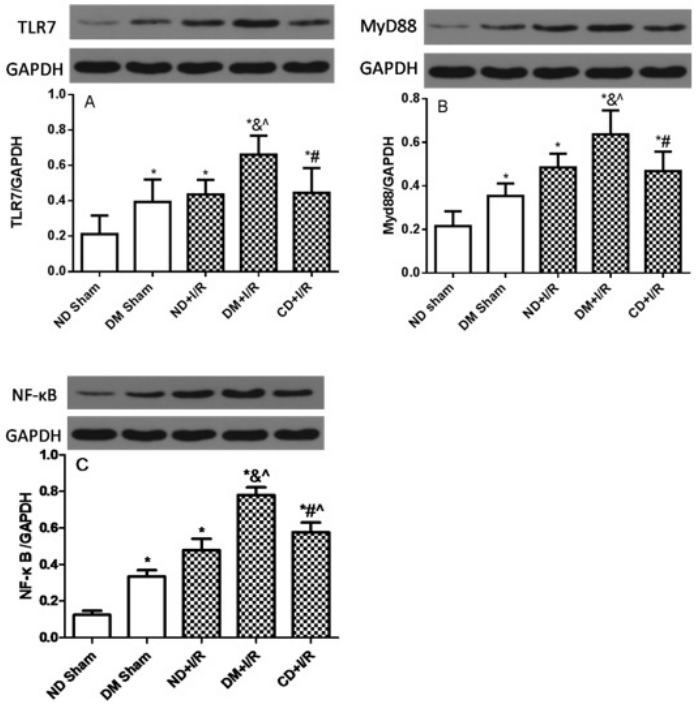

FIGURE 5 - Western blot analysis of TLR7 (**A**), MyD88 (**B**) and NF-κB (**C**) proteins expression (n=5). $^{*}p<0.05$ *vs*. NG Sham group; $^{\&}p<0.05$ *vs*. NG+I/R group; $^{\wedge}p<0.05$ *vs*. DM Sham group; $^{\#}p<0.05$ *vs*. DM+I/R group (n=5).

Alleviation of renal injury after inhibition of TLR7

As TLR7 antagonist, Chloroquine was used in this study. Chloroquine treatment for 6 days did not have any effect on the increase weight of rat body and hyperglycemia, compared with DM+I/R group. However, CD+I/R group was partially exhibited weaker renal dysfunction as reflected by lower levels of BUN and Cr, improvement of Paller scores and apoptosis, compared with DM+I/R rats (Figure 1A-D, $p<0.05$). Also, productions of both IL-6 and TNF-α were significantly lower levels in CD+I/R group when compared with DM+I/R group, detected by ELISA in the blood serum (Figure 1 E-F). All the observations showed that the inhibition of TLR7 reduced the damage of diabetic renal I/R.

Downstream products of TLR7 include MyD88 and NF-κB proteins. TLR7 antagonist treatment of rats indicated the decrease of TLR7, MyD88 and NF-κB in CD+I/R group, compared with DM+I/R group, respectively (Figure 5-A,B,C).

Discussion

Diabetes is an increased risk factor for the development of acute kidney I/R injury in clinical studies involving patients with diabetes[2]. Studies in rats with streptozotocin (STZ)-induced type 1 diabetes (T1DM) indicated that diabetic rats had significantly greater histological damage, tubular apoptosis, and long-term fibrosis and higher mortality after ischemia than nondiabetic rats[3].The mechanisms underlying this susceptibility are not well understood. Previous work has demonstrated an increase in the expression of certain inflammatory cytokines in the diabetic kidney[2,3]. In this study, we showed that the pathological changes of kidney were increased, the apoptosis was enhanced, the IL-6 and TNF-α were raised up in the diabetic I/R model. The results possibly implicate that diabete may increase the susceptibility to renal I/R injury and implicate a hyperresponsive inflammatory response as a cause of this susceptibility. TLRs have been implicated in the pathogenesis of acute and chronic renal disorders. They may promote renal injury in renal ischemia-reperfusion injury, AKI, acute allograft rejection, renal fibrosis, and antibody-mediated glomerulonephritis, whereas TLR7 predominantly contribute to inflammatory response in immune complex-mediated renal nephropathysuch as lupus nephritis[16,24]. However, the role of TLR7 in renal I/R injury in the setting of diabetes has not been examined.

To our knowledge, no longitudinal studies have assessed the impact of TLR7 viewing on renal I/R injury problems in T1DM. Lee *et al*.[14] showed that TLR7 stimulation increased the levels of proinflammatory cytokines and type 1/2 IFNs and in the pancreatic lymph nodes of young prediabetic NOD mice. TLR7 stimulation of NOD bone marrow-derived dendritic cells increased activation and production of proinflammatory cytokines. Similarly our study found that the level of IL-6 and TNF-α were significant increased in DM+I/R animals when compared with ND+I/R group. Many studies showed that TLR7 was expressed in kidney, but not specific positioning in SD rats, for example, renal tubular or glomerular. In our study, we confirmed the expression of TLR7 in renal tissue (Figure 5). Before analyzing the data, we aimed to know where the TLR7 was expressed in kidney. For this purpose, we observed the expression of TLR7 by immunohistochemical staining. As a result, we obtained that TLR7 was expressed predominantly in adjacent renal tubules cells, minimal in renal glomerulus. Location of TLR7 is beneficial for the later phase in vitro. We found that a high expression of TLR7 in I/R diabetic rats confers I/R nondiabetic rats in kidney. We hypothesized that TLR7 may involves the injury in acute kidney I/R of STZ-induced diabetic rats. In the present study, We hypothesized that the activation of TLR7 may be one of predisposing factor to ischemic injury in autoimmune diabetes. To test whether kidney function might already be impaired after 48 h of reperfusion, we measured serum BUN, Cr and histological damage. And we could showed that BUN, Cr and Paller scores in DM+I/R group were significantly higher than ND+I/R group after 48h of reperfusion. Most importantly however, the levels of BUN and Cr in the antagonist of Chloroquine therapeutical rats were partially protected against renal dysfunction as reflected by significantly lower levels of BUN and Cr compared with DM+I/R rats 48 hours after I/R injury, which is due to other mechanisms that might trigger the TLR7 pathway later, as for example other TLR pathways[25]. Leemans *et al.*[26], Patrick Paulus as well as Wu *et al.*[25] could show, that TLRs play an important role in the development of kidney I/R, but only after 1 to 10 days post ischemia. All the observations showed that the inhibition of TLR7 reduced the damage of diabetic renal I/R. The fact strengthens our hypothesis.

TLR7 is one of the important ways to mediate the inflammatory reaction in the TLR family. Recent studies on a murine lupus model suggest that TLR7 may have opposing inflammatory and regulatory roles[27]. The data presented in this paper also demonstrate that TLR7 stimulation accelerates the acute renal I/R injury of autoimmune diabetes in SD rats. These studies suggest that TLR7 activation of the innate immune system may aggravate preexisting kidney disease. TLRs, their associated signaling molecules, and their triggered cytokines, are prime candidates for future research in type 1 diabetes and autoimmunity[28]. They participate in renal tubulointerstitial

damage induced by the immune response[29]. When over-activated, TLRs induce body damage, specifically fibrosis. All TLRs except for TLR3 are thought to share the MyD88-dependent pathway that activates NF-κB and mitogen-activated protein (MAP) kinases, leading to the induction of inflammatory cytokine genes[30]. Taken together, these observations provide hypothesis into the signaling pathway of TLR7. In this study, it showed that TLR7 and its downstream effector MyD88/NF-κB were significantly upregulated in the kidney of I/R of diabetic rats compared with the diabetic group, at protein levels. Decreased TLR7 expressions in the cytoplasm appear to result in low activation of MyD88 and NF-κB at protein levels. These findings suggest that the TLR7 was activated in renal I/R of T1DM, targeting renal tubular epithelial cells, among others. Overall, the injury reaction observed in renal tissue may be the result of overactivation of the TLR7/MyD88/NF-κB-dependent innate immunity under renal I/R with T1DM, and might be involved in occurrence and progression of renal I/R injury with diabetic patients and future studies will define the exact mechanisms. In Chloroquine-treated group, TLR7 signaling pathway was blocked, associated with downregulation of MyD88 and NF-κB. In addition, although chloroquine is not the specific inhibitor of TLR7, it plays an important role in the study of TLR. This is one of the deficiency in this reseach. Using knockout mice or gene silencing technique will make it more convincing. TLR7 siRNA in cell experiments is our further study.

In summary, our findings shown the impact of diabetes on kidney I/R injury and examined the role of TLR7 in I/R-induced acute kidney injury in diabetic rats. TLR7 produced in response to ischemia exacerbates renal dysfunction in T1DM. These results demonstrate an important role for TLR7 in I/R kidney injury in diabetes. Additional studies to investigate the related mechanism of TLR7 in human diabetic nephropathy are warranted.

Conclusion

Toll-like receptor 7 seems to participate in the pathogenesis of kidney injury of renal ischemia/reperfusion in T1DM. Inhibition of TLR7 reduces the damage of diabetic renal ischemia/reperfusion.

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