# TLR7 mediates increased vulnerability to ischemic acute kidney injury in diabetes

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## SUMMARY

OBJECTIVE: *Diabetes is a risk factor for acute kidney injury (AKI). However, its mechanism of pathogenesis has not been elucidated. The aim of the study was to investigate the role of inflammation and the toll-like receptor 7 (TLR7) in ischemic AKI for diabetes.*

METHODS: *A high glucose hypoxia-reoxygenation model of human renal tubular epithelial (HK-2) cells was used to generate AKI induced by ischemia-reperfusion in diabetes. The activity of cells was measured by CCK-8 assay and LDH activity. Inflammatory cytokines were assessed by ELISA. TLR7, MyD88, and NF-κB expressions were examined by western blotting. Apoptosis was evaluated by flow cytometry.*

RESULTS: *The high glucose group and low glucose group were subjected to hypoxia-reoxygenation. The low glucose group developed only mild cell damage, apoptosis, and inflammatory response. In contrast, an equivalent hypoxia-reoxygenation injury provoked severe cell damage, apoptosis, and inflammatory response in the high glucose group. Expression of TLR7 and its related proteins were measured in the high glucose group before and after hypoxia-reoxygenation. The high glucose group exhibited more significant increases in TLR7 expression following hypoxia-reoxygenation than the low glucose group. In addition, the expression of TLR7 and its related proteins after hypoxia-reoxygenation were higher in the high glucose group than in the low glucose group. Inhibition of TLR7 provides significant protection against ischemic injury in diabetes.*

CONCLUSION: *Our results suggest that diabetes increases the vulnerability to ischemia-induced renal injury. This increased vulnerability originates from a heightened inflammatory response involving the TLR7 signal transduction pathway.*

KEYWORDS: *Health Vulnerability. Ischemia. Acute kidney injury. Diabetes mellitus. Toll-Like Receptor 7.*

## INTRODUCTION

Diabetes mellitus is a chronic immune response disease that affects millions of people worldwide and leads to progressive whole-body organ damage, especially of the kidney. The discovery of toll-like receptors (TLRs) has provided great contributions in the field of diabetes research. TLRs are an important part of the innate immune system and play a crucial role in the detection of microbial infections and the antibacterial response defense of the host. In recent years, the role of TLRs in the cardiovascular and nervous system, liver, spleen, and particularly in kidney disease has attracted more attention. After activation,

TLRs promote cell proliferation, differentiation, apoptosis, secretion of inflammatory factors through signal transduction pathways, and are involved in the occurrence and development of multiple diseases[1]. The association of TLRs with inflammatory reactions and cell apoptosis has become a topic of concern for many researchers[2,3]. Therefore, in this study, we seek to address the hypothesis that diabetes increases the vulnerability to ischemic acute kidney injury (AKI) by enhancing the production of inflammatory cytokines and expression of TLR7 within the kidney.

## METHODS
### Antibodies and reagents

The following antibodies and antagonists were used in this study: TLR7 (NBP2-24906, Novus Biologicals, USA), MyD88 (ab2064, Abcam, Cambridge, UK), NF-κBP65 (8242S, Cell Signaling Technology, USA) and chloroquine diphosphate (CQ) (Sigma-Aldrich Inc., St. Louis, MO, USA). TLR7 siRNA plasmid (sc-40266, Santa Cruz Biotechnology, Inc, California, USA) and control siRNA plasmid were from Santa Cruz Biotechnology. Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) were evaluated by commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (Elabscience Biotechnology, Wuhan, China). Chloroquine was dissolved in calcium- and magnesium-free phosphate-buffered saline at a concentration of 10 mM. Stock solutions were diluted to the desired final concentrations with growth medium just before use.

### Cell culture and experimental conditions

Experiments were performed with human renal tubular epithelial (HK-2) cells (ATCC, American Type Culture Collection, Manassas, VA, USA), which are human proximal tubular epithelial cells. Cells were cultured in MEM medium (#31985, Gibco, Grand Island, NY, USA), which was supplemented with 10% fetal bovine serum (FBS, #10099-141, Gibco) and 1% Penicillin-Streptomycin solution in 5% $CO_2$.

### Transfection of siRNA and experimental grouping

HK-2 cells were seeded in 6-well plates ($2 \times 10^5$ per well) and transfected with Lipofectamine 2000 reagent (#11668-019, Invitrogen, USA) according to the manufacturer's instructions. Cells were used for further experiments 48 hours after transfection. After this time period, the HK-2 cells were randomly divided into eight groups (n = 6): 1- low-glycemic group (stimulated with LG (5.6 mM), LG group); 2 - high glucose group (stimulated with LG (30 mM), HG group); 3 - low glucose + mannitol group (24.4 mM, M group); 4 - hypoxia complex oxygen group (hypoxia for four hours and reoxygenation for two hours, LH/R group); 5 - hypoxia complex oxygenation group (HH/R group); 6 - high glucose + anoxic complex oxygen + TLR7 gene silencing group (HH/R-siRNA group); 7 - high glucose + hypoxia + reoxygenation, RNA interference in the control group (HH/R-Scrambled siRNA group); and 8 - a pre-treatment group (HH/R-CQ group) of high sugar + anoxic + hypoxic + chloroquine (50 μM)[4].

### Cell in vitro simulated ischemia/reperfusion model

For the hypoxic treatment, after 72 h HG stimulation in the absence or presence of Chloroquine[5], HK-2 cells were incubated in glucose-free Krebs-Ringer bicarbonate buffer for four hours in a hypoxic chamber equilibrated with 5% CO2, 1% O2, and 94% N2. After hypoxic incubation, the cells were returned to complete medium and reoxidized for two hours with 5% CO2, 21% O2, and 74% N2. Control cells were incubated in a regular cell culture incubator with 21% O2[6].

### Cell viability and lactate dehydrogenase (LDH) activity

Cell viability was determined by using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions. HK-2 cells ($1 \times 10^5$ cells/well) were seeded into 96-well plates and pretreated with various conditions as described[6]. 10μL CCK-8 (Beyotime: C0037, Beijing, China) was then added and cells were incubated for four hours (37°C, 5% CO2), and the absorbance was measured at 450 nm with an ELISA assay plate reader. The LDH content was measured by an LDH Cytotoxicity Assay Kit (Jiancheng Biotech, Nanjing, China).

### Measurement of inflammatory cytokines

IL-6 and TNF-α levels in HK-2 cells were assessed using a rat ELISA kit (Elabscience Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's instructions.

### Western blotting analysis

Expression of TLR7, MyD88, and NF-κB was examined using western blotting. The protein content was

determined by a Bicinchoninic Acid (BCA) protein assay. The protein samples were separated by polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% milk and incubated overnight with the appropriate primary antibodies (anti-TLR3 1:500, anti-MyD88 1:600, and anti-NF-κB 1:1000 antibody) at 4°C. The next day, membranes were incubated with HRP-labeled secondary antibodies (anti-rabbit, 1:5000) for 1 h and then washed with TBST. The membranes were developed on ECL prime solution, and the chemiluminescent signal was measured by BandScan 5.0 software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

### Apoptosis assay

After reoxygenation, the cells were trypsinized, washed twice with PBS, and resuspended in binding buffer. The percent apoptosis was assessed by using the Annexin V-APC/7-AAD detection kit (Nanjing Key-Gen Biotech, Nanjing, China) according to the manufacturer's instructions. Samples were determined by flow cytometry with the FACScan system (BD Biosciences). Apoptotic cells were defined as the cells located in the right two quadrants of each figure, and the percentages were determined by flow cytometry.

### Statistical Analysis

The data were reported as means ± SE. All statistical analyses were performed using SPSS19.0 software. Statistical significance was analyzed by one-way or two-way ANOVA.

### RESULTS
### Detection of cell injury

Hypoxia reoxygenation was induced in the high glucose group and low glucose group. As seen in Figure 1A and B ($p < 0.05$), the high glucose group subjected to four hours of hypoxia developed marked cell damage while the low glucose group maintained relatively little cell damage. TLR7 expression is inhibited by TLR7 siRNA in HK-2 cells. Moreover, the HH/R group enhanced the effects described above compared to the HH/R-siRNA group, whereas transfection of TLR7-siRNA and treatment with chloroquine inhibited the injury of HK-2 under high glucose and hypoxia-reoxygenation. Flow cytometry showed that the apoptosis rate of the HG, LH/R and HH/R groups was higher than the LG group (Figure 1C, D) ($p < 0.05$).

The apoptosis rate of the LH/R group was significantly lower than the HH/R group ($p < 0.05$). Our results demonstrate that TLR7-siRNA transfection or chloroquine pretreatment can significantly reduce apoptotic cells under high glucose hypoxia-reoxygenation.

### Inflammatory cytokines in HK-2 cells exposed to high glucose and hypoxia-reoxygenation

Since inflammation is an important mediator of ischemic injury and since diabetes is characterized by an increase in inflammatory mediators, we examined the inflammatory factors IL-6 and TNF-α before and after hypoxia-reoxygenation (Figure 2A, B). As shown in Figure 2, the activation of inflammatory factors was significantly greater in the HH/R group than in the LH/R group ($p < 0.05$). TLR7-siRNA transfection or chloroquine pretreatment can significantly reduce the release of inflammatory factors under high glucose hypoxia-reoxygenation (HH/R group vs. HH/R-siRNA group; HH/R group vs. HH/R-CQ group) ($p < 0.05$).

### Expression of TLR7 and related protein (MyD88 and NF-κB) in HK-2 cells impaired by high glucose and hypoxia-reoxygenation

First, the mannitol group (24.4 mM) was used as an osmotic control group, and there was no significant difference between the LG group and LG plus mannitol group (data not shown). However, after 72 hours of high glucose (30 mM) stimulation, TLR7 protein expression was significantly upregulated ($p < 0.05$) (Figure 3A, B) (LG group vs. HG group). Following four hours of hypoxia and two hours of reoxygenation, TLR7 protein expression was further increased (LG group vs. HH/R group; HG group vs. HH/R group). Meanwhile, MyD88 protein expression (Figure 3A, C) and NF-κB protein expression (Figure 3 A, D) also showed the same trend as TLR7 protein expression. Compared with the LH/R group, TLR7 protein, MyD88 protein, and NF-κB protein of the HH/R group were more significantly increased. Our data demonstrate that renal ischemia induces a significantly greater accumulation of TLR7 and its related proteins in the renal tubular epithelial cells in diabetic kidneys compared with nondiabetic kidneys.

After successful transfection of specific TLR7-siRNA, the cells were divided into groups according to the experimental steps. TLR7 protein expression (Figure 3A, B), MyD88 protein expression (Figure 3A, C) and NF-κB protein expression (Figure 3A, D) were significantly lower in the HH/R-siRNA group than in
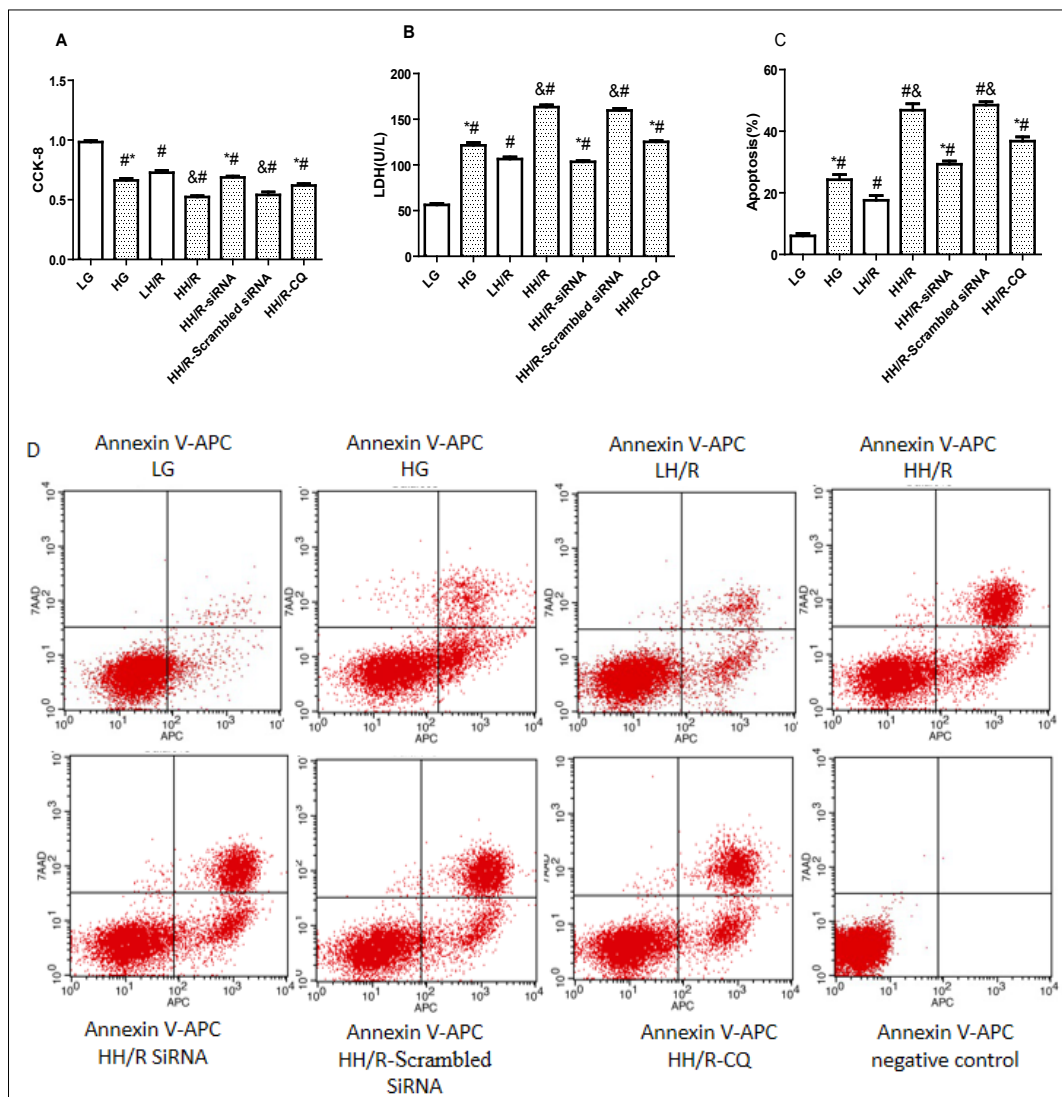
**FIGURE 1.** DETECTION OF CCK-8 AND LDH LEVELS IN EACH GROUP OF CELLS. EACH GROUP OF HK-2 CELLS WAS TESTED FOR (A) CCK-8 AND (B) LDH AFTER HYPOXIA FOR 4 HOURS AND REOXYGENATION FOR 2 HOURS (N=6). APOPTOSIS(C) OF HK-2 CELLS IN EACH GROUP. (D) APOPTOSIS RATE = UPPER RIGHT QUADRANT CELL (LATE APOPTOTIC CELL) LOWER RIGHT QUADRANT CELL (EARLY APOPTOTIC CELL), APOPTOSIS RATE WAS DETECTED BY FLOW CYTOMETRY AND STATISTICAL ANALYSIS (N = 6). THE HG GROUP WAS MORE VULNERABLE TO ISCHEMIC ACUTE KIDNEY INJURY (AKI) (COMPARED WITH THE LG GROUP, #P < 0.05; COMPARED WITH THE LH/R GROUP, &P < 0.05; COMPARED WITH THE HH/R GROUP, *P < 0.05).

the non-transfected group. TLR7 inhibitor chloroquine pretreatment showed the same trend as TLR7-siRNA transfection. TLR7-siRNA transfected cells and chloroquine pretreatment can inhibit the expression of TLR7, MyD88, and NF-κB proteins under high glucose hypoxia-reoxygenation conditions.

## DISCUSSION

The basal expression of TLR7, under conditions of immune mechanism balance, functions to initiate protective signaling mechanisms that protect the immune mechanism during innate immunity from kidney injury. However, imbalances in immune mechanisms, whether at the receptor level or at the level of downstream effectors, can result in severe kidney damage. Our study elucidated several of the downstream effectors involved in TLR7-mediated damage in high glucose and hypoxia-reoxygenation injury of HK-2 cells. Previous animal experiments by Yayi et al.[7] showed that TLR7 protein expression is mainly concentrated in renal tubular epithelial cells. Renal ischemia-reperfusion in diabetic rats causes renal function decline, pathological renal damage, and release of inflammatory factors[7]. Our study further confirmed from HK-2 cell culture that inhibition of TLR7 can
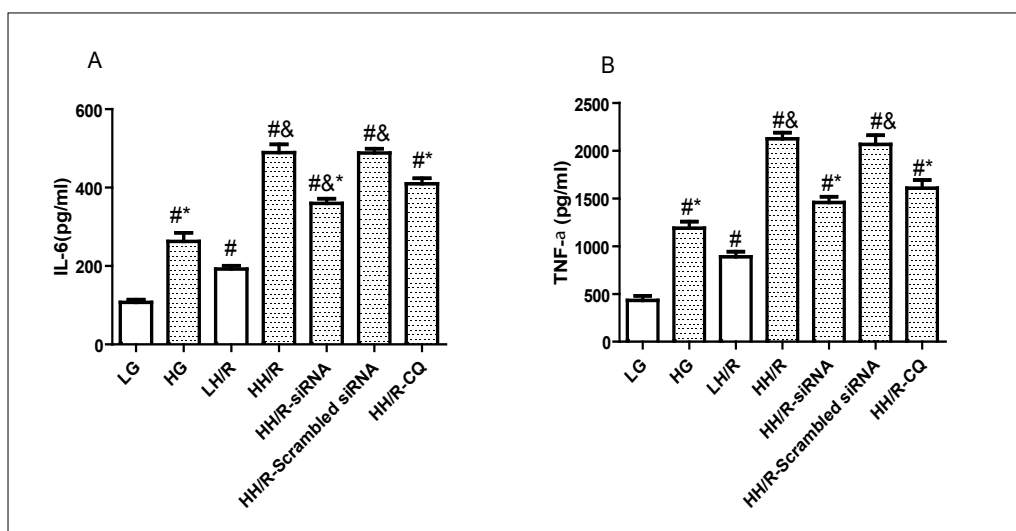
**FIGURE 2.** IL-6 AND TNF-α EXPRESSION LEVELS IN EACH GROUP. THE CONCENTRATION OF (A) IL-6 AND (B) TNF-α IN HK-2 CELL SUPERNATANT WAS DETERMINED BY ELISA (N = 6) (COMPARED WITH THE LG GROUP, #P < 0.05; COMPARED WITH THE LG/R GROUP, &P < 0.05; COMPARED WITH HH/R GROUP, *P < 0.05).
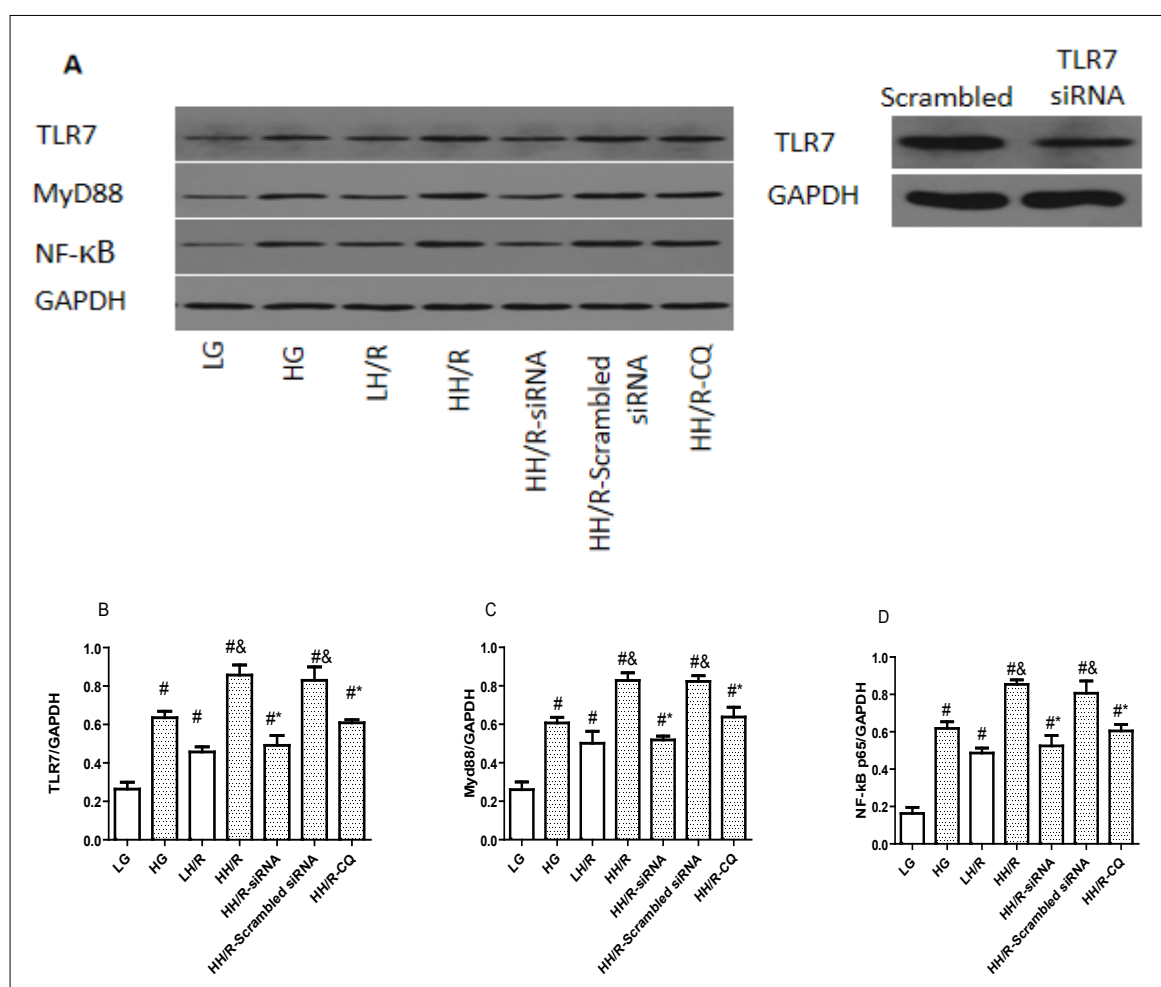


**FIGURE 3.** TLR7, MYD88, AND NF-κB PROTEIN EXPRESSION IN HK-2 CELLS OF EACH GROUP (N = 6). THE PROTEIN BANDS AND WESTERN BLOTTING WERE USED TO DETECT THE EXPRESSION OF TLR7, MYD88, AND NF-κB PROTEINS IN HK-2 CELLS.

improve the inflammatory response induced by high glucose and hypoxia-reoxygenation, reduce the apoptosis rate and enhance its viability, all of which alleviates the vulnerability of HK-2 cells to high glucose hypoxia-reoxygenation injury. This mechanism may occur by inhibiting TLR7/MyD88/NF-κB signaling to achieve renal protection.

Kidney vulnerability to ischemic injury has been reported in experimental models of diabetes and in clinical studies of diabetic patients, but the mechanism remains unclear[8]. This study showed that high glucose is a key factor in increasing the vulnerability of acute ischemia-reperfusion injury in the kidney. In vitro experiments showed that high glucose reduces cell activity, aggravates cell apoptosis, and increases inflammatory factor release. The results reported in an in vitro model of diabetes confirm that diabetes increases the vulnerability to ischemic AKI and implicates a serious inflammatory response as a cause of this vulnerability. Specifically, we found that upregulation of TLR7 markedly enhances renal injury in diabetes after ischemic AKI. We, therefore, hypothesize that the abnormal expression of TLR7 is associated with the vulnerability of high glucose and hypoxia-reoxygenation in human renal tubular epithelial cells. Our experiments confirmed that the upregulation of TLR7 is an important factor of vulnerability to renal injury.

Recent studies have shown increased expression of certain inflammatory cytokines such as TNF-α, IL-6, CCL2/MCP-1, CXCL1, and CXCl10/IP-10 in diabetic kidneys[9-12]. Renal ischemia in AKI animals results in upregulation of several inflammatory cytokines and chemokines, and some leukocytes infiltrate the kidneys, including dendritic cells, natural killer T cells, T and B lymphocytes, neutrophils, and macrophages[13]. Deletion or inhibition of certain cytokines or leukocyte subtypes may be one of the causes of diabetic renal insufficiency or ischemic AKI. Therefore, the highly reactive inflammatory response of diabetes is one of the causes of increased renal vulnerability to diabetes. The reason for this vulnerability requires further investigation. Our experiments showed that the levels of inflammatory factors IL-6 and TNF-α in the diabetic renal ischemia-reperfusion group were much higher than in the nondiabetic ischemia/reperfusion group. We speculate that high glucose-induced inflammatory factor release is one cause of diabetic renal injury vulnerability.

In this study, we used HK-2 cells to simulate a diabetic renal ischemia-reperfusion injury model with high glucose for 72 hours, hypoxia for four hours, and reoxygenation for two hours. The data obtained in this study indicate that downregulation of TLR7 attenuates acute renal injury induced by hypoxia-reoxygenation in HK-2 cells, which is characterized by increased cell activity, decreased apoptosis, and decreased release of inflammatory factors. Our study suggests that hyperactivation of TLR7 may exacerbate existing kidney damage. Inhibition of TLR7 expression reduces renal damage. TLR7 protein, MyD88 protein, and NF-κB protein were detected by western blotting. Studies have shown that high glucose stimulation and hypoxia-reoxygenation can significantly stimulate TLR7 MyD88 and NF-κB protein expression. TLR7-siRNA transfected cells and chloroquine pretreatment can downregulate TLR7, MyD88, and NF-κB protein expression under high glucose and hypoxia-reoxygenation conditions, reduce apoptosis, and increase cell viability. We speculate that TLR7 activates immune cells by relying on the signaling pathway of MyD88 to produce a cascade inflammatory response, releasing a large number of inflammatory factors. The release of these inflammatory factors further aggravates kidney damage.

In summary, the upregulation of TLR7 in the kidney might account for the enhanced inflammatory response and vulnerability of the diabetic kidney to ischemia. Our results may be helpful to find new targets for the treatment of diabetic ischemic AKI and provides a theoretical basis and new clinical strategies for reducing the incidence of perioperative diabetic renal ischemic AKI.

## Acknowledgments

## Conflict of interests

The authors declare that they have no conflict of interests.

## RESUMO

OBJETIVO: *O diabetes é um fator de risco para a lesão renal aguda (LRA). No entanto, seu mecanismo de patogênese não foi elucidado. O objetivo do estudo foi investigar o papel da inflamação e do receptor Toll-like 7 (TLR7) na LRA isquêmica no diabetes.*

MÉTODOS: *Um modelo de hipóxia-reoxigenação de células epiteliais tubulares renais humanas (HK-2) na presença de concentrações altas de glicose foi utilizado para gerar LRA induzida por isquemia-reperfusão em diabetes. A atividade das células foi medida pelo ensaio Cell Counting Kit-8 (CCK-8) e pela atividade da lactato desidrogenase (LDH). As citocinas inflamatórias foram avaliadas por ensaio imunoenzimático (Elisa). A expressão de TLR7, do fator de diferenciação mieloide 88 (MyD88) e do fator de transcrição nuclear-κB (NF-κB) foi examinada por Western blotting. A apoptose foi avaliada por citometria de fluxo.*

RESULTADOS: *Os grupos glicose alta e glicose baixa foram submetidos à hipóxia-reoxigenação. O grupo de baixa glicose desenvolveu apenas danos celulares ligeiros, apoptose e uma resposta inflamatória. Em contraste, no grupo de alta glicose, uma lesão equivalente de hipóxia-reoxigenação provocou danos celulares graves, apoptose e uma resposta inflamatória. A expressão de TLR7 e suas proteínas relacionadas foi medida no grupo de alta glicose antes e após a hipóxia-reoxigenação. O grupo de alta glicose exibiu maiores aumentos na expressão de TLR7 após hipóxia-reoxigenação do que o grupo de baixa glicose. Além disso, a expressão de TLR7 e suas proteínas relacionadas após a hipóxia-reoxigenação foi maior no grupo com alto nível de glicose do que no grupo com baixo nível de glicose. A inibição do TLR7 fornece proteção significativa contra a lesão isquêmica no diabetes.*

CONCLUSÃO: *Nossos resultados sugerem que o diabetes aumenta a vulnerabilidade à lesão renal induzida por isquemia. Essa vulnerabilidade acrescida tem por origem uma resposta inflamatória aumentada envolvendo a via de transdução de sinal do TLR7.*

PALAVRAS-CHAVE: *Vulnerabilidade em saúde. Isquemia. Lesão renal aguda. Diabetes mellitus. Receptor 7 Toll-Like.*

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