Ulinastatin Alleviates Rhabdomyolysis-Induced Acute Kidney Injury by Suppressing Inflammation and Apoptosis via Inhibiting TLR4/NF-κB Signaling Pathway

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Received 3 March 2022; accepted 20 April 2022

Abstract—Acute kidney injury (AKI) is an important complication of rhabdomyolysis (RM), but there is lack of effective treatments. Ulinastatin (UTI) is a broad-spectrum serine protease inhibitor isolated and purified from human urine with strong anti-inflammatory and cytoprotective actions. The aim of this research was to investigate the effect and potential mechanism of UTI on RM-induced AKI (RM-AKI). We established RM-induced AKI model and myoglobin (Mb)-stimulated NRK-52E cell model. In vivo, twenty-four rats were randomly divided into three groups (n = 8): control, RM-AKI, and RM-AKI + UTI. In vitro, the NRK-52E cells were divided into six groups according to the different treatment method. Mb-stimulated NRK-52E cells were treated with UTI or si-TLR4 transfection to characterize the mechanisms of UTI in RM-AKI. Indicators of the kidney injury, cell viability, cell cycle, oxidative stress, inflammation, apoptosis, and TLR4/NF-κB signaling pathway were assessed. In vivo and in vitro, UTI significantly decreased the expression of TLR4 and p65. In vivo, UTI significantly improved renal function and reduced inflammatory reaction and kidney injury. In vitro, UTI protected NRK-52E cells from Mb stimulation by suppressing cell cytotoxicity, cell cycle inhibition, overproduction of ROS, inflammation, and apoptosis. Additionally, UTI played a protective role by downregulating the TLR4 expression. The results indicate that UTI alleviates RM-AKI by suppressing the inflammatory response and apoptosis via inhibiting TLR4/NF-κB signaling pathway. Our study provides a new mechanism for the protective effect of UTI on RM-AKI.

KEY WORDS: ulinastatin; rhabdomyolysis; acute kidney injury; TLR4/NF-κB; inflammation; apoptosis

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INTRODUCTION

Rhabdomyolysis (RM) is a syndrome that occurs when striated muscle cell membrane integrity disrupt due to crush, ischemia, inflammation or intoxication, and release electrolyte, myoglobin (Mb), creatine kinase (CK) into the circulation, leads to the electrolyte disorder, effective circulating blood volume reduction, even multiple organ dysfunction, and severe cases might even be fatal [1]. About 10–60% of RM patients eventually lead to acute kidney injury (AKI) because of hypermyoglobinemia [2, 3]. The pathological mechanism of RM-induced AKI(RM-AKI) is apoptosis or necrosis of tubular epithelial cells (TECs), included renal tubular obstruction, tubular oxide injury, vasoconstriction, and low blood volume [4–6]. Nowadays, there is no targeted drug that can relieve RM-AKI, and the prognosis of patients is poor [7]. Therefore, investigation of the pathogenesis and treatment of RM-AKI has important clinical significance.

Toll-like receptors (TLRs) are the members of the membrane protein family which can identify pathogen-associated molecules and mediate innate immune response [8]. TLR4 is one of the most important TLRs family members and belongs to I transmembrane receptor, which is involved in immune inflammatory response, apoptosis, AKI-related regeneration, and repair [9–11]. Binding of stimulating factors to the TLR4 receptor is the first step in activating the innate immune system. After a series of phosphorylation cascades, the transcription factor nuclear factor kappa-B (NF-κB) is transferred from cytoplasm to nucleus, and a series of inflammatory mediators are released, including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) [12, 13]. Teng et al. [14] found that the expression of TLR4 in renal tubular epithelial cells was upregulated in ischemia–reperfusion rat model. Inhibition of TLR4 reduced the release of downstream pro-inflammatory factors and improved renal function in rats [15].

Ulinastatin (UTI), a glycoprotein derived from human urine, has a molecular weight of 67 kDa and functions as an inhibitor of various inflammatory proteases, including trypsin, chymotrypsin, elastase, and various pancreatic enzymes [16]. UTI has anti-inflammatory effect, and its mechanisms are relevant to oxygen-free radical scavenging and anti-lipid peroxidation, stabilizing lysosome membrane and inhibition the formation of inflammatory cytokines [17, 18]. Clinical studies and animal experiments have reported that the application of UTI can play an important role in the protection against septic shock, intensive pancreatitis, ischemia–reperfusion organ injury, and multi-organ dysfunction [19, 20]. Li et al. [21] demonstrated that UTI could alleviate the renal dysfunction and injury associated with ischemia–reperfusion of the kidney. Although UTI has been widely used in clinical practice, studies investigating the effect of UTI on the renal protection of RM-AKI patients are limited, and the underlying mechanisms remain unclear. Based on these findings, we constructed an RM-AKI rat model and an in vitro RM-AKI model by incubating NRK-52E cells with Mb. Furthermore, we investigated whether UTI alleviated RM-AKI by suppressing inflammation and apoptosis via inhibiting TLR4/NF-κB signaling pathway, which will provide new strategy for the treatment of RM-AKI.

MATERIALS AND METHODS

RM-AKI Rat Model

Twenty-four male Sprague–Dawley (SD) rats (weighing 300 g ± 10 g) were obtained from the Experimental Animal Center of Tianjin Medical University. All rats were housed and fed in a temperature- and humidity-controlled environment with a 12-h light/dark cycle and had free access to food and water. All rats were forbidden to drink for 24 h before establishment of model and then were randomly divided into three groups (n = 8): control, glycerol-treated (RM-AKI), and RM-AKI + UTI. All rats were anesthetized with 1% pentobarbital (40 mg/kg) injection via tail vein. To establish the RM-AKI model, rats were injected tonally with 100,000U/kg UTI immediately after modeling RM‑AKI Rat Model

In the RM-AKI + UTI group, the rats were intraperitoneally with 100,000U/kg UTI immediately after modeling [23]. In the control group, the rats received an equal amount of saline only. All rats were sacrificed when glycerol injection for 24 h. Blood samples were obtained from the rats and centrifuged to produce serum. Then the blood, the kidney, and muscle tissue were stored at −80°C for further analysis. All procedures performed in this study involving animals were approved by the ethical committee of the Tianjin Medical University General Hospital (Ethical No. IRB2020-DWFL-083). The animal experiments were performed according to the Guideline of Animal Care and Use Committee of the Tianjin Medical University General Hospital.
Histological Analysis

The kidney and muscle tissue were fixed in 10% formalin, dehydrated, embedded in paraffin, and sectioned into 5-μm sections. The sections were stained with hematoxylin–eosin (HE) staining. All stained images were photographed using a light microscope (Nikon Eclipse E100, Nikon, Japan) at 200 × magnification. Tubular injury/regeneration was defined as including vacuolization, luminal cell casts, and acellular/atrophic changes, and scoring was as follows: 0 = none detected; 1 = 1–10% tubules involved; 2 = 10–25% tubules involved; 3 = 25–50% tubules involved; 4 = greater than 50% tubules involved [24].

Assessments of Biochemical Parameters in Serum

The serum creatinine (Cr), blood urea nitrogen (BUN), and Mb and CK levels were detected by the BK-200VET automatic biochemical analyzer (Laibao Medical Instrument Co., Ltd, Shandong, China).

Cell Culture, Transfection, and RM-AKI Cell Model

NRK-52E cells (Cell Bank of Chinese Academy of Sciences) were cultured in DMEM medium containing 10% fetal bovine serum at 37°C, 5% CO₂, and 95% air in constant temperature incubator. And cells in logarithmic growth phase were harvested for cell biology experiments. For cell transfection, we cultured 3 × 10⁴ NRK-52E in 24-well plates overnight. When the NRK-52E cells were 50% confluent in fresh serum-free medium, they were transiently transfected with small interfering RNAs targeting TLR4 (si-TLR4) lentivirus (the virus titer was 1 × 10⁸TU/ml) or negative control (si-NC) lentivirus at a multiplicity of infection (MOI) of 30 (Shanghai GenePharma Co., Ltd, Shanghai, China). All groups were treated 48 h for subsequent experiment.

To construct a RM-AKI cell model, nontransfected or transfected NRK-52E cells were pretreated with 5000U/ml UTI for 2 h; after which, they were stimulated with 100 μM Mb at 37 ℃ in a 5% CO₂ atmosphere for 24 h [25, 26]. Cells were divided into the following six groups: control, control + UTI, control + Mb, Mb + si-TLR4, Mb + si-NC, and Mb + UTI.

Cell Viability Assay

Cell viability was measured using a cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan). NRK-52E cells were seeded into 96-well plates at a density of 3 × 10³ cells per well and then incubated with 10 μl of CCK-8 solution for 24, 48, and 72 h. At each time point, the absorbance at a wavelength of 450 nm (OD 450 nm) was measured with a microplate reader (Bio-Rad, Hercules, CA).

Measurements of Cell Cycle and Intracellular ROS by FCM

Cells were washed twice with PBS and resuspended with precooled PBS in 1 ml ice. Precooling 95% ethanol in 4 ml ice was shocked in low vortex and at the same time by dripping with 1 ml cell suspension (operated on ice), fixed at 4°C for 12–24 h after mixing uniformly, precipitated at 1000 rpm/5 min and washed once with precooled PBS in ice. 0.4 ml propidium iodide staining solution was added into each tube, slow and fully resuspended cell precipitation, then with warm bath method for 30 min at 37°C away from light. Detection was operated in PE channel, and cell cycle proportion was analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

To determine the intracellular levels of ROS, NRK-52E cells from different groups were washed with PBS and then incubated with DCFH-DA (10 μM, final concentration) for 15 min in the dark. Next, the cells were washed with PBS and suspended by trypsinization. Following centrifugation (4°C, 3000 rpm/5 min), the cells were resuspended in 1 ml of PBS and analyzed by flow cytometry.

Apoptosis Analysis

NRK-52E cells were first fixed with 4% paraformaldehyde for 25 min, followed by 0.2% TritonX-100 membrane transportation, Tdt reaction mix incubation in the dark, DAPI staining for 3 min, anti-fluorescence quenching mounting, and apoptosis detection by fluorescence microscopy. The apoptosis index (%) was calculated as TUNEL positive cells/total cells × 100%.
Quantitative Real-Time PCR Analysis (qRT-PCR)

The expression of the TLR4 mRNA, IL-6, and TNF-α was assessed. Total RNA was extracted from cells and kidney tissue by TRIzol method according to the manufacturer’s protocol. Then, cDNA template by RNA reverse transcription synthesis was generated with the Prime-Script RT Reagent Kit (Takara Bio Inc.; Shiga, Japan). The threshold cycle was obtained from triplicate samples and averaged. β-Actin was used as an internal control to normalize the samples. The expression level of the target gene was calculated according to the $2^{-\Delta\Delta CT}$ formula. The primer sequences are listed in Table 1. All primers were designed and synthesized by TAKARA co., Ltd. (Dalian, China).

Western Blot Analysis

The kidney tissues were weighed and centrifuged at 12000 rpm for 30 min at 4℃. The supernatants were immediately collected to detect the concentration of the proteins with the bicinchoninic acid (BCA) kit and then to separate the denatured proteins with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer onto polyvinylidene difluoride (PVDF) membranes. Put into a diluent containing corresponding primary antibodies against TLR4, p-p65, p65, and GAPDH, incubate with corresponding secondary antibody horse-radish peroxidase (HRP) in tris-buffered saline (TBS)-T for 1 h at room temperature, place in the exposure box, developing and fixing. Reagent antibodies were purchased from (proteintech) Wuhan Three Eagles Biotechnology Co., Ltd. Semi-quantitative analysis was finished with ImageJ 1.8.0 image analysis software.

| Gene  | Primer sequence (5’ to 3’) |
|-------|---------------------------|
| TLR4  | Forward TATCATCAGTGATCGGTTG<br>Reverse CAGTCTCATCCTGGCTCG |
| IL-6  | Forward GTCCCTCAGTCAAATCCA<br>Reverse TAAAGCTAGCCTGGCGA |
| TNF-α | Forward CACCTCTTCCTCATTTG<br>Reverse GTGCCTGCTGCTCTTTGTA |
| β-Actin | Forward AGGAAATCTGCGGTGA CAT<br>Reverse CCTCGCGCATCGGAAGACA |

Cell samples were achieved by NRK-52E cells washed with PBS and centrifuged. According to the manufacturer’s protocols, the nucleus/plasma protein was extracted from the cells using cell plasma protein extraction reagent. All other procedures of western blot were exactly the same as previous trails.

Immunohistochemical Staining

The formalin-fixed kidney tissue was taken for dehydration, transparency, wax dipping, embedding, sectioning, dewaxing, and hydration. The levels of TLR4, p65, and p-p65 antibody were measured. Fifty microliters of the corresponding primary antibody was added dropwise at 4℃ overnight. Add the EnVision reagent dropwise, and let it stand at room temperature for 30 min. The results were detected by light microscopy with 200× magnification. Five fields were observed in each sample, and the integrated optical density (IOD) was calculated.

Immunofluorescence

NRK-52E cells from the different groups were fixed with 1 ml of 4% PFA for 25 min, permeated with 0.2% Triton X-100 for 5 min, and blocked with goat serum for 1 h. Next, the cells were incubated overnight with primary antibodies against TLR4, followed by incubation with secondary antibodies. The cellular nuclei were stained with DAPI. Cells were sealed with 4 μl resistance to fluorescence quenching sealing liquid. Images of the stained cells were obtained with a fluorescence microscope (Olympus).

Enzyme-Linked Immunosorbent Assay (ELISA)

Inflammatory cytokines IL-6 and TNF-α were detected by using conventional ELISA kits (Haling Biotechnology Co., Ltd, Shanghai, China). Operating procedures were strictly finished according to the manufacturer’s instructions.

Statistical Analysis

Statistical analysis was finished with SPSS 20.0 (IBM Corp., Armonk, NY, USA). All data conform to normal distribution, and measurement data is expressed as mean ± standard deviation (SD). Differences among groups were analyzed by one-way ANOVA, followed by post hoc pairwise comparison (LSD) tests for analysis.
Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, Inc.; San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant.

RESULTS

**UTI Attenuates Histopathological Changes of the Kidney Tissue in RM-AKI Rats**

Pathological changes in kidney and muscle tissues were evaluated by HE staining. As shown in Fig. 1a, there was glomerular congestion and edema, and renal tubular epithelial cells were swollen and degenerated in the RM-AKI group. UTI treatment could lessen the glomerular congestion and edema and improve the swelling of renal tubular epithelial cells. The injury score of kidney tissues was significantly higher in the RM-AKI group (Fig. 1b). We also found that UTI significantly reduced the injury score compared with the RM-AKI group. As shown in Fig. 1c, we found the muscle tissue destruction and muscle fiber dissolution in the RM-AKI group. UTI treatment reduced the muscle tissue damage. Therefore, we inferred that UTI had a protective effect on kidney and muscle tissue in RM-AKI rats.

**UTI Improved the Kidney Function and Inhibited Inflammation in RM-AKI Rats**

The serum Cr, BUN levels are considered important biochemical markers of the severity of kidney injury, while MB and CK levels are considered to be important indicators to reflect the severity of muscle tissue damage. As shown in Fig. 2a–d, the levels of serum Cr, BUN, Mb, and CK were significantly reduced after UTI treatment.

Proinflammatory factors IL-6 and TNF-α are considered to be the important evaluation indicators in inflammatory response. As shown in Fig. 2e, f, the levels of IL-6 and TNF-α were significantly increased in the RM-AKI group. The results showed that inflammatory reaction occurred in rats after modeling. Inflammatory factors were significantly decreased after UTI treatment. So we confirmed that UTI inhibited the inflammatory response in RM-AKI rats.

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**Fig. 1**  
**a** HE staining and histological observation of the kidney tissues in each group. **b** The injury score of the kidney tissues in each group. **c** HE staining and histological observation of the muscle tissues in each group. Data are expressed as the mean ± SD ($n=8$ per group). *$p<0.05$, **$p<0.01$, compared with control group. *$p<0.05$ compared with RM-AKI group. Scale bars, 100 μm.
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Fig. 2  a–d The serum Cr, BUN, Mb, and CK levels were measured by automatic biochemical analyzer. e, f The levels of IL-6 and TNF-α were measured by ELISA. Data are expressed as the mean ± SD (n = 8 per group). *p < 0.05, **p < 0.01, compared with control group, #p < 0.05 compared with RM-AKI group.

UTI Inhibited RM-AKI by Suppressing the TLR4/NF-κB Signaling Pathway In Vivo

The expression of TLR4, NF-κBp65(p65) and phosphorylated-p65 (p-p65) was analyzed by western blot (Fig. 3a, b) and immunohistochemical analysis (Fig. 4a). GAPDH was used as internal reference. The levels of TLR4 and p-p65 were significantly increased in the RM-AKI rats. The results showed that the expression of intermediates in the TLR4/NF-kB signaling pathway and p65 phosphorylation was upregulated after modeling. In other words, the TLR4/NF-κB signaling pathway was activated in vivo. UTI treatment significantly reduced the levels of TLR4 and p-p65. The TLR4mRNA level was analyzed by qRT-PCR and was normalized to β-actin. As shown in Fig. 3f, the level of TLR4mRNA was significantly increased in RM-AKI rats. UTI treatment significantly reduced the level of TLR4mRNA.

The results demonstrated that UTI significantly downregulated TLR4 expression and prevented p65 phosphorylation. So we concluded that UTI suppressed the TLR4/NF-κB signaling pathway in RM-AKI rats.

UTI Protected NRK-52E Cells Against Mb-Induced Cytotoxicity

NRK-52E cells were subjected to different concentrations of Mb (0, 50, 100, and 150 μmol/L) for 24 h. And we found that with the increase of Mb concentration, cell viability decreased gradually (Fig. 5a). The results indicated Mb damaged NRK-52E cells in a dose-dependent manner. In order to select moderate cell
viability, we selected the treatment of 100 μmol/L Mb as the condition for use in the subsequent experiments. As shown in Fig. 5b, c, Mb exposure significantly inhibited cell viability and cell proliferation. In Mb-stimulated NRK-52E cells, transfection with si-TLR4 or UTI treatment significantly increased the cell viability and cell proliferation. Additionally, no significant differences were found between the Mb + si-TLR4 and Mb + UTI group. The results suggested that UTI played the similar role as TLR4 knockdown. So we concluded that UTI protected NRK-52E cells from Mb-induced cytotoxicity by downregulating TLR4 expression.

UTI Attenuated Mb-Induced Apoptosis in NRK-52E Cells

As shown in Fig. 5d, Mb exposure significantly increased cell apoptosis. In Mb-induced NRK-52E cells, transfection with si-TLR4 or treatment with UTI significantly reduced apoptosis. The results showed that UTI reduced apoptosis by downregulating TLR4 expression.

UTI Reduced Mb-Induced Inflammation in NRK-52E Cells

qRT-PCR and ELISAs analyses revealed that the levels of inflammatory factors, including IL-6 and TNF-α, were significantly increased in Mb-induced NRK-52E cells (Fig. 6a, b). The results showed the inflammatory response occurred in NRK-52E cells by Mb stimulation. And transfection with si-TLR4 or UTI treatment significantly reduced inflammatory factors levels. So we concluded that UTI decreased the levels of inflammatory factors by downregulating TLR4 expression.
UTI Suppressed Cell Cycle Inhibition and the Production of ROS in Mb-Induced NRK-52E Cells

The cell cycle was evaluated by flow cytometry. As shown in Fig. 7a, Mb exposure significantly increased the proportion of cells in G0/G1 phase. The results suggested NRK-52E cells experienced the cell cycle inhibition in G0/G1 phase by Mb stimulation. And transfection with si-TLR4 or UTI treatment could suppress the cell cycle inhibition.

As shown in Fig. 7b, the intracellular levels of ROS in NRK-52E were assessed using a DCFH-DA probe. The results showed that the intracellular ROS production was significantly increased in Mb-induced NRK-52E cells. Transfection with si-TLR4 or UTI treatment effectively suppressed ROS overproduction.

UTI Inhibited Mb-Activated TLR4/NF-κB Signaling Pathway In Vitro

The TLR4mRNA level was analyzed by qRT-PCR and was normalized to β-actin. As shown in Fig. 8a, the TLR4mRNA level was significantly increased in Mb-induced NRK-52E cells. Transfection with si-TLR4 or UTI treatment significantly reduced the TLR4mRNA level. Immunofluorescence results suggested that the expression of TLR4 increased significantly in Mb-induced NRK-52E cells (Fig. 8b). And transfection with si-TLR4 or UTI treatment effectively reduced this expression. The findings showed that UTI significantly downregulated TLR4 expression.

The expressions of TLR4, p65, and p-p65 was analyzed by western blot. GAPDH was used as internal reference. As shown in Fig. 9a and b, the expression levels of TLR4 and p-p65 were significantly increased in Mb-induced NRK-52E cells. Mb challenge contributed to the upregulation of p-p65 in nucleus. The results showed that the TLR4/NF-κB signaling pathway was activated in Mb-induced NRK-52E cells. Transfection with si-TLR4 or UTI treatment significantly reduced the levels of TLR4 and p-p65. So we concluded that UTI suppressed the TLR4/NF-κB signaling pathway in Mb-induced NRK-52E cells.

DISCUSSION

In our study, injection with glycerol disrupted the structure and function of the kidney tissues, stimulated with Mb-injured NRK-52E cells and initiated the inflammatory
response, demonstrating that our RM-AKI models had been generated successfully. Our data further confirmed that UTI suppressed TLR4 expression and decreased the levels of pro-inflammatory cytokines IL-6 and TNF-α in RM-AKI rats. The results showed that UTI attenuated AKI progression by decreasing the kidney tissue destruction, improving the kidney function, and reducing the kidney injury scores and inflammatory reaction. Consistent with our results, Li et al. [21] demonstrated that UTI could alleviate the renal dysfunction and injury associated with ischemia–reperfusion of the kidney.

UTI is a serine protease inhibitor that plays a protective role in multiple organs [27]. With its strong anti-inflammatory and cytoprotective actions in various cell and animal models, UTI can modulate innate immunity and proinflammatory responses [28–30]. Yang et al. [23] reported that UTI ameliorates acute kidney injury induced by crush syndrome inflammation by modulating Th17/Treg cells. To confirm the cell protective effect of UTI against the progression of RM-AKI, we mimicked RM renal tubular epithelial cell in vitro by incubating the NRK-52E cells with Mb. Mb has been implicated consistently as the primary nephrotoxin in RM, with proposed mechanisms of toxicity that include tubular obstruction, oxidant injury, and vasoconstriction [31].

Our data demonstrated that in Mb-stimulated NRK-52E cells, transfection with si-TLR4 or treatment with UTI could significantly increase the cell viability and cell proliferation. We concluded that UTI could protect NRK-52E cells from Mb-induced cytotoxicity by
downregulating TLR4 expression. Similarly, we found that UTI effectively suppressed the cell cycle inhibition in G1/S phase and production of ROS in Mb-induced NRK-52E cells. There are two important regulatory points in the cell cycle: G1/S and G2/M [32]. When the positive regulatory factor in G1 reaches a certain degree, the cell cycle can cross the G1/S junction to continue the cell cycle process. Otherwise, the cell cycle is inhibited in G1/S phase and blocked cell cycle progression. So we concluded that UTI suppressed the cell cycle inhibition and promoted cell cycle procession. ROS can oxidatively impair cell function [33]. The results showed that UTI alleviated the oxidative stress response of NRK-52E cells by inhibiting MB-induced intracellular ROS overproduction.

It was noteworthy that we subsequently observed that transfection with si-TLR4 or UTI treatment could reduce the level of proinflammatory factor and cell apoptosis. Our study suggested that UTI had similar protective effects with downregulation of TLR4 expression. So we concluded that UTI protected NRK-52E cells against Mb-induced inflammation and apoptosis by downregulating TLR4 expression.
It has been reported that TLR4/NF-κB signaling pathway mediates inflammation and immune response in various diseases [34]. Chen et al. [35] reported that UTI attenuated LPS-induced inflammation and inhibited endoplasmic reticulum stress-induced apoptosis in renal tubular epithelial cells via regulation of the TLR4/NF-κB and Nrf2/HO-1 pathways. In addition, Cao et al. [36] reported that ulinastatin mediated suppression of regulatory T cells through TLR4/NF-κB signaling pathway in murine sepsis. Therefore, our study focused on the changes of TLR4 and p65 in vivo and in vitro, especially in verifying whether UTI could alleviate RM-AKI process by inhibiting the TLR4/NF-κB signaling pathway.

Our research showed that when glycerol was injected into rats, the expression levels of TLR4 and p-p65 were significantly increased, and the results suggested that the TLR4/NF-κB signaling pathway was activated in rats after modeling. Meanwhile, we found that the expression levels of TLR4 and p-p65 were decreased significantly after UTI treatment, and the results suggested that UTI suppressed the TLR4/NF-κB signaling pathway in RM-AKI rats.

To further confirm our conclusion, we found that the expression levels of TLR4 and p-p65 were significantly increased in Mb-induced NRK-52E cells. The results showed that the TLR4/NF-κB signaling pathway was activated in NRK-52E cells by Mb stimulation.
**Fig. 8**  
**a** The TLR4 mRNA level was determined by qRT-PCR. β-Actin was used as an internal control to normalize the samples. 
**b** The expression of TLR4 was visualized by immunofluorescence. The nuclei were counterstained with DAPI (blue). Data are expressed as the mean ± SD. *p < 0.05, **p < 0.01, compared with control group, *p < 0.05 compared with control + Mb group. Scale bars, 100 μm.

**Fig. 9**  
**a, b** The TLR4, p-p65, and p65 expression levels were detected by WB. GAPDH was used as internal reference. The results of the TLR4 (c) and p-p65 (d) with GraphPad Prism 6.0 software were analyzed. Data are expressed as the mean ± SD. *p < 0.05, **p < 0.01, compared with control group. *p < 0.05 compared with control + Mb group.
Transfection with si-TLR4 or UTI treatment significantly reduced the levels of TLR4 and p-p65. We also found that p65 in the cytoplasm was phosphorylated and transferred into the nucleus in Mb-induced NRK-52E cells by immunofluorescence analysis. The results showed that UTI downregulated the expression of TLR4 and suppressed the TLR4/NF-κB signaling pathway in Mb-induced NRK-52E cells. According to the research results in vitro, we concluded that UTI carries out the cell protective functions by suppressing Mb-induced cytotoxicity, the cell cycle inhibition, intracellular ROS overproduction, the inflammation, and apoptosis via inhibiting the TLR4/NF-κB signaling pathway.

CONCLUSIONS

In summary, our present study provides the evidence that UTI exerts its therapeutic effects on RM-AKI by inhibiting the inflammation and apoptosis, which may be involved with TLR4/NF-κB signaling pathway. The study uses an animal model, and findings may or may not apply to humans, and rhabdomyolysis is simulated in the study model and may or may not apply to the acute kidney injury in the clinical setting of rhabdomyolysis. It should be noted that the requirement for treatment with UTI limits its clinical relevance and remains a limitation of this study. Moreover, whether UTI alleviates the inflammatory reaction of RM-AKI via other signaling pathways remains to be further explored. Overall, our findings may provide new insights into the pathways involved in effection of UTI on the treatment of RM-AKI.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1007/s10753-022-01675-4.

ACKNOWLEDGEMENTS

The authors are grateful for the technical assistance of the Wenzhou Safety (Emergency) Institute of Tianjin University.

AUTHOR CONTRIBUTION

Jinxiang Wang: data curation, formal analysis, and writing—original draft. Guowu Xu: conceptualization and writing—review and editing. Heng Jin: investigation and writing—review. Xinyue Yang: investigation. Yanfen Chai: investigation. Ziquan Liu: investigation. Shike Hou: project administration and supervision. Haojun Fan: conceptualization and writing—review and editing.

FUNDING

This work was supported by the Scientific Research Translational Foundation of Wenzhou Safety (Emergency) Institute of Tianjin University (grant number TJUWY2022022), the Scientific Research Program of Tianjin Education Commission (grant number 2021KJ211), and the National Natural Science Foundation of China (grant number 82072222).

DATA AVAILABILITY

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

DECLARATIONS

Ethics Approval All procedures performed in this study involving animals were approved by the ethical committee of the Tianjin Medical University General Hospital. The animal experiments were performed according to the Guideline of Animal Care and Use Committee of the Tianjin Medical University General Hospital.

Consent to Participate Not applicable.

Consent for Publication All authors consent to the publication of this manuscript.

Competing Interests The authors declare no competing interests.

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