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# Effect of lncRNA CRNDE on sepsis-related kidney injury through the TLR3/NF-κB pathway

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**Abstract. – OBJECTIVE: The aim of this study was to detect the expression of long non-coding ribonucleic acid (lncRNA) colorectal neoplasia differentially expressed gene (CRNDE) in the kidney tissues of mice with sepsis-induced acute kidney injury (AKI) and its effect on KI, and to further explore its mechanism.**

**MATERIALS AND METHODS: A total of 60 male C57 mice were randomly divided into 3 groups based on a random number table, including the control group (Sham group, *n*=20), sepsis-related KI group [lipopolysaccharide (LPS) group, *n*=20] and CRNDE inhibition group [LPS + CRNDE small interfering ribonucleic acid (siRNA) group, *n*=20]. Mice in LPS and LPS + CRNDE siRNA groups were intraperitoneally injected with 5 mg/kg LPS, while the tail vein was injected with 5 μL CRNDE siRNAs. After 12 h, the expression level of lncRNA CRNDE in kidney tissues of mice in each group was detected *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR). At the same time, 2 mL of orbital blood was collected from each mouse, and the levels of creatinine and blood urea nitrogen were detected. Subsequently, kidney tissue samples were collected from mice in each group. Periodic acid Schiff (PAS) staining was used to assess the injury of renal tubulointerstitium, followed by scoring. Hematoxylin and eosin (H&E) staining was applied to detect cell injury in kidney tissues. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was adopted to examine the apoptosis in kidney tissues in mice of each group. Meanwhile, the distribution and expression of p65 in kidney tissues of mice in each group were determined *via* immunohistochemical staining. Finally, the expression of Toll-like receptor 3 (TLR3)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway in kidney tissues of mice in each group was detected using Western blotting.**

**RESULTS: Compared with the Sham group, lncRNA CRNDE level in the kidney of the LPS group was remarkably up-regulated ($p<0.05$). The levels of creatinine and blood urea nitrogen in LPS + CRNDE siRNA group were notably lower than those of the LPS group ($p<0.05$). PAS staining results manifested that renal tubulointerstitial injury in the LPS group was significantly more serious than that of the LPS + CRNDE siRNA group ($p<0.05$). According to H&E staining results, serious edema, rupture and necrosis observed in kidney tissue cells of the LPS group. However, after the intervention of CRNDE siRNA, cell edema and necrosis were markedly relieved. In addition, TUNEL staining results indicated that the apoptotic level of kidney tissue cells in the LPS + CRNDE siRNA group was significantly lower than that of the LPS group ($p<0.05$). Subsequent immunofluorescence staining demonstrated that p65 expression in the LPS group increased significantly, which was markedly inhibited by CRNDE siRNA intervention ($p<0.05$). Furthermore, Western blotting displayed that CRNDE siRNA could effectively inhibit the activation of TLR3 and p65 in mouse kidney tissue induced by LPS ($p<0.05$).**

**CONCLUSIONS: Inhibition of CRNDE can reduce sepsis-induced KI by blocking the activation of the TLR3/NF-κB pathway. Moreover, CRNDE is expected to become a target for clinical treatment of sepsis-related KI.**

*Key Words:*
Sepsis-related kidney injury, LncRNA CRNDE, TLR3/NF-κB.

## Introduction

Sepsis is a systemic inflammatory response syndrome (SIRS) caused by infection. Currently, it has become the leading cause of death in intensive care units worldwide[1]. Sepsis is a persistent and uncontrolled inflammatory response triggered by various causes, eventually leading to multiple organ dysfunction syndrome (MODS)[2]. At present, the principle of clinical treatment of sepsis is to maintain the stability of important organs and prevent further infec-

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tion of diseased organs, so there are great limitations[3]. In addition, no satisfactory results are achieved by targeted therapy for certain specific inflammatory factors in improving the prognosis of sepsis. Meanwhile, the mortality rate remains high[4]. It is known to all that kidney is one of the organs most vulnerable to sepsis[5]. Acute kidney injury (AKI) is the most common and potentially fatal complication of sepsis. Eventually, it will develop into severe sepsis in more than half of the patients[6]. Once AKI in patients develops into severe sepsis, the mortality rate is as high as 70%[7,8]. Timely and effective inhibition of AKI occurrence and development contributes to the improvement of sepsis prognosis. Therefore, searching for new targets to improve sepsis is still of great significance to enhance the prognosis and survival of sepsis patients.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is an important transcription factor for many inflammatory genes, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6. Therefore, NF-κB plays an important role in the occurrence and development of various inflammatory diseases[8,9]. Toll-like receptor 3 (TLR3)[10] is one of the crucial pattern recognition receptors on the cell membrane. Meanwhile, it serves as a crucial bridge between natural immunity and adaptive immunity. Carmody and Chen[11] have shown that lipopolysaccharide (LPS) can further activate NF-κB by activating TLR3, thereby leading to transcriptional activation of various inflammatory factors and cell damage.

Long non-coding ribonucleic acid (lncRNA) refers to a kind of long-chain RNA molecule with over than 200 nucleotides in length[12]. Although lncRNAs themselves cannot encode corresponding proteins in cells, they can regulate the expression of target genes at various levels, such as (post-) transcriptional level and epigenetic modification. Ultimately, this may affect the progression of diseases[13]. Colorectal neoplasia differentially expressed gene (CRNDE) is a member of the lncRNA family. The expression and mechanism of CRNDE in the sepsis-related KI model have not been fully elucidated. In this work, the model of sepsis-related KI was successfully established in mice. The expression of lncRNA CRNDE in control group and disease group was detected. Meanwhile, the effect and mechanism of CRNDE knockout on KI in mice with sepsis were further discussed by small interfering RNA (siRNA) transfection.

## Materials and Methods

### Animal Grouping and Modeling

A total of 60 male C57 mice weighing (25.13±1.31) g were divided into 3 groups using the random number table method, including the control operation group (Sham group, n=20), sepsis-related KI group (LPS group, n=20) and CRNDE inhibition group (LPS + CRNDE siRNA group, n=20). No statistically significant differences were observed in age and weight among the three groups. Mice in LPS and LPS + CRNDE siRNA group were intraperitoneally injected with 5 mg/kg LPS, while the tail vein was injected with 5 μL CRNDE siRNAs. After 12 h, blood samples were collected from orbital veins, and kidney specimens were collected from mice in each group. All operations against animals were approved by the Animal Ethics Committee of Liaocheng People's Hospital Animal Center.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Detection

TRIzol assay (Invitrogen, Carlsbad, CA, USA) was adopted to extract total RNA in kidney tissues. The concentration and purity of extracted RNAs were detected by an ultraviolet spectrophotometer. When the ratio of absorbance at 260 to that at 280 ($A_{260}/A_{280}$) was 1.8-2.0, the RNA could be used. Subsequently, extracted messenger RNAs (mRNAs) were reverse transcribed into complementary deoxyribonucleic acids (cDNAs) through RT and stored in a refrigerator at -80°C for use. Specific Reverse Transcription-Polymerase Chain Reaction (RT-PCR) system was: 2.5 μL 10× buffer, 2 μL cDNAs, 0.25 μL forward primers (20 μmol/L), 0.25 μL reverse primers (20 μmol/L), 0.5 μL deoxy-ribonucleotide triphosphates (10 mmol/L), 0.5 μL Taq enzymes ($2\times10^6$ U/L) and 19 μL double distilled water. The amplification system of RT-PCR was the same. Primer sequences used in this study were as follows: lncRNA CRNDE, F: 5'-CCGTGCACGCTGTGAGAGTGTGCTC-3', R: 5'-TGTGCTCTGCTTGAGAGGA-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

### Hematoxylin and Eosin (H&E) Staining

Kidney tissues obtained from each group were placed in 10% formalin overnight, dehydrated and embedded in wax blocks. Subsequently, all kidney tissues were cut into 5 μm slices, fixed on glass slides and dried for staining. According

to relevant instructions, the sections were soaked in xylene, ethanol at gradient concentration and hematoxylin, followed by sealing with resin. After drying in the air, observation and photographing were conducted under an optical microscope (Nikon, Tokyo, Japan). Finally, the morphologies of kidney cells, tubules and the interstitium were observed.

### Immunohistochemical Staining

Kidney tissue sections were baked in an oven at 60°C for 30 min and dewaxed with xylene (5 min × 3 times), followed by dehydration with 100%, 95% and 70% ethanol, respectively (3 times each). Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide methanol, and the tissues were sealed with sheep serum for 1 h. Antibodies against superoxide dismutase 2 (SOD2) were diluted at 1:200 [Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA)]. Then, the sections were incubated with diluted antibodies at 4°C overnight, followed by washing with PBS 4 times in a shaker. After incubation with the secondary antibody, the color was developed with diaminobenzidine. 6 samples were randomly selected from each group, and 5 fields of view were randomly selected for each sample. Finally, photographing was performed under 200× and 400× optical microscopes.

### Periodic Acid Schiff (PAS) Staining and Scoring

Kidney tissues were embedded in paraffin and cut into 4-μm thick slices attaching to glass slides. Subsequently, the slices were dewaxed with xylene, stained with PAS, and observed under a microscope (Olympus BX51, Olympus, Tokyo, Japan). Five consecutive areas were observed and photographed under a 200× optical microscope. The tissue damage of each slice was then scored. Renal tubular necrosis (injury) scores in PAS-stained sections were as follows: 0 point: normal, 1 point: less than10%, 2 points: 10-25%, 3 points: 26-75% and 4 points: more than 75%.

### The Expression of p65 in Tissues via Immunofluorescence

Kidney tissue sections were baked in an oven at 60°C for 30 min and dewaxed with xylene (5 min × 3 times), followed by dehydration with 100%, 95% and 70% ethanol, respectively (3 times each). Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide methanol, and the tissues were sealed with sheep serum for 1 h. Antibodies against p65 were diluted at 1:200 (PBS) and incubated at 4°C overnight, followed by washing with PBS 4 times in a shaker. After incubation with fluorescein isothiocyanate (FITC) secondary antibody, the cells were incubated at 37°C for 1 h. The nuclei were then stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). After color development, 6 samples were randomly selected from each group, and 5 fields of view were randomly selected for each sample. Finally, photographing was performed under a 200× fluorescence microscope.

### Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining

Kidney tissues were sliced, baked in an oven at 60°C for 30 min, dewaxed with xylene (5 min × 3 times) and dehydrated with 100%, 95% and 70% ethanol, respectively (3 times each). The slices were then incubated with protein kinase K for half an hour. After washing with PBS, terminal deoxynucleotide transferase and Luciferase-labeled deoxyuridine triphosphate were added, followed by for 1 h at 37°C. Subsequently, specific antibody labeled with horseradish peroxidase (HRP) was added for 1 h of incubation at 37°C again. After staining of the nucleus, DAPI was photographed and counted under a fluorescence microscope.

### Western Blotting

Kidney tissues of mice in each group were fully ground in lysis buffer and ultrasonically lysed, followed by centrifugation. Subsequently, the supernatant was collected and successively transferred into Eppendorf tubes (EP; Hamburg, Germany). The concentration of extracted protein was measured *via* the bicinchoninic acid (BCA) assay and ultraviolet spectrophotometry. The protein volume of all samples was set constant to equal concentration. After sub-packaging, the samples were placed in a refrigerator at -80°C for use. Next, the protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The membranes were incubated with primary antibody at 4°C overnight, and goat anti-rabbit secondary antibody for 1 h in the dark. Finally, immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) method.

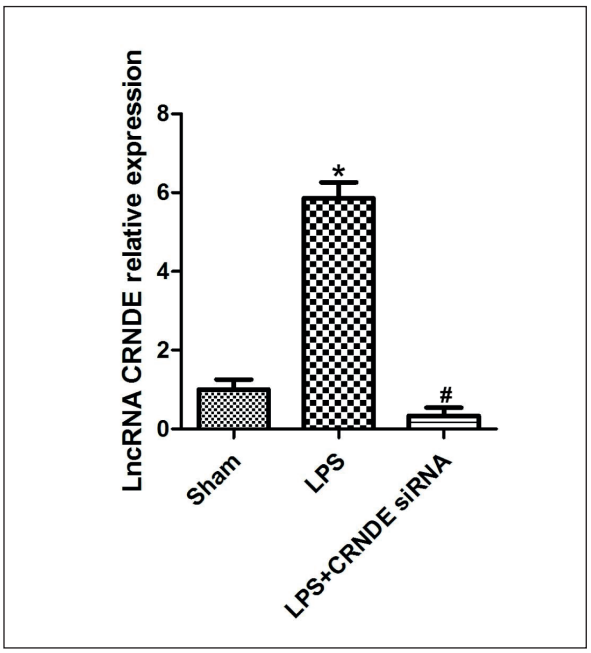


**Figure 1.** Expression level of lncRNA CRNDE in kidney tissues of mice in each group. Sham: control group, LPS: sepsis-related KI group, and LPS + CRNDE siRNA group: sepsis-related KI + CRNDE inhibition group. *$p$<0.05 *vs.* Sham group and $^{\#}p$<0.05 *vs.* LPS group.

### *Statistical Analysis*

Statistical Product and Service Solutions 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation. *t*-test was used to compare the difference between the two groups. $p$<0.05 was considered statistically significant.

## Results

### *Expression of LncRNA CRNDE in Kidney Tissues of Mice in Each Group*

RT-PCR results revealed that the expression of lncRNA CRNDE in kidney tissues of mice in the LPS group increased significantly when compared with the Sham group ($p$<0.05). After intravenous injection of lncRNA CRNDE into mouse tail for 12 h, the expression level of lncRNA CRNDE in kidney tissues was significantly down-regulated ($p$<0.05). The results indicated that the model in lncRNA CRNDE knockout group was successfully induced (Figure 1).

### *Evaluation of Renal Function in Mice of Each Group*

The expression levels of creatinine and blood urea nitrogen in mice were detected by automatic blood biochemical analyzer. The results found that the levels of creatinine and blood urea nitrogen in the LPS group increased markedly ($p$<0.05). Meanwhile, the renal function of mice was significantly improved. However, the levels of creatinine and urea nitrogen decreased remarkably after knockout of lncRNA CRNDE ($p$<0.05; Figure 2).

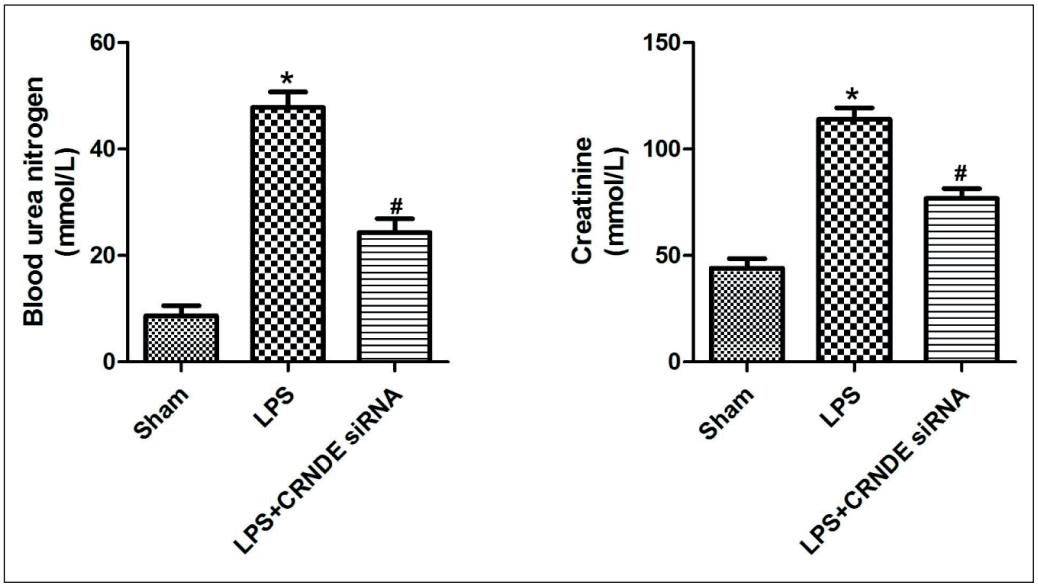


**Figure 2.** Renal function of mice in each group. Sham: control group, LPS: sepsis-related KI group, and LPS + CRNDE siRNA group: sepsis-related KI + CRNDE inhibition group. *$p$<0.05 *vs.* Sham group and $^{\#}p$<0.05 *vs.* LPS group.

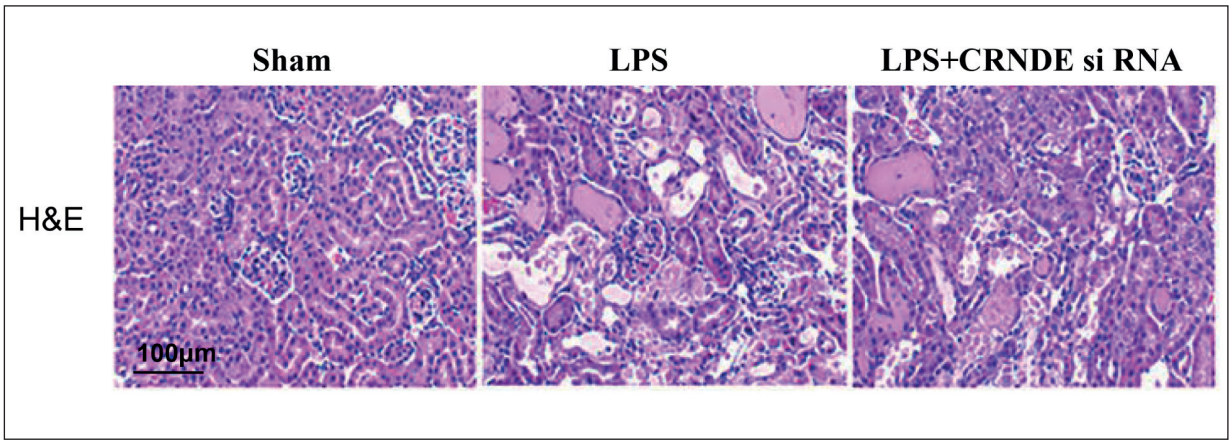


**Figure 3.** H&E staining of kidney tissues of mice in each group (magnification × 40). Sham: control group, LPS: sepsis-related KI group, and LPS + CRNDE siRNA group: sepsis-related KI + CRNDE inhibition group.

### *H&E Staining of Kidney Tissues of Mice in Each Group*

H&E staining results manifested that kidney tissues in mice of the LPS group showed marked abnormal pathological changes, such as cell edema, necrosis, abnormal arrangement and basement membrane damage when compared with the Sham group. However, the histopathological injury of kidney tissues in the LPS + lncRNA CRNDE siRNA group was remarkably reduced when compared with the LPS group (Figure 3).

### *PAS Staining of Kidney Tissues of Mice in Each Group*

To further evaluate the renal tubular injury in kidney tissues of mice, PAS staining and scoring were performed in each group. The results demonstrated that renal tubular cells in the LPS group displayed significant necrosis, vacuolization and flattening. Meanwhile, the deletion of the brush border appeared. After knockout of lncRNA CRNDE, these pathological changes in mouse renal tubules were markedly suppressed, whereas the renal tubular injury score increased remarkably ($p<0.05$; Figure 4).

### *Evaluation of Cell Apoptosis in Kidney Tissues of Mice in Each Group*

TUNEL staining was conducted to evaluate cell apoptosis in kidney tissues of mice in each group. The number of apoptotic cells in the LPS group was significantly larger than that of the Sham group ($p<0.05$). However, the number of apoptotic cells in kidney tissues of lncRNA CRNDE siRNA group was evidently smaller than that of the LPS group ($p<0.05$). The above results suggested that lncRNA CRNDE siRNA could inhibit KI caused by sepsis (Figure 5).

### *Immunohistochemical Staining of p65 in Kidney Tissues of Mice in Each Group*

Considering the important role of NF-κB in septic KI, the distribution and expression of p65 in kidney tissues of the three groups were detected using the immunohistochemical technique. The results demonstrated that the level of p65 in kidney tissues of mice in lncRNA CRNDE siRNA group was markedly lower than that of the LPS group ($p<0.05$; Figure 6).

### *Effect of LncRNA CRNDE Knockout on the TLR3/NF-κB Signaling Pathway*

The effect of lncRNA CRNDE knockout on the TLR3/NF-κB signaling pathway in KI caused by sepsis was further investigated. It was found that the expression levels of TLR3 and p65 in LPS group were significantly higher than those of the Sham group ($p<0.05$). However, the expression levels of the above molecules were notably inhibited after lncRNA CRNDE knockout ($p<0.05$; Figure 7). These results indicated that the damage of lncRNA CRNDE siRNA to sepsis-related KI was mediated by the TLR3/NF-κB signaling pathway.

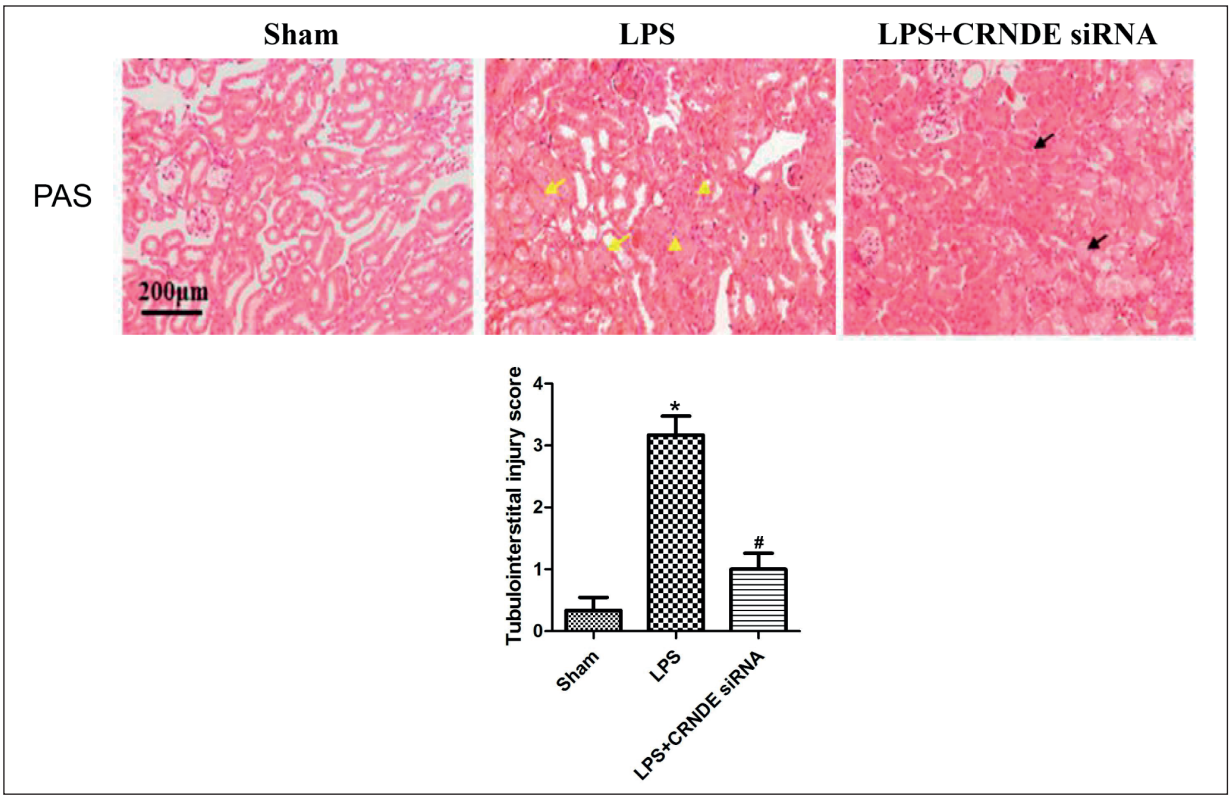


**Figure 4.** PAS staining of kidney tissues of mice in each group (magnification × 200). Sham: control group, LPS: sepsis-related KI group, and LPS + CRNDE siRNA group: sepsis-related KI + CRNDE inhibition group. *$p$<0.05 *vs.* Sham group and $^{\#}p$<0.05 *vs.* LPS group.

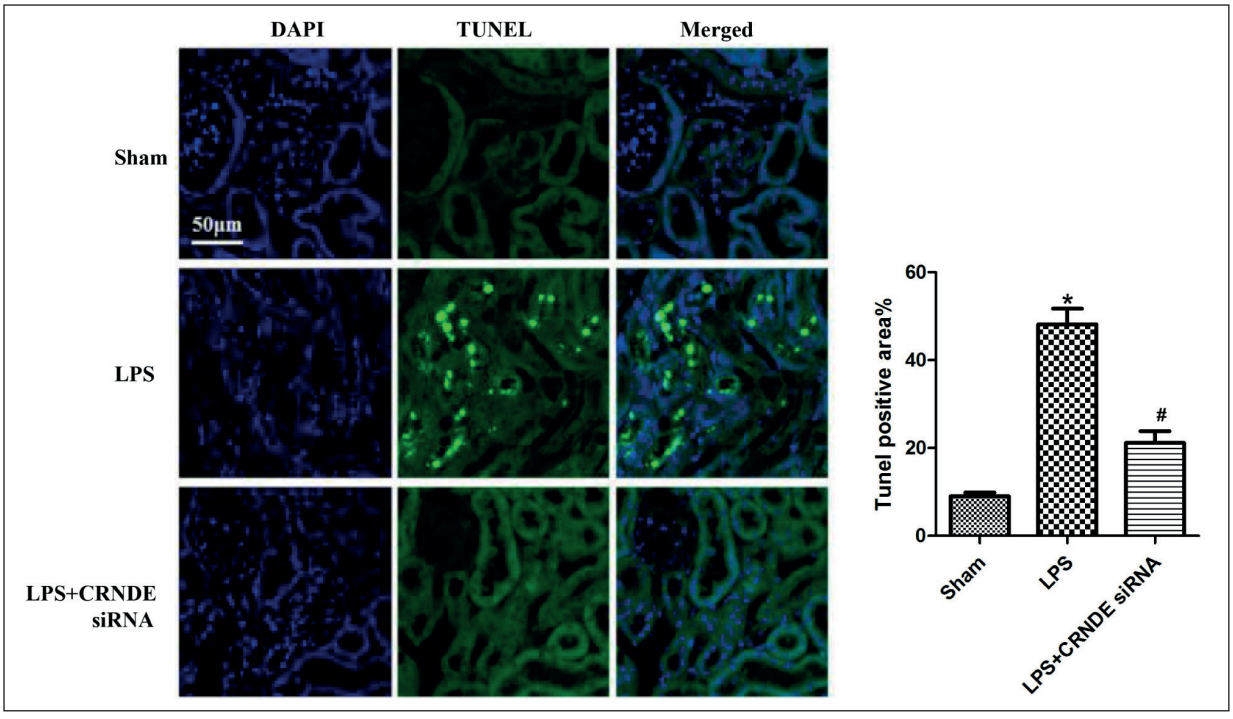


**Figure 5.** TUNEL staining of kidney tissues of mice in each group (magnification × 200). Sham: control group, LPS: sepsis-related KI group, and LPS + CRNDE siRNA group: sepsis-related KI + CRNDE inhibition group. *$p$<0.05 *vs.* Sham group and $^{\#}p$<0.05 *vs.* LPS group.

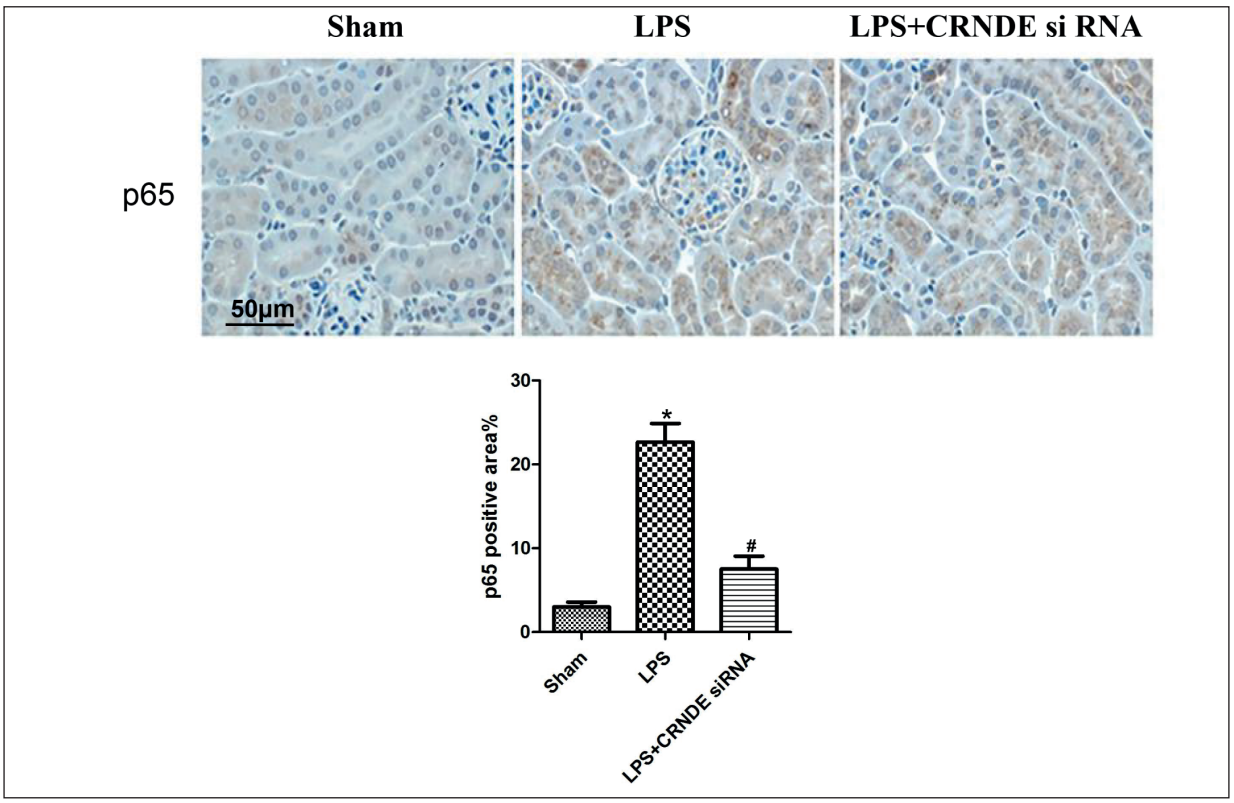


**Figure 6.** Immunohistochemical staining of p65 in kidney tissues of mice in each group (magnification × 200). Sham: control group, LPS: sepsis-related KI group, and LPS + CRNDE siRNA group: sepsis-related KI + CRNDE inhibition group. *$p$<0.05 *vs.* Sham group and #$p$<0.05 *vs.* LPS group.

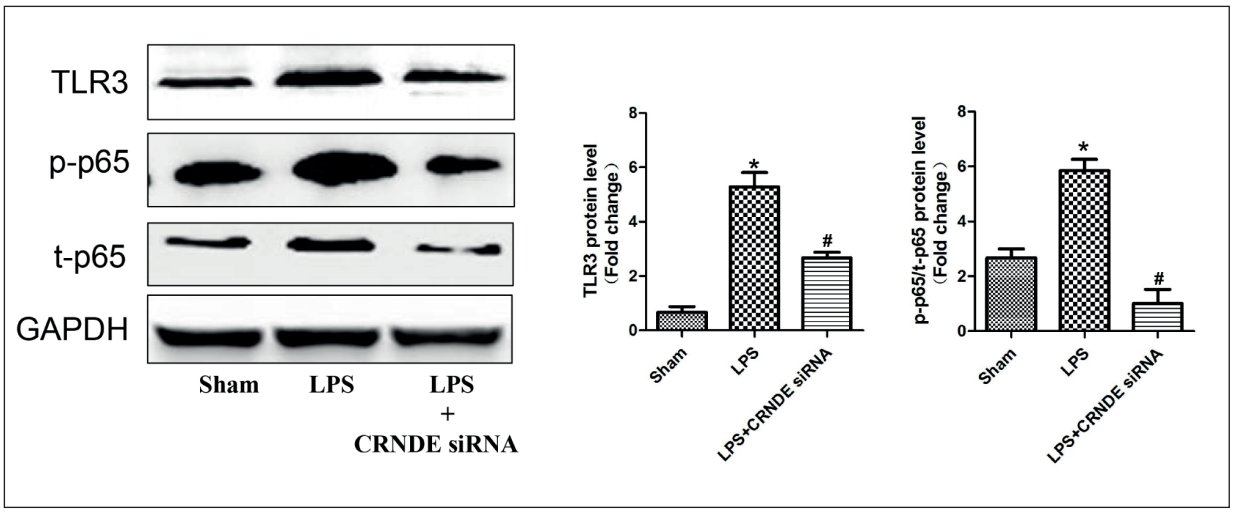


**Figure 7.** Expression of the TLR3/NF-κB signaling pathway in kidney tissues of mice in each group. Sham: control group, LPS: sepsis-related KI group, and LPS + CRNDE siRNA group: sepsis-related KI + CRNDE inhibition group. *$p$<0.05 *vs.* Sham group and #$p$<0.05 *vs.* LPS group.

## Discussion

In February 2016, sepsis was newly defined as a life-threatening OD caused by the disorder of host's response to infection. This new definition emphasizes that OD is an important risk factor for poor prognosis in sepsis and should be treated in time before infection control. KI is the most common OD in patients with severe sepsis, which leads to high mortality rate[14,15]. Therefore, more attention should be paid to the treatment of KI caused by sepsis.

The inflammatory response is considered to be one of the main causes of sepsis-related KI[16]. Zou et al[17] have revealed that the activation of the TLR3/NF-κB signaling pathway is an important molecular mechanism leading to inflammation activation, independent of MyD88. NF-κB (p65) is mainly located in the cytoplasm and normally binds to the inhibitor of NF-κB (IκB). The activation of NF-κB (p65) is regulated by IκB kinase (IKK), which can phosphorylate NF-κB (p65) and induce nuclear translocation of NF-κB (p65)[18]. NF-κB (p65) is combined with κB co-recognition sequence to further transcriptionally activate corresponding pro-inflammatory genes. Currently, Zheng et al[19] have shown that the activation of TLR3 receptors can activate IKKs and further activate NF-κB (p65). Djafarzadeh et al[20] have manifested that the activation of TLR3 in human hepatocytes promotes apoptosis by inducing NF-κB (p65) activation and Caspase-8-dependent mitochondrial dysfunction. Naringin has been observed to alleviate sepsis-induced small intestine injury by up-regulating tight junction proteins, ZO-1 and claudin-1. The mechanism may be related to the regulation of Naringin on the RhoA/ROCK/NF-κB/MLCK/MLC signaling pathway[21]. Additionally, by inhibiting NF-κB (p65), the down-regulation of aquaporin-2/V2 receptor protein can be effectively reduced. Ultimately, this can reduce pathological damage to the kidney[22]. The above results indicate that suppressing the activation of the TLR3/NF-κB signaling pathway in sepsis-related KI is an important target for the treatment of AKI.

CRNDE was first found to be highly expressed in colorectal cancer new organism. Meanwhile, it was found to be abnormally highly expressed in glioma[23]. Previous studies have found that CRNDE promotes the proliferation and migration, whereas inhibits the apoptosis of glioma cells and neuroblastoma. However, CRNDE can be inhibited by insulin and insulin-like growth factors (IGFs) in a manner dependent on the PI3K/Akt/mTOR and Raf/MAPK signaling pathways[24,25]. Recently Li et al[26] have verified that CRNDE can induce inflammation in astrocytes and glioma cells by activating TLR3 and phosphorylating NF-κB (p65). In the present work, the expression of CRNDE in kidney tissues of sepsis mice and normal mice was detected. The results revealed that the expression level of CRNDE in kidney tissues increased significantly in sepsis-related KI model ($p<0.05$). Furthermore, the expression of CRNDE in the mouse kidney was suppressed by gene knockout. The subsequent experiment demonstrated that the renal function of mouse kidney was evidently improved after LPS injection for 12 h. This was manifested as a significant decrease in the levels of creatinine and urea nitrogen ($p<0.05$). Additionally, the injury of kidney tissues in mice was detected by histological staining. The results manifested that CRNDE knockout could markedly alleviate edema, necrosis, renal tubular injury, disorder of arrangement and destruction of the brush border in the kidney of mice. Our data also discovered that CRNDE knockout could inhibit LPS-induced renal cell apoptosis and p65 activation. Furthermore, the protein expression levels of TLR3 and NF-κB in the mouse kidney were measured *via* Western blotting. The results showed that CRNDE knockout could significantly inhibit the activation of the TLR3/NF-κB signaling pathway. However, there were still some limitations in this study: 1) No cell experiments were designed to verify the results. 2) No direct target of CRNDE affecting the TLR3/NF-κB signaling pathway was found. 3) Whether CRNDE had any effect on the TLR4/NF-κB signaling pathway relying on MyD88 was not verified.

## Conclusions

These results showed that CRNDE can induce KI caused by sepsis by activating the TLR3/NF-κB signaling pathway. Therefore, targeted inhibition of CRNDE is expected to be a potential target for the treatment of sepsis-related KI.

### Conflict of Interests

The authors declare that they have no conflict of interest.

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