European Review for Medical and Pharmacological Sciences 2019; 23: 8011-8017

Regulatory effect of lncRNA NKILA on autophagy induced by sepsis kidney injury

Y.-M. YANG, Y.-H. LI, L.-L. DING, Y. FU, N. LI

Department of Intensive Care Unit, First Hospital of Jilin University, Changchun, China

Abstract. – OBJECTIVE: The aim of this study was to investigate the regulatory effect of long non-coding RNA (lncRNA) NKILA on autophagy in sepsis-induced kidney injury.

MATERIALS AND METHODS: Sepsis model was successfully established in rats by cecal ligation and puncture (CLP). Hematoxylin and eosin (HE) staining was performed to evaluate the pathological lesions in rat kidney tissues. Subsequently, serum samples of sepsis rats were collected. The levels of blood urea nitrogen (BUN) and serum creatinine (SCr) were determined. Western blot and quantitative real time-polymerase chain reaction (qRT-PCR) were conducted to detect the protein and mRNA expression levels of LC3, Beclin-1, activated caspase-3, p-Akt (308), p-Akt (472), Akt and NKILA in kidney tissues of sepsis rats at different time points, respectively. Subsequently, HK-2 cells were induced with different doses of lipopolysaccharide (LPS) for different time points. The expression levels of the above genes in cells were detected as well. Finally, changes in autophagy and apoptosis in LPS-induced HK-2 cells with the treatment of PI3K pathway inhibitor or Akt inhibitor were observed.

RESULTS: Typical pathological lesions were observed in kidney tissues of sepsis rats, with increased serum levels of BUN and Scr. This indicated the successful construction of the sepsis model in rats. The expression levels of LC3, Beclin-1, and NKILA/Akt significantly increased in kidney tissues of sepsis rats. *In vitro* experiments revealed that NKILA expression in HK-2 cells was gradually up-regulated with the increase of LPS dose and prolongation of LPS induction. The expressions of LC3 and NKILA/Akt were higher after 4 h of LPS treatment, and 8 h of LPS induction. Furthermore, the treatment of PI3K pathway inhibitor or Akt inhibitor remarkably down-regulated LPS-induced LC3 expression, while it accelerated cell apoptosis and up-regulated NKILA expression.

CONCLUSIONS: Autophagy occurs at sepsis-induced kidney injury, which can be regulated by NKILA/Akt pathway.

Key Words:
Autophagy, NKILA, PI3K/Akt pathway, Sepsis, Kidney injury.

Introduction

Sepsis is a systemic inflammatory response syndrome caused by infection. It is also a common cause of acute kidney injury (AKI) in ICU patients[1]. AKI is an independent risk factor for predicting mortality in septic patients[2]. Therefore, it is of great significance to identify the key molecules that can mediate sepsis-induced renal tubular epithelial cell damage. This is conducive to the development of early intervention of sepsis-induced AKI. In recent years, some studies have found that bacterial endotoxin and its inflammatory factors are direct and important causes of kidney damage. Lipopolysaccharide (LPS) stimulation leads to a severe inflammatory response, thus producing a large number of inflammatory factors such as tumor necrosis factor-α (TNF-α) and reactive oxygen species (ROS). Ultimately, oxidative stress, mitochondrial damage, and energy depletion lead to the apoptosis of renal tubular epithelial cells[3]. During this process, autophagy may exert an essential role in the damage and repair of renal tubular epithelium.

Autophagy is a programmed death progress, which is different from cell apoptosis. Under the state of hypoxia, starvation or infection of eukaryotic cells, damaged organelles and macromolecular substances will be degraded by lysosomes. This may further provide materials and nutrients for cell repair and renewal[4].

Long non-coding RNAs (lncRNAs) regulate gene expressions at multiple levels. Previous studies have established that lncRNAs are closely associated with tumorigenesis, viral replication, and inflammatory response. Inflammatory injury secondary to sepsis involves multiple factors and pathways. In recent years, the significant role of lncRNAs in the pathogenesis of sepsis has been identified[5-7]. However, whether autophagy is involved in sepsis-induced renal tubular epithelial cell injury remains unclear. Moreover, whether certain lncRNAs are involved in autophagy of re-

Corresponding Author: Nan Li, MD; email: leenan79@163.com

nal tubular epithelial cells in the pathogenic progression of sepsis has not been fully elucidated. In this study, we first established a sepsis model in rats by cecal ligation and puncture (CLP) and observed the autophagy of renal tubular epithelial cells. Subsequently, *in vitro* experiments were conducted to investigate the potential roles of lncRNA NKILA and PI3K pathway in the regulation of sepsis-induced autophagy. The aim of this study was to provide a new direction in the clinical treatment of AKI.

Materials and Methods

Cell Culture

HK-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C and 5% CO_2. Cell passage was performed using 0.25% trypsin. For *in vitro* experiments, cells were first seeded into 6-well plates at 2 × 10^8/L. Until 70-80% of confluence, the cells were incubated with serum-free Keratinocyte-SFM for 24 h to synchronize cell growth. After that, HK-2 cells were induced with different doses of LPS (0, 0.1, 10, or 20 mg/L) for 12 h or induced with 10 mg/L LPS for different time points.

Establishment of Sepsis-Induced Model in Rats

Sprague Dawley (SD) rats were anesthetized with 10% chloral hydrate (diluted in normal saline) by intraperitoneal injection at 4 mL/kg. A 3-4 cm incision was made in the midline of the abdomen to expose the cecum. The mesentery between the cecum was carefully cut in avoidance of blood vessels on the membrane. A 5-0 suture was used for ligation of the cecum away from the cecum, where 4 repeated punctures were performed using an 18 G needle. 1 mm feces were squeezed from the puncture site, and the incision was sutured using sutures. For CLP procedures, pre-warmed saline was intraperitoneally injected at 50 mL/kg. 6 h later, 4 mg/kg imipenem/cilastatin diluted in 10 mL of pre-warmed saline was subcutaneously injected in rats. The rats were then sacrificed, and blood and kidney samples were harvested. This study was approved by the Animal Ethics Committee of Jilin University Animal Center.

Hematoxylin and Eosin (HE) Staining

Kidney tissues were placed in formaldehyde solution for 48 h, dehydrated in an automatic dehydrator for 12 h, paraffin-embedded, and sliced. All obtained slides were preserved at 4°C for subsequent use. For HE staining, the slides were dewaxed and stained with hematoxylin-eosin. After dehydration with gradient ethanol, vitrification with dimethylbenzene, and sealing with neutral gum, pathological changes in renal interstitial and renal tubules were observed and captured under a light microscope.

Detection of Serum Levels of Blood Urea Nitrogen (BUN) and Serum Creatinine (SCr)

Blood samples were harvested from the heart, followed by centrifugation for collecting serum. The serum levels of BUN and SCr were determined as previously described[8].

Western Blot

Total protein was first extracted from cell lysis or tissue homogenate. The concentration of protein samples was quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Subsequently, extracted proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocking with skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. On the next day, the membranes were washed with Tris-buffered Saline with Tween 20 (TBST) and incubated with corresponding secondary antibody at room temperature for 1 h. Immuno-reactive bands were developed by enhanced chemiluminescence (ECL) method.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Tissue or cells were first lysed in TRIzol on ice (Invitrogen, Carlsbad, CA, USA). The lysate was then incubated with chloroform for extraction and isopropanol for precipitation. After being washed with 75% ethanol, the precipitate was air dried and diluted in 20 μL of diethyl pyrocarbonate (DEPC) water. The extracted RNA of each sample was reversely transcribed into complementary deoxyribose nucleic acid (cDNA). QRT-PCR was detected according to the instructions of SYBR® Green Master Mix (TaKaRa, Otsu, Shiga, Japan). The specific procedure was as follows: denatur-

ation at 95°C for 1 min, 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, for a total of 40 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as an internal reference. PCR primers were listed in Table I. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method as previously described[9].

Double-Staining of Hoechst 33342 and Annexin V/Propidium Iodide (PI)

Cells were first harvested and washed with phosphate-buffered saline (PBS) twice. After that, the cells were stained with 5 μL Hoechst 33342 and 5 μL PI at 4°C for 20-30 min. After the PBS washing, cell apoptosis was observed using a fluorescence microscope.

Other cells were collected and re-suspended in binding buffer. 100 μL cell suspension was transferred into a 5 mL flow cytometry tube, followed by incubation with 5 μL Annexin V and 10 μL PI in dark for 10 min. Finally, 300 μL PBS was supplied for the determination of normal, apoptotic and necrotic cells by flow cytometry.

Statistical Analysis

Statistical Product and Service Solu (SPSS) 13.0 software (IBM, Armonk, USA) was utilized for all statistical analys Quantitative data were represente mean standard deviation ($\bar{x}\pm s$). Chi- st and LSD *t*-test were performed f compa g differences. $p<0.05$ was co si d significant.

Result

Pathologi Lesi s in Kidney Tissues of Sepsis

HE ining icated at compared with the sha rats i CLP procedures which wed ev nt kid injury. In particular, patho- al l kidney aggravated with the prolon of sepsis. Renal tubular epithelial cell swell brush border loss, vacuolar degeneration, ne is, tubular formation, cell shedding, and other pathological changes were significantly pronounced at 12 h of CLP procedures when compared with those at 6 h (Figure 1A). Besides, the serum levels of BUN and SCr remarkably increased in sepsis rats in a time-dependent manner (Figure 1B and 1C). All the above pathological changes indicated the successful construction of the sepsis model in rats.

Expression Changes of Autophagy-Related Genes and NKILA/AKT in Kidney Tissues of Sepsis Rats

Western blot analysis showed that autophagy indicators (LC3 and Beclin-1) were lowly expressed in the sham group. LC3 and Beclin-1 were up-regulated at 6 h and reached the peak at 12 h, whereas they were both down-regulated at 18 h. Protein expression of activated caspase-3 incre d with the prolongation of CLP procedures. T d that sepsis-induced AKI accelerated pase-3-m ted apoptosis in a time-dependent r er (Figur A). Both qRT-PCR and Wes blot lts re ed that the relative expre s of NKI -Akt were significantly u egulat after C proce- dures and reache t 6 h. However, they began to decre at 12 gure 2 nd 2C).

Express C ges of A ophagy-Related Genes and NK AKT in LPS-Induced H lls

K-2 cells were induced with different doses PS for 12 Western blot results showed that L and p-A 472) gradually increased with an inc of LPS, which achieved the peak at 10 mg/L and started to decrease at 20 mg/L. protein level of p-Akt (308) gradually in- cre d and reached the peak at 20 mg/L (Figure 3A). QRT-PCR results revealed that the highest level of NKILA was at 10 mg/L, which decreased at 20 mg/L (Figure 3B). Subsequently, HK-2 cells were induced with 10 mg/L LPS for 0, 4, 8 and 12 h, respectively. The expression levels of LC3, p-Akt (472) and NKILA achieved the highest levels at 8 h and decreased at 12 h. On the contrary, the expression level of p-Akt (308) still remained high at 12 h (Figure 3C and 3D).

Regulatory Effect of PI3K Pathway Inhibitor on Autophagy and NKILA in LPS-Induced HK-2 Cells

LPS-induced HK-2 cells were treated with PI3K pathway inhibitor 3-MA or Akt inhibitor, respectively. Both the proteins and the mRNA levels of LC3 and NKILA were significantly inhibited by 3-MA or Akt inhibitor (Figure 4A and 4B). More importantly, treatment of 3-MA or Akt inhibitor remarkably accelerated the apoptosis of LPS-induced HK-2 cells (Figure 4C).

Discussion

The mortality of sepsis patients combined with AKI is remarkably higher than those with-

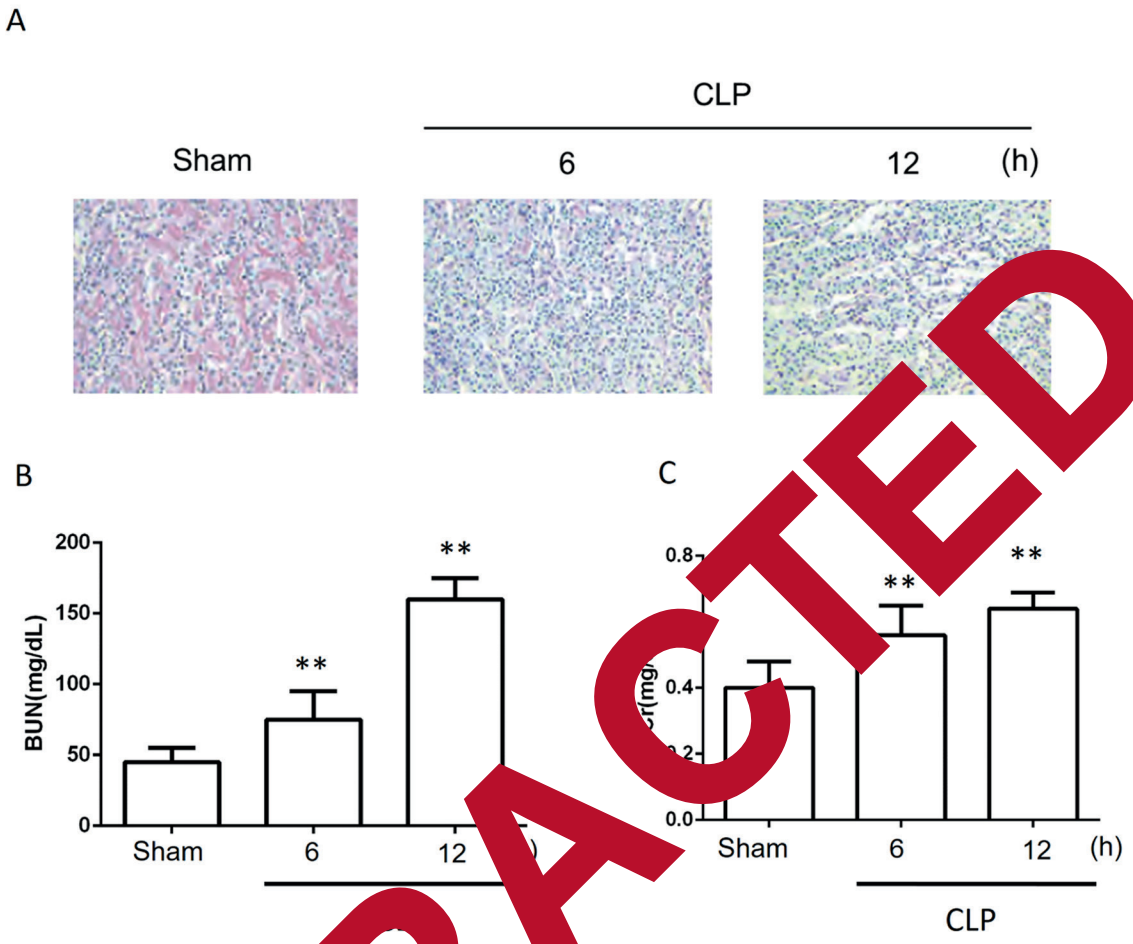

Figure 1. Pathological lesions in k tissu sis rats. **A,** HE staining of rat kidney tissues in sham group and CLP group (magnification 40×). **B,** S n l N l of sham group, 6 h CLP group and 12 h CLP group. **C,** Serum level of SCr in rats of sham group CLP gr d 12 h CLP group. $^{**}p<0.01$ *vs.* Sham group.

out AKI, a untin r over 70%. Meanwhile, it has beco on the most urgent problems to be olved ritical mergency medicine[1]. Isch peri the pathogenesis of sis-ind ed AK it has been found that the l hy ion resulted from renal tubular ascular dysfunction and the acute tubular rosis (ATN) after renal ischemia are the maj athogenic factors of sepsis-induced AKI. However, renal pathology in death cases of sepsis patients with AKI shows that ATN occurs in more than 70% of patients. However, renal tubular epithelial cell apoptosis is more pronounced[9]. An *in vivo* experiment determined renal hemodynamics in sepsis mice. The results have shown that nearly two-thirds of sepsis mice experienced renal ischemia. However, the remaining showed unchangeable or increased renal blood flow, which is more similar to hyper-dynamic septic shock in humans[9]. In the case of constant or even increased blood flow to the kidney, renal tubular epithelial cells still undergo apoptosis. It is suggested that there are other factors responsible for sepsis-induced AKI, except for renal hemodynamic changes. Bacterial endotoxin and its inflammatory factors are direct and important causes of sepsis-induced kidney injury. Meanwhile, LPS stimulates severe inflammatory reactions, thereby producing a large number of inflammatory factors such as TNF-α and ROS. As a result, oxidative stress, mitochondrial damage, and energy depletion ultimately lead to the apoptosis of renal tubular epithelial

cells. During this process, autophagy may be significant in renal tubular epithelial damage and repair.

Hypoxia, starvation and infection conditions can lead to the degradation of damaged organelles and macromolecular substances triggered by autophagy-related genes in eukaryotic cells. This degradation process contributes to maintain energy homeostasis, which differs from cell apoptosis. Such a programmed death progression is known as autophagy. The final outcome of apoptosis is cell death, while autophagy is a double-edged sword for cell survival and death[10]. Meanwhile, autophagy is closely related to apoptosis. Autophagy is required for and occurs before apoptosis. An appropriate degree of autophagy protects cells from apoptosis and necrosis, while excessive autophagy accelerates cell death accompanied by apoptosis[11]. Currently, ischemia-reperfusion, drug-induced renal injury and obstructive nephropathy have been extensively studied in researches of renal tubular epithelial cell autophagy[12-14]. The crucial role of autophagy in sepsis has been widely clarified in recent years. Autophagosomes and mitochondrial oxidative stress can be obs... in the heart and liver tissues of sepsis pati... confirming the occurrence of autophagy in s... sis[6-8]. In addition, LC3 and Becli... deficie... cy in sepsis animal models established by both CLP or LPS administration can significantly up-regulate the levels of inflammatory factors. These results suggest that autophagy inhibition may aggravate cellular inflammation[15,16]. Therefore, we suggested that autophagy is greatly involved in the pathogenesis and inflammatory response of sepsis.

PI3K/Akt is a classical survi... pathway, which is involved in apoptosis, ... genesis. PI3K has been found t... a down... am molecule of lncRNA NKILA... ecent ye... it has been demonstrated ... t the ... K/Akt ... th-way regulates autop... y via the ... eam molecular mTOR. ... his st..., we su... ssfully established a se... si... d... n rats by CLP. The autophagy of ... al tu... r epit... al cells was observed ... ll. Patho... lesions of kidney agg... ate... ith the pr... ngation of sepsis, characterized by ... al tubular epithelial cell sw... g, brush bor... loss, vacuolar degenera-t..., necrosis, tubular formation, cell shedding, a... other pat... ogical changes. Meanwhile, the s... n levels ... BUN and SCr significantly increa... esting the successful construction of the sepsis model in rats. Subsequently, the ... ein and mRNA expression levels of autop... -related genes and NKILA/p-Akt in renal tubular epithelial cells were determined by

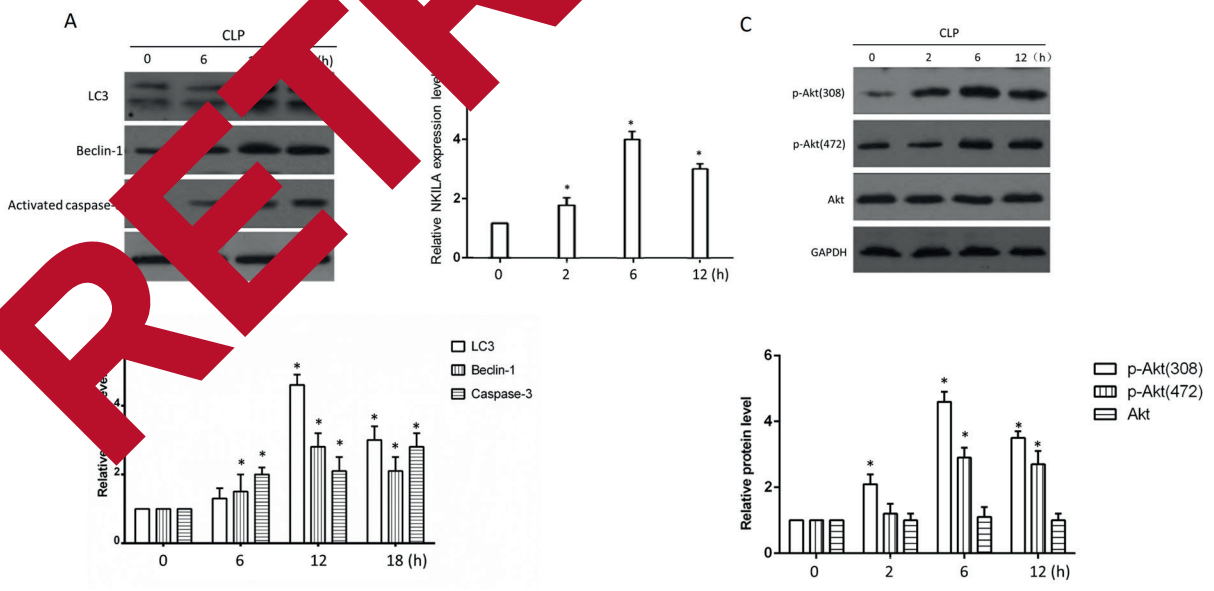

Figure 2. Expression changes of autophagy-related genes and NKILA/AKT in kidney tissues of sepsis rats. **A,** Western blot analysis of LC3, Beclin-1 and activated caspase-3 in rat kidney tissues at 0, 6, 12 and 18 h after CLP procedures. **B,** Relative expression of NKILA in rat kidney tissues at 0, 2, 6 and 12 h after CLP procedures by qRT-PCR. **C,** Western blot analysis of p-Akt (308), p-Akt (472) and Akt in rat kidney tissues at 0, 2, 6 and 12 h after CLP procedures. $^{*}p<0.05$ *vs.* 0 h group.

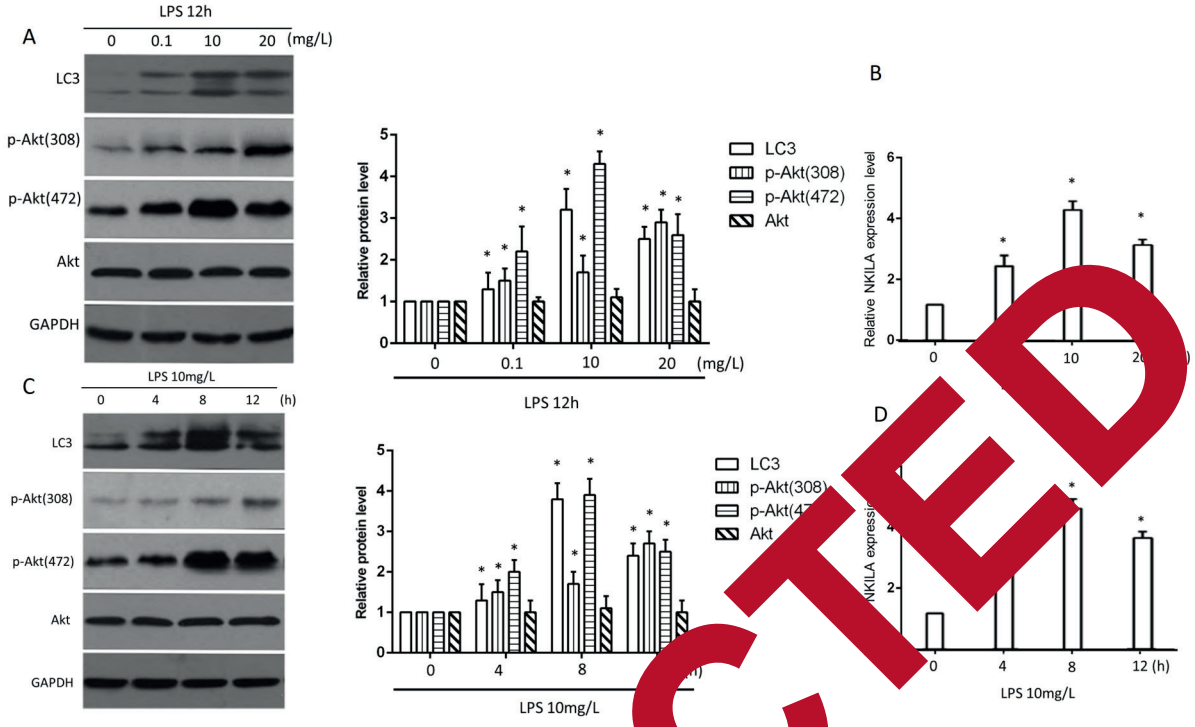

Figure 3. Expression changes of autophagy-related genes and NKILA/AKT in LPS-induced HK-2 cells. **A,** Western blot analysis of LC3, p-Akt (308), p-Akt (472) and Akt in HK-2 cells induced with 0, 0.1, 10 and 20 mg/L LPS for 12 h, respectively. **B,** Relative expression of NKILA in HK-2 cells induced with 0, 0.1, 10 and 20 mg/L LPS for 12 h, respectively. **C,** Western blot analysis of LC3, p-Akt (308), p-Akt (472) and Akt in HK-2 cells induced with 10 mg/L LPS for 0, 4, 8 and 12 h, respectively. **D,** Relative expression of NKILA in HK-2 cells induced with 10 mg/L LPS for 0, 4, 8 and 12 h, respectively. *p<0.05 *vs.* 0 h group.

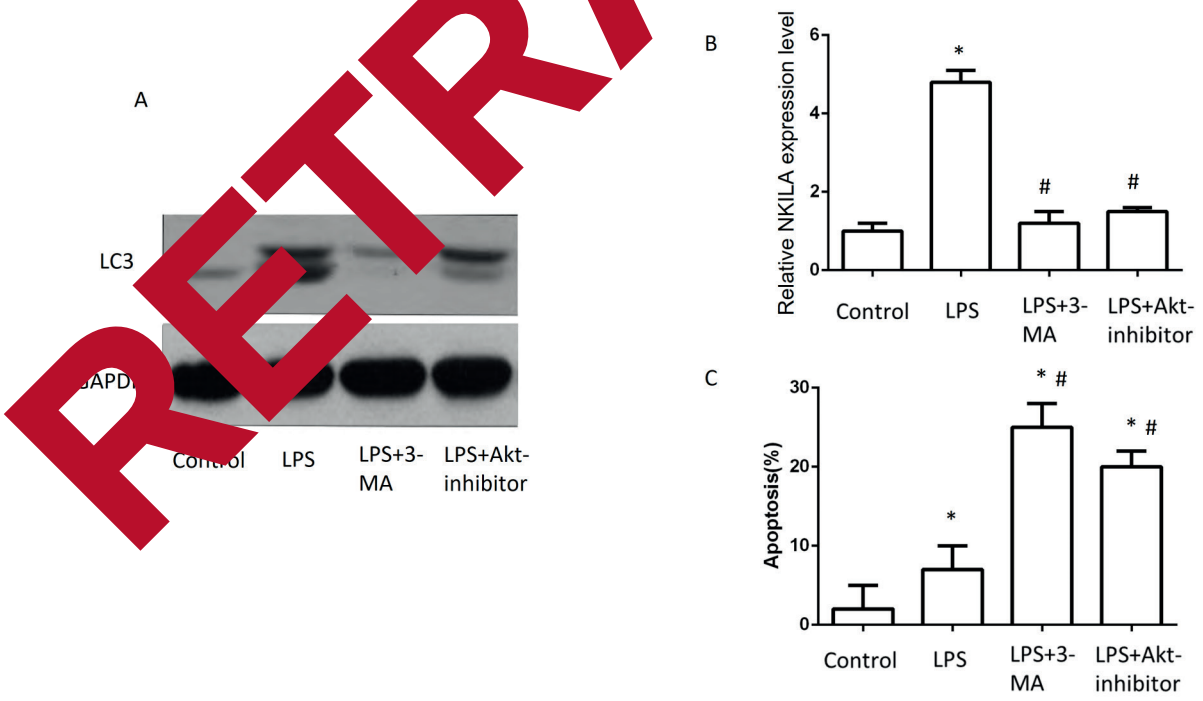

Figure 4. Regulatory effect of PI3K pathway inhibitor on autophagy and NKILA in LPS-induced HK-2 cells. HK-2 cells were induced with LPS, followed by treatment with PI3K pathway inhibitor 3-MA or Akt inhibitor. **A,** Western blot analysis of LC3 in HK-2 cells. **B,** Relative expression of NKILA in HK-2 cells. **C,** Apoptosis of HK-2 cells. *p<0.05 *vs.* control; #p<0.05 *vs.* LPS group.

Western blot and qRT-PCR, respectively. The results indicated that the expression levels of LC3, Beclin-1, and NKILA/p-Akt remarkably increased with the prolongation of sepsis. Interestingly, Beclin-1 expression started to up-regulate at 6 h and achieved the peak at 12 h. However, Beclin-1 was significantly downregulated at 18 h. This might be explained by the abundant apoptosis resulted from long-term LPS induction that over-activated caspase-3 and cleaved Beclin-1[16]. Furthermore, in LPS-induced HK-2 cells, the expressions of LC3 and NKILA/p-Akt were significantly up-regulated as well. Both PI3K pathway inhibitor and Akt inhibitor could down-regulate the expressions of autophagy-related genes and accelerate cell apoptosis. Our results suggested that NKILA/p-Akt could regulate autophagy in sepsis-induced AKI.

In this study, we established classical *in vivo* and *in vitro* sepsis models in rats and HK-2 cells, respectively. The autophagy occurring in renal tubular epithelial cells during sepsis was determined as well. Furthermore, we elucidated the protective roles of NKILA/Akt and autophagy in sepsis-induced AKI. Our results might help to provide a new strategy for clinical interventi sepsis-induced AKI.

Conclusions

We demonstrated that au oc sis-induced kidney injur ich regulated by NKILA/Akt pathw

Conflict of in est

The authors d e no c icts of interest.

Ac gem

T study supp by Health Commission Of Jilin ince.

References

1) Haddy FJ. Acute renal failure and sepsis. N Engl J Med 2004; 351: 2347-2349.
2) Bagshaw SM, Uchino S, Bellomo R, Morimatsu H, Morgera S, Schetz M, Tan I, Bouman C, Macedo E, Gibney N, Tolwani A, Oudemans-van Straaten HM, Ronco C, Kellum JA, BEST Kidney Investigators. Septic acute kidney injury in critically ill patients: clinical characteristics and outcomes. Clin J Am Soc Nephrol 2007; 2: 431-439.
3) Wang Y, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR. Downregulation of liver X receptor-alpha in mouse kidney and HK-2 proximal tubular cells by LPS and cytokines. J Lipid Res 2005; 46: 2377-2387.
4) Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. Science 2000; 290: 1717-1721.
5) Zhang L, Wang DL, Yu P. LncRN s the expression of its target gene XA10 in e ne-trial carcinoma through co g with mi 2. Eur Rev Med Pharmacol Sci 2 2: 4820 27.
6) Chen H, Wang X, Ya Cheng X, W. LncRNA MALAT1 gulates sepsis d cardiac inflammati nd dy ction via teraction with miR-125 ar PK/NF ppaB. Int Immunophar ol 20 : 69-7
7) Huang K, Zhu Y, Luo Q, Qing C. Diag c e of the In A NEAT1 in peripheral blood mo clear cells of patients with sepsis Markers 7: 2017: 7962836.
8) Wan L, Bagshaw SM, Langenberg C, Saotome T, May C, Bellomo R. Pathophysiology of septic acute kidney injury: at do we really know? Crit Care Med 008; 36: 98-S203.
9) C, Wan L, Egi M, May CN, Bellomo R. Renal blood flow in experimental septic acute renal failure. Kidney Int 2006; 69: 1996-2002.
10) llai T, Tóth ML, Kovács AL. Janus-faced autophagy: a dual role of cellular self-eating in neurodegeneration?. Autophagy 2007; 3: 461-463.
11) Hsieh YC, Athar M, Chaudry IH. When apoptosis meets autophagy: deciding cell fate after trauma and sepsis. Trends Mol Med 2009; 15: 129-138.
12) Isaka Y, Kimura T, Takabatake Y. The protective role of autophagy against aging and acute ischemic injury in kidney proximal tubular cells. Autophagy 2011; 7: 1085-1087.
13) Bolisetty S, Traylor AM, Kim J, Joseph R, Ricart K, Landar A, Agarwal A. Heme oxygenase-1 inhibits renal tubular macroautophagy in acute kidney injury. J Am Soc Nephrol 2010; 21: 1702-1712.
14) Li L, Zepeda-Orozco D, Black R, Lin F. Autophagy is a component of epithelial cell fate in obstructive uropathy. Am J Pathol 2010; 176: 1767-1778.
15) Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, Englert JA, Rabinovitch M, Cernadas M, Kim HP, Fitzgerald KA, Ryter SW, Choi AM. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol 2011; 12: 222-230.
16) Carchman EH, Rao J, Loughran PA, Rosengart MR, Zuckerbraun BS. Heme oxygenase-1-mediated autophagy protects against hepatocyte cell death and hepatic injury from infection/sepsis in mice. Hepatology 2011; 53: 2053-2062.