



Nuclear factor-kappa B p50-induced microRNA-20a-3p plays a detrimental role in sepsis-induced acute kidney injury

Weihong Mao¹, Xiaoming Wang², Yongchen Zhang³, Huding Zhu^{4*}, Li Dai⁴, Juanjuan Chen⁴

¹Department of Clinical Laboratory, The People's Hospital of Danyang, Affiliated Danyang Hospital of Nantong University, Danyang, 212300, China

²Department of Clinical Laboratory, Jiangsu Cancer Hospital, Affiliated Cancer Hospital of Nanjing Medical University, Nanjing, 210009, China

³Department of Clinical Laboratory, The Second Hospital of Nanjing, Affiliated Nanjing Hospital of Nanjing University of Chinese Medicine, Nanjing, 210003, China

⁴Department of Clinical Laboratory, Danyang Hospital of Traditional Chinese Medicine, Danyang, 212300, China

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ABSTRACT

Sepsis is the most common cause of acute kidney injury (AKI). Based on microarray-based clustering analysis of miRNAs altered in the kidneys of LPS-AKI mice, miR-20a-3p was upregulated in the kidney tissues of lipopolysaccharide (LPS)-treated mice. We aimed to reveal the functions of miR-20a-3p in septic AKI by establishing the LPS-stimulated mouse model of AKI and constructing the LPS-stimulated HK-2 cells. Reverse transcription-quantitative PCR was used to quantify miR-20a-3p expression and inflammation-associated factors including MCP-1, TNF- α , and IL-6. Silencing of miR-20a-3p reduced inflammation and apoptosis in kidneys as well as alleviated AKI symptoms in mice. The LPS-induced inflammatory response and apoptosis in HK-2 cells were rescued by miR-20a-3p silence. Moreover, nuclear factor-kappa B (NF- κ B) is a transcriptional factor for miR-20a-3p to increase its expression. The binding of NF- κ B p50 and miR-20a-3p promoter was verified by ChIP assay. To sum up, miR-20a-3p is transcriptionally activated by NF- κ B p50, playing a harmful role in sepsis-induced AKI by inducing inflammation and apoptosis.

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Introduction

Acute kidney injury (AKI) is an acute disease process with diverse aetiology, complex pathogenesis, and uncertain outcomes. Sepsis is a common cause of AKI in critically ill patients (1). The development of AKI followed by sepsis increases the death risk of in-hospital patients by 6-8 folds (2, 3) and enhances the risk of chronic kidney diseases in survivors (4). Prevention of septic AKI is usually impossible (5). Blood purification and pharmacologic therapy are experimental therapies for septic AKI, but their effects were not fully ideal (6). Therefore, understanding the pathogenesis of septic AKI is important.

Regardless of species, disease stage or severity, and organs, three mechanisms including microcirculatory dysfunction, inflammation, and metabolic reprogramming were consistently observed during sepsis-associated organ injury (6). During inflammation, the expression of ion transporters is downregulated in renal tubules, and tubular solute transport is decreased (7, 8). Renal tubular cells not only are victims of inflammation but also deliver intrarenal inflammation in sepsis-associated AKI (9, 10). In this context, lipopolysaccharides (LPSs) directly interact with Toll-like receptors on renal tubule cells to increase the production of proinflammatory cytokines that induce the infiltration of leukocytes into the kidneys, leading to a vicious cycle of inflammation and tissue damage (6, 10). In sepsis-induced AKI, the raised inflammation response

was found with enhanced MCP-1, TNF- α and IL-6 levels.

MicroRNAs (miRNAs) are small noncoding RNAs with a length of 19 and 25 nucleotides, well-conserved among species, and modulate gene expression via binding target mRNAs for degradation or translational repression (11). MiRNAs are relatively easy to be detected and have great potential to regulate cellular differentiation and cell cycle, which makes them promising biomarkers in ill-defined conditions like AKI. MiRNAs as mediators in AKI were first revealed in a transgenic mouse model that exhibits renal proximal tubular-specific silencing of a miRNA-producing enzyme (12). In recent years, more and more miRNAs were identified to regulate septic AKI by suppressing inflammation or enhancing inflammation (13-15). Based on microarray-based clustering analysis of miRNAs altered in the kidneys of LPS-AKI mice (annotated as GSE172038 in GEO accession), miR-20a-3p was dramatically upregulated in the kidney tissues of LPS-treated mice. This study designed assays to reveal the upstream mechanism of miR-20a-3p and to explore its functions in septic AKI using a transgenic mouse model and an LPS-stimulated human proximal tubular cell line (HK-2).

Materials and Methods

Animals

Under a protocol approved by the Animal Ethical Committee of Danyang Hospital of Traditional Chinese Medi-

* Corresponding author. Email: zhuhuding@163.com

cine, experiments were conducted on mice. C57BL/6 mice at the age of 8–10 weeks of both sexes were purchased from Vital River (Beijing, China). Septic AKI was induced by an intraperitoneal injection of LPS in mice ($n = 24$) at a dosage of 10 mg/kg body weight (16). Control mice ($n = 8$) were treated with the same volume of normal saline by intraperitoneal injection. For silencing of miR-20a-3p, miR-20a-3p antagomir (GenePharma, Shanghai, China) was intraperitoneally administrated into mice ($n = 8$) at a dosage of 20 mg/kg one day before LPS injection (17). Mice ($n = 8$) were intraperitoneally injected with the same volume of NC antagomir to serve as the controls for mice with miR-20a-3p antagomir. Blood and renal cortex tissues were collected from mice after the modeling for 24 h. To evaluate renal function, serum creatinine and blood urea nitrogen (BUN) levels were measured using a Creatinine Assay kit (ab65340, Abcam) and a Urea Nitrogen (BUN) Colorimetric Detection Kit (EIABUN, Invitrogen).

Histology and TUNEL assay

Kidney tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin-eosin (H&E). The staining results were observed under an optical microscope (TE2000-U; Nikon, Japan) and was used for histological evaluations. A One-step TUNEL In Situ Apoptosis Kit (E-CK-A320) was used to assess apoptosis in renal tissues based on the manufacturer's instructions. TUNEL-staining pictures were photographed using fluorescence microscopy (Nikon).

Cell cultures and chemical treatment

The HK-2 cells (CRL-2190, ATCC) were cultured in DMEM containing 10% FBS at 5% CO₂, 37°C. 5 μ g/mL of LPS was used to treat HK-2 cells for 24 h to establish a sepsis model *in vitro* (18). To suppress NF- κ B, 100 μ M TPCA-1 was added concurrently with LPS. In some experiments, HK-2 cells were transfected with 200 nM miR-20a-3p inhibitor using Lipofectamine 2000 (1803712; Invitrogen) for silencing miR-20a-3p expression.

RNA isolation and reverse transcription quantitative PCR

Total RNA was isolated from the kidney cortex and HK-2 cells using the TRIzol reagent (R0016, Beyotime). PCR was conducted for measurement of MCP-1, TNF- α , and IL-6 expression using SYBR Green PCR Master Mix (4309155, Applied Biosystems) on an ABI PRISM 7500 Sequence Detection System. For miRNA expression assessment, RNA was reverse transcribed into cDNA using a miRNA Reverse Transcription kit (EZB-miRT4-S, EZBioscience), and PCR was performed using a TaqMan miRNA assay kit (Applied Biosystems). Relative RNA expression was evaluated using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with normalization to the expression of GAPDH (for mRNAs) or U6 spliceosomal RNA (for miR-20a-3p). Primers were listed as follows:

Human MCP-1, CCCAAAGAAGCTGTGATGTG, AAGGAAGAACTTCAGGCCA,
 Mouse MCP-1, CAACTCTCACTGAAGCCAG, TTAACTGCATCTGGCTGAG.
 Human TNF- α , CTCTAATCAGCCCTCTGGC, GAGG-GTTTGCTACAACATGG,
 Mouse TNF- α , TTCTCATTCTGCTTGTGG, TTGG-GAACTTCTCATCCCT.

Human IL-6, GATTCAATGAGGAGACTTGCC, TGTTCTGGAGGTACTCTAGGT,
 Mouse IL-6, TACCACTTCACAAGTCGGA, AATTGC-CATTGCACAATC.
 Human GAPDH, TCATTTCTGGTATGACAACGA, GTCTTACTCCTTGGAGGCC,
 Mouse GAPDH, GAGAGTGTTCCTCGTCCC, ACTT-TGCCACTGCAAATGG.
 Human miR-20a-3p, ACTGCATTATGAGCACT-TAAAGCG, CTCTACAGCTATATTGCCAGCCAC,
 Mouse miR-20a-3p, ACTGCATTACGAGCACT-TAAAGG, CTCTACAGCTATATTGCCAGCCAC.
 Human U6, ATACAGAGAAAGTTAGCACGG, GGAA-TGCTTCAAAGAGTTGTG,
 Mouse U6, CTCGCTTCGGCAGCACA, ACGCTTCAC-GAATTTGCGT.

Western blotting

RIPA reagent (P0013B, Beyotime) was used for extracting proteins from HK-2 cells. Protein concentration was determined using a BCA Protein Assay kit (P0011, Beyotime). Protein samples were separated with 12% SDS-PAGE gel, transferred onto PVDF membranes, and blocked in 5% skim milk for 2 h at 37°C. Next, samples were treated with primary antibodies including anti-cleaved caspase-3 (1/500, ab32042, Abcam), anti-p50 (1/2000, ab283688, Abcam), anti-p-p50 (1/200, ab28849, Abcam), and anti-GAPDH (1/5000, ab8245, Abcam) overnight at 4°C and the secondary antibody (1/1000, ab96899, Abcam) at 37°C for 2 h. GAPDH is an internal control.

ChIP assay

After fixation with 1% formaldehyde and neutralization with glycine, cell samples were sonicated to shear the DNA, and the supernatants containing DNA were harvested. Equal amounts of DNA were incubated with an anti-p50 antibody (1/30, ab283688). The immunoprecipitated products were subjected to qPCR amplification of putative NF- κ B binding sequences. The value of qPCR was normalized with input DNA.

Cell apoptosis evaluation

Cell apoptosis was assessed by double staining with Annexin V and propidium iodide (PI) followed by analysis using flow cytometry. After treatment by LPS and miR-20a-3p inhibitor, HK-2 cells were seeded in a 6-well plate, washed with PBS two times, and stained with Annexin V-FITC/PI at room temperature in the dark using an Annexin V-FITC/PI Apoptosis Detection Kit (40302ES20, YEASEN). After staining for 15 min, HK-2 cells were analyzed with flow-cytometry (Attune NxT, Invitrogen). The apoptotic rate (%) was determined as a percentage of early apoptotic cells (Q2) + a percentage of late apoptotic cells (Q3).

Statistical analysis

Data from three technical repeats are expressed as the mean \pm SD. P values were calculated from unpaired independent Student's t-test or one-way analysis of variance (ANOVA) using the GraphPad prism 8.2. The correlation between miR-20a-3p and creatine or BUN levels was determined by Spearman's correlation analysis. A $P < 0.05$ was set as the threshold to indicate statistical significance.

Results

Increase of miR-20a-3p in septic AKI in mice

Based on the GSE172038 dataset, miR-20a-3p shows dramatic upregulation in the kidneys of the LPS-induced mouse model of AKI (Figure 1A). In our study, LPS-treated mice also showed higher expression of miR-20a-3p in kidney tissues than control mice. After injecting with miR-20a-3p antagomir, renal miR-20a-3p expression was decreased (Figure 1B). LPS induced the upregulation of serum creatinine and BUN levels as well as kidney damage in mice, and these effects were rescued by miR-20a-3p antagomir (Figure 1C-E). Moreover, we identified a positive correlation between miR-20a-3p and serum creatinine or BUN levels (Figure 1F).

MiR-20a-3p silencing alleviates apoptosis and inflammation in LPS-induced AKI

A TUNEL assay was performed to reveal renal apoptosis. Renal apoptosis can be hardly seen in sham-operated mice and was observed in LPS-treated mice. MiR-20a-3p antagomir reduced renal apoptosis in an LPS-induced mouse model of AKI (Figure 2A). Expression of MCP-1, TNF-α, and IL-6 in renal tissues was increased by LPS treatment, while miR-20a-3p antagomir rescued the LPS-induced MCP-1, TNF-α, and IL-6 levels in mice (Figure 2B-D).

NF-κB mediates the upregulation of miR-20a-3p

As predicted by JASPAR (relative profile score threshold: 80%) (19), NFKB1 potentially targets the promoter regions of miR-20a-3p at site 1: GGGAGTTCAC and site 2: AGGACATTCC. A ChIP assay was conducted and verified the binding of NF-κB on both sites 1 and 2 of the

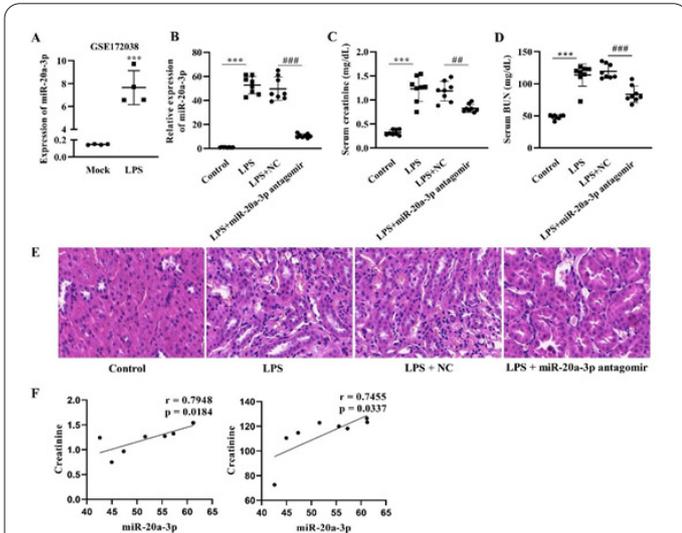


Figure 1. Increase of miR-20a-3p in septic AKI in mice. (A) Expression of miR-20a-3p in the kidneys of LPS-induced AKI in mice based on GSE172038 dataset. ***P<0.001, LPS vs. Mock group. (B) MiR-20a-3p expression in the kidneys of mice of the sham, LPS, LPS + NC antagomir, LPS + miR-20a-3p antagomir groups. ***P<0.001, LPS vs. Control group; ###P<0.001, LPS+miR-20a-3p antagomir vs. LPS+NC group. (C-D) Serum creatinine and BUN levels in mice. (E) Mice kidney H&E staining results. ***P<0.001, LPS vs. Control group; ###P<0.01, ###P<0.001, LPS+miR-20a-3p antagomir vs. LPS+NC group. (F) Correlation of miR-20a-3p level and serum creatinine and BUN levels in LPS-treated mice. There are 8 mice in each group. ***P<0.001, LPS vs. Mock group.

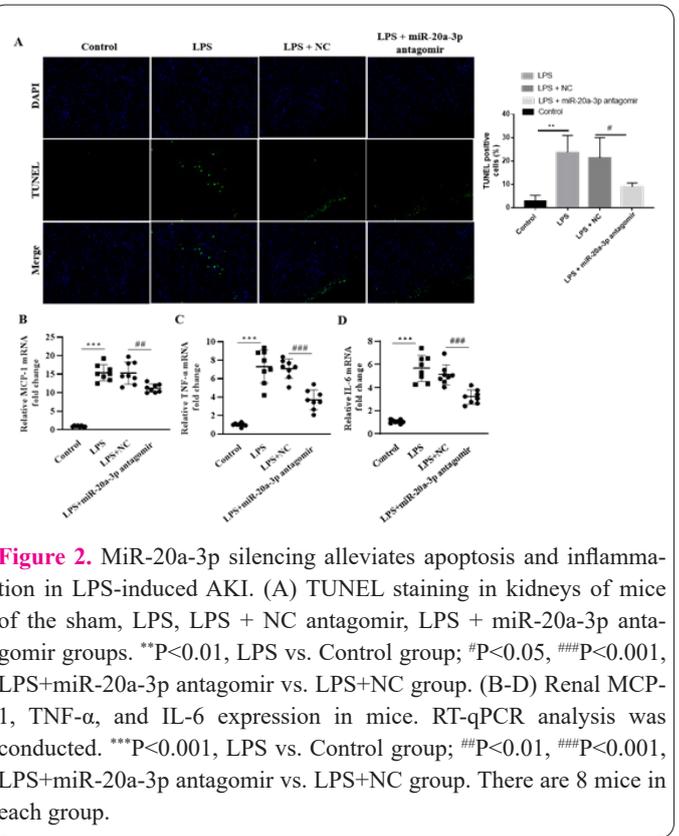


Figure 2. MiR-20a-3p silencing alleviates apoptosis and inflammation in LPS-induced AKI. (A) TUNEL staining in kidneys of mice of the sham, LPS, LPS + NC antagomir, LPS + miR-20a-3p antagomir groups. **P<0.01, LPS vs. Control group; #P<0.05, ###P<0.001, LPS+miR-20a-3p antagomir vs. LPS+NC group. (B-D) Renal MCP-1, TNF-α, and IL-6 expression in mice. RT-qPCR analysis was conducted. ***P<0.001, LPS vs. Control group; #P<0.01, ###P<0.001, LPS+miR-20a-3p antagomir vs. LPS+NC group. There are 8 mice in each group.

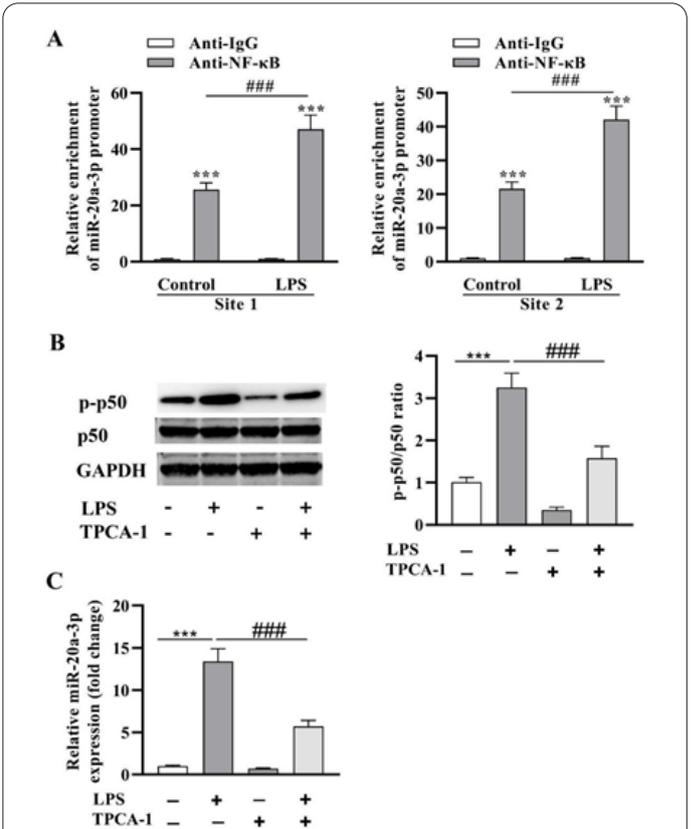


Figure 3. NF-κB mediates the upregulation of miR-20a-3p. (A) A ChIP assay followed by RT-qPCR analysis was performed for revealing the binding of NF-κB p50 on the miR-20a-3p promoter in control or LPS-stimulated HK-2 cells. ***P<0.001, Anti-NF-κB vs. Anti-IgG group; ###P<0.001, LPS vs. Control group. (B) p-p50 and p50 protein bands in HK-2 cells after treatment of LPS or (and) TPCA-1. ***P<0.001, LPS vs. Control group; ###P<0.001, LPS + TPCA-1 vs. LPS group. (C) MiR-20a-3p expression was quantified by RT-qPCR. ***P<0.001, LPS vs. Control group; ###P<0.001, LPS + TPCA-1 vs. LPS group.

miR-20a-3p promoter. LPS increased the binding of NF- κ B to sites 1 and 2 of the miR-20a-3p promoter (Figure 3A). LPS induced a higher ratio of p-p50/p50 protein and higher miR-20a-3p expression in HK-2 cells. After treatment with 100 μ M TPCA-1, p-p50 protein and miR-20a-3p expression were decreased (Figure 3B-C).

MiR-20a-3p inhibitor reduced apoptosis and inflammatory response in LPS-treated HK-2 cells

Effects of miR-20a-3p on apoptosis and inflammatory response in HK-2 cells were subsequently investigated. LPS increased cell apoptotic rate and enhanced cleaved caspase-3 expression, while miR-20a-3p inhibitor had adverse effects (Figure 4A-C). MCP-1, TNF- α , and IL-6 expression was enhanced by LPS and reduced by miR-20a-3p inhibitor in HK-2 cells. MiR-20a-3p inhibitor rescued the stimulative effects of LPS on the apoptosis and inflammatory response in HK-2 cells (Figure 4D).

Discussion

This study verified the upregulation of miR-20a-3p in the kidneys of an LPS-induced mouse model of AKI. Many studies focused on miR-20a-3p in cancers to reveal its potential to suppress or enhance cancer cell line apoptosis (20-22). MiR-20a-3p is also associated with inflammation-associated conditions, for example, increased miR-20a-3p can suppress *Ltbp2* in human immunodeficiency virus-infected individuals, causing dysregulated inflammation (23). MiR-20a-3p exhibits higher expression in the peripheral plasma of patients with Hashimoto's thyroiditis than healthy controls (24). TNF- α and IL-6 are major pro-inflammatory cytokines in sepsis (25). The bacterial clearance in septic mice was mediated by MCP-1 (26). Our findings demonstrated that miR-20a-3p silence alleviated AKI symptoms and suppressed expression of MCP-1, TNF- α and IL-6 in LPS-treated mice. The LPS-induced apoptosis in renal tissues was also suppressed by miR-20a-3p silencing. In addition, in an *in vitro* model of LPS-stimulated HK-2 cells, miR-20a-3p silence reduced cell apoptosis and suppressed the expression of inflammation-associated factors. These findings indicated that miR-20a-3p silence has a beneficial role in septic AKI by reducing apoptosis and inflammation, especially through suppressing cleaved caspase-3 and pro-inflammatory factors MCP-1, TNF- α , and IL-6.

NF- κ B has five subunits including RelA (p65), RelB, c-Rel, NF- κ B1 (p50/p105), and NF- κ B2 (p52/p100) and is a significant transcription factor for inflammatory cytokines such as IL-1 β and TNF- α in AKI (27-29). Activated NF- κ B transcriptionally increased the expression of inflammatory factors and exacerbates renal injury (30). The NF- κ B1 is an essential regulator of AKI but does not influence chronic renal injury (31). In our study, NF- κ B p50 was activated in LPS-stimulated HK-2 cells. NF- κ B1 promoted the transcription of miR-20a-3p to induce its upregulation in AKI. Inhibition of NF- κ B using TPCA-1 rescued the LPS-induced increase of miR-20a-3p expression in HK-2 cells. Intriguingly, miR-20a-3p can in turn target the NF- κ B pathway to modulate the host immune response, contributing to the mycobacterium tuberculosis infection (32), which indicates the mutual interaction between miR-20a-3p and NF- κ B pathway in inflammation-related diseases.

However, our research of miR-20a-3p is at the discove-

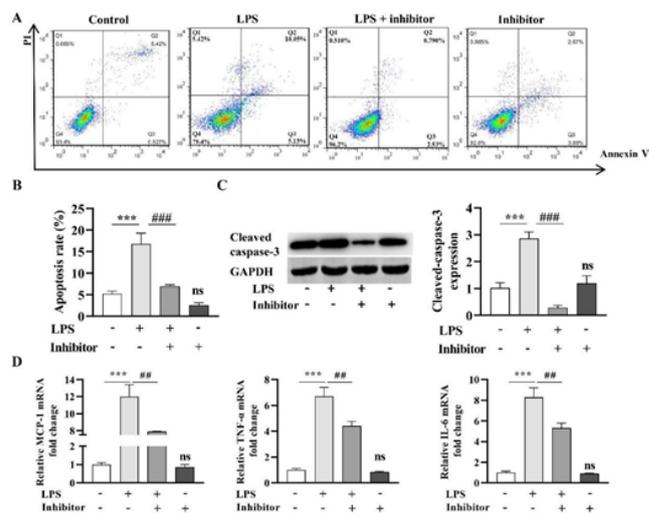


Figure 4. MiR-20a-3p inhibitor reduced apoptosis and inflammatory response in LPS-treated HK-2 cells. (A) Flow cytometry results of Annexin V/PI staining in HK-2 cells of the control, LPS, miR-20a-3p inhibitor, and LPS + miR-20a-3p inhibitor groups. (B) Quantitative results of the apoptotic cells. *** P <0.001, LPS vs. Control group; ### P <0.001, LPS + Inhibitor vs. LPS group. (C) Cleaved caspase 3 protein band in HK-2 cells. *** P <0.001, LPS vs. Control group; ### P <0.001, LPS + Inhibitor vs. LPS group. (D) MCP-1, TNF- α , and IL-6 expression in HK-2 cells. RT-qPCR analysis was conducted. *** P <0.001, LPS vs. Control group; ## P <0.01, ### P <0.001, LPS + Inhibitor vs. LPS group.

ry phase, and further studies in patients with septic AKI is needed to confirm the upregulation of sepsis-induced miR-20a-3p and to verify our findings. The location of miR-20a-3p in renal tissues needs to be measured by RNA-fluorescence in situ hybridization assay. Importantly, the downstream targets of miR-20a-3p in septic AKI will be studied in future experiments.

In conclusion, NF- κ B p50 increased miR-20a-3p expression in septic AKI at the transcriptional level. MiR-20a-3p plays a detrimental role in sepsis-induced AKI by regulating apoptosis and inflammation. This study is beneficial for understating the pathogenesis of septic AKI and developing corresponding molecular targeted therapy.

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Interest conflict

Conflict of interest exists when an author (or the author's institution) has financial or personal relationships that inappropriately influence (bias) his or her actions (such relationships are also known as dual commitments, competing interests, or competing loyalties).

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this

published article

Authors' contribution

All authors had equal roles in study design, work, statistical analysis and manuscript writing.

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Ethics approval and consent to participate

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