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Inhibition of microRNA-543 alleviates sepsis-induced acute kidney injury *via* targeting Bcl-2

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Abstract. – OBJECTIVE: Sepsis has a high morbidity and mortality and is prone to cause acute kidney injury (AKI). Here, we aimed to demonstrate the function and molecular mechanism of microRNA-543 (miR-543) in septic AKI.

MATERIALS AND METHODS: MiR-543 inhibitor or NC was transfected into LPS-treated HK-2 cells to observe lipopolysaccharide (LPS)-induced inflammation and apoptosis. The detection of inflammation and apoptosis of HK-2 cells relies on Western blot, quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR), enzyme-linked immunosorbent assay (ELISA), Cell Counting Kit-8 (CCK-8) assay, flow cytometry, and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining.

RESULTS: MiR-543 expression was increased in LPS-treated HK-2 cells. By transfecting miR-543 inhibitor into HK-2 cells, miR-543 expression was dramatically reduced. The downregulation of miR-543 remarkably inhibited the inflammation and apoptosis, which was manifested by the reduction of inflammatory cytokines (TNF-α, IL-6, IL-1β), the reversal of apoptosis-related proteins expression (Bcl-1, Bax), the increase of cell viability and the decrease of the proportion of apoptotic cells. The result of Luciferase activity assay demonstrated that miR-543 directly targets Bcl-2.

CONCLUSIONS: MiR-543 expression was increased in LPS-treated HK-2 cells, and silencing miR-543 could inhibit LPS-induced inflammation and apoptosis in HK-2 cells *via* targeting Bcl-2.

Key Words:
Sepsis, Acute kidney injury, MiR-543, Bcl-2, Lipopolysaccharide, Inflammation, Apoptosis.

Introduction

Sepsis is a systemic inflammatory response syndrome induced by infection. Uncontrolled inflammation can cause multiple organ dysfunction syndrome and affect the clinical prognosis of patients[1]. It has a high morbidity and mortality and is the most common cause of acute kidney injury (AKI). Studies[2-4] have shown that among sepsis patients, the incidence of AKI is 40%-50%, and the hospitalization mortality of sepsis patients with AKI can increase by 6-8 times, and the long-term risk of chronic kidney disease and cardiovascular death also increases correspondingly. Septic AKI is characterized by acute renal failure, which is characterized by inadequate blood filtration, imbalance of water and electrolytes, and disturbance of urine production. Since the pathogenesis of septic AKI has not been fully elucidated, symptomatic supportive treatment is currently the main clinical treatment for patients with septic AKI, so it is of great theoretical value and clinical significance to explore the pathogenesis of septic AKI and develop a new therapeutic approach based on this target.

MicroRNA (miRNA) is a type of endogenous, single-chain, non-coding RNA found in eukaryotes that participates in regulating various physiological processes in cells at the post-transcriptional level[5]. MiRNA has strong tissue specificity, is easy to amplify the signal pathway, and has an average half-life of up to 5 days[6]. It can be repeatedly frozen and thawed and exists in blood and other body fluids in a very stable form[7]. Its clinical detection is highly operable. MiRNA is widely involved in

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the pathogenesis of AKI, including apoptosis, inflammatory response, ischemia/reperfusion (I/R) injury, angiogenesis, fibrosis repair and so on, which are closely related to the occurrence, development, and prognosis of AKI[8-10]. Currently known miRNAs related to AKI are miR-21, miR-24, miR-92a, miR-122, miR-126, miR205, miR-494 and so on. They are involved in the regulation of the occurrence, development and prognosis of AKI caused by different etiologies, and are of great significance to clinicians' early intervention and treatment.

MiR-543 is an evolutionarily conserved miRNA between species. MiR-543 has been extensively studied in tumors, but has been poorly studied in other diseases, especially septic AKI. In this paper, we constructed an *in vitro* model of septic AKI by LPS to study the function of miR-543 in inflammation and apoptosis in septic AKI. Our results suggested that miR-543 could be a potential therapeutic target for septic AKI.

Materials and Methods

Cell Culture

HK-2 cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were cultured in complete medium composed of Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F-12; Gibco, Rockville, MD, USA) and 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA). The incubator used to culture HK-2 cells was constant at 37°C and contained 5% CO_2. When the cells grow to 70%-80% confluence, the cells were passaged with 0.25% trypsin digestion. To build an *in vitro* model of septic AKI, HK-2 cells were treated with 500 ng/mL lipopolysaccharide (LPS).

Cell Transfection

According to the protocols, miR-543 inhibitor, negative control (NC) inhibitor (RiboBio, Guangzhou, China), miR-543 mimic and NC mimic were transfected into HK-2 cells using Transfection Kit (RiboBio, Guangzhou, China).

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA of HK-2 cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 20 μL of diethyl pyrocarbonate (DEPC)-treated Water (Beyotime, Shanghai, China) was used to dissolve the total RNA and stored at -80°C. 1 μL of RNA sample was took to measure the optical density (OD) value on the UV-1750 ultraviolet spectrophotometer and calculate the RNA concentration. The ratio of OD260/OD280 is between 1.8 and 2.0, indicating that the prepared RNA has high purity. To obtain a miRNA complementary deoxyribose nucleic acid (cDNA) library and a mRNA cDNA library, reverse transcription was performed using miRNA 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) and HiScript™ 1st strand cDNA synthesis kit (Vazyme, Nanjing, China) in accordance with the protocols, respectively. The amplification reaction was performed Prism 7900 System using miRNA Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). 20 μL reaction system: 0.4 μL of Forward Primer (10 μM), 0.4 μL of Reverse Primer (10 μM), 10 μL of 2 × miRNA Universal SYBR qPCR Master Mix, 8.2 μL of ribonase-free water, 1 μL of cDNA template. U6 was the internal control of miR-543, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal control of mRNAs. All the primers were listed in Table I.

Table I. Real-time PCR primers.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
Bax	CAGTTGAAGTTGCCATCAGC	CAGTTGAAGTTACCATCAGC
Bcl-2	GACTGAGTACCTGAACCGGCATC	CTGAGCAGCGTCTTCAGAGACA
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
IL-6	ACAGAAGGAGTGGCTAAGGA	AGGCATAACGCACTAGGTTT
IL-1β	TTGAGTCTGCCCAGTTCC	TTTCTGCTTGAGAGGTGCT
miR-543	GAAACATTCGCGGTGCA	GAGAGGAGAGGAAGAGGGAA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative Reverse-Transcription Polymerase Chain Reaction.

Western Blot

Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) was utilized to isolate total protein of HK-2 cells and protein concentration was measured using the bicinchoninic acid (BCA) method. 5 × sodium dodecyl sulphate (SDS) protein loading buffer (at a ratio of 1:4) (Beyotime, Shanghai, China) was added to the protein sample, and cooked at 100°C for 5 to 10 minutes and stored at -20°C for later use. The proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), then, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After that, the membranes were incubated with 5% skimmed milk for 1-2 hours. Then, the membranes were incubated with primary antibodies (TNF-α, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IL-6, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IL-1β, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Bax, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Bcl-2, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; GAPDH, Abcam, Cambridge, MA, USA, Rabbit, 1:1000) at 4°C overnight. Subsequently, the membrane was incubated with secondary antibody (Abcam, Cambridge, MA, USA, Rabbit, 1:5000) for 2 hours. Blots were developed with Super enhanced chemiluminescence (ECL) Detection Reagent (YEASEN, Shanghai, China). The integrated optical density value (IOD) of the blots was measured with Gel-pro software.

Enzyme-Linked Immunosorbent Assay (ELISA) Assay

The HK-2 cell supernatant was collected and centrifuged with a centrifugal force of 200 g for 5 minutes to remove cell debris. The content of inflammatory cytokines (TNF-α, IL-6, IL-1β) in the cell supernatant were detected by corresponding ELISA detection kits (Bestbio, Shanghai, China).

Flow Cytometry

Trypsin Solution without EDTA (ethylenediaminetetraacetic acid; Beyotime, Shanghai, China) was used to harvest HK-2 cells. Then, the cells were washed 3 times using phosphate-buffered saline (PBS) and collected by centrifugation. Then, the cells were resuspended in 100 μL of Binding Buffer, and 5 μL of Annexin V-FITc (KeyGen, Shanghai, China) and PI (KeyGen, Shanghai, China) were added in the dark. The apoptosis rate was analyzed by flow cytometry.

Cell Counting Kit-8 (CCK-8) Assay

2000 HK-2 cells were placed to each well of the 96-well plates with 100 μL complete medium. After the cells were treated in line with the experimental requirements, 10 μL of CCK-8 solution (Beyotime, Shanghai, China) was added to each well and continue to incubate for 2 hours in the cell incubator. The absorbance at 450 nm was detected by a spectrophotometer.

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick End Labeling (TUNEL) Staining

HK-2 cells were first fixed using 4% paraformaldehyde. The cell membrane was disrupted with 0.1% Triton X-100 for 20 minutes. Then, the cells were incubated with the prepared TUNEL reagent (Roche, Basel, Switzerland) at 37°C for 1 hour. After that, 4',6-diamidino-2-phenylindole (DAPI) (Roche, Basel, Switzerland) was used to stain the nucleus. The TUNEL positive cells were observed with a fluorescence microscope.

Luciferase Activity Assay

Luciferase reporter plasmids (RiboBio, Guangzhou, China) containing wild-type (WT) or mutant (MUT) 3'UTR of Bcl-2 mRNA were constructed. Then, miR-543 mimic or NC mimic were co-transfected into HEK293T cells with WT plasmids or MUT plasmids using Lipofectamine® 3000 (Thermo Fisher Scientific, Waltham, MA, USA). Luciferase activities were detected using the Dual-Luciferase Reporter Assay System kit (YEASEN, Shanghai, China) according to the instructions.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). Data were represented as mean ± Standard Deviation (SD). The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated the significant difference.

Results

MiR-543 Expression Was Increased in LPS-Treated HK-2 Cells

HK-2 cells were treated with LPS at the concentration of 500 ng/ml. By RT-PCR analysis, miR-543 expression was significantly increased in HK-2 cells treated with LPS (Figure 1A). After transfection with miR-543 inhibitor, the level of miR-543 in HK-2 cells was markedly reduced (Figure 1B).

Downregulation of MiR-543 Inhibited LPS-Induced Inflammation of HK-2 Cells

After using miR-543 inhibitor to inhibit miR-543 expression in HK-2 cells, we detected the expression of TNF-α, IL-6 and IL-1β in the cells by Western blot (Figure 2A). Compared with the control group, the expression of three inflammatory cytokines in the LPS group was greatly increased. However, the downregulation of miR-543 notably reduced their expression. The mRNA levels of the three inflammatory cytokines were also detected, and the results were consistent with protein levels (Figure 2B). Compared with the LPS + NC group, it was found that the contents of these three inflammatory cytokines in the cell supernatant of the LPS + inhibitor group was remarkably reduced by using the ELISA detection kits (Figure 2C-2E).

Downregulation of MiR-543 Inhibited LPS-Induced Apoptosis of HK-2 Cells

First, Western blot was used to detect the expression of apoptosis-related proteins (Bcl-2, Bax) in HK-2 cells (Figure 3A). LPS treatment dramatically reduced Bcl-2 expression and increased Bax expression in HK-2 cells. However, downregulation of miR-543 reversed these effects. Downregulation of miR-543 also reversed the decrease in cell viability caused by LPS (Figure 3B). And, consistent with protein expression results, silencing miR-543 can restore Bcl-2 mRNA and Bax mRNA expression (Figure 3C). In addition, inhibition of miR-543 significantly reduced LPS-induced increase in apoptosis of HK-2 cells (Figure 3D and 3E).

Downregulation of MiR-543 Inhibited LPS-Induced Inflammation and Apoptosis by Targeting Bcl-2

We first used the StarBase database to predict the possible binding sites of miR-543 and Bcl-2 (Figure 4A). Through Western blot, we found that Bcl-2 expression was negatively correlated with the level of miR-543 (Figure 4B). Finally, the Luciferase activity assay proved that miR-543 directly targets Bcl-2 (Figure 4C).

Discussion

Sepsis is a common clinical syndrome in critically ill patients, with a high case fatality rate and poor clinical prognosis. Although great progress has been made in anti-infective therapy, the mortality rate from sepsis is still high[11]. AKI is a common clinical syndrome characterized by rapid decline in renal function caused by multiple etiologies and pathological mechanisms. AKI is a common and critically

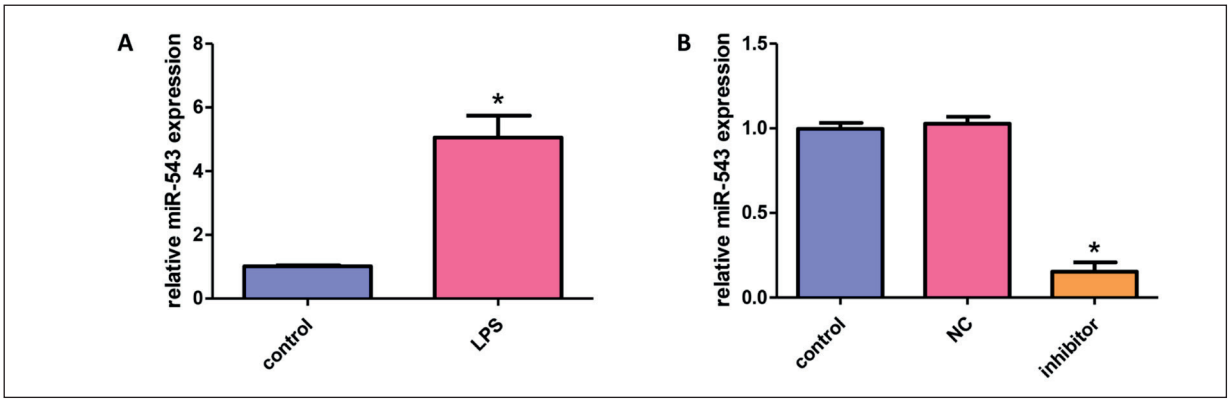

Figure 1. MiR-543 expression was increased in LPS-treated HK-2 cells. **A,** MiR-543 expression in LPS-treated HK-2 cells was detected by RT-PCR ("*" $p<0.05$ *vs.* control, n = 3). **B,** MiR-543 expression in HK-2 cells transfected with miR-543 inhibitor was detected by RT-PCR ("*" $p<0.05$ *vs.* NC, n = 3).

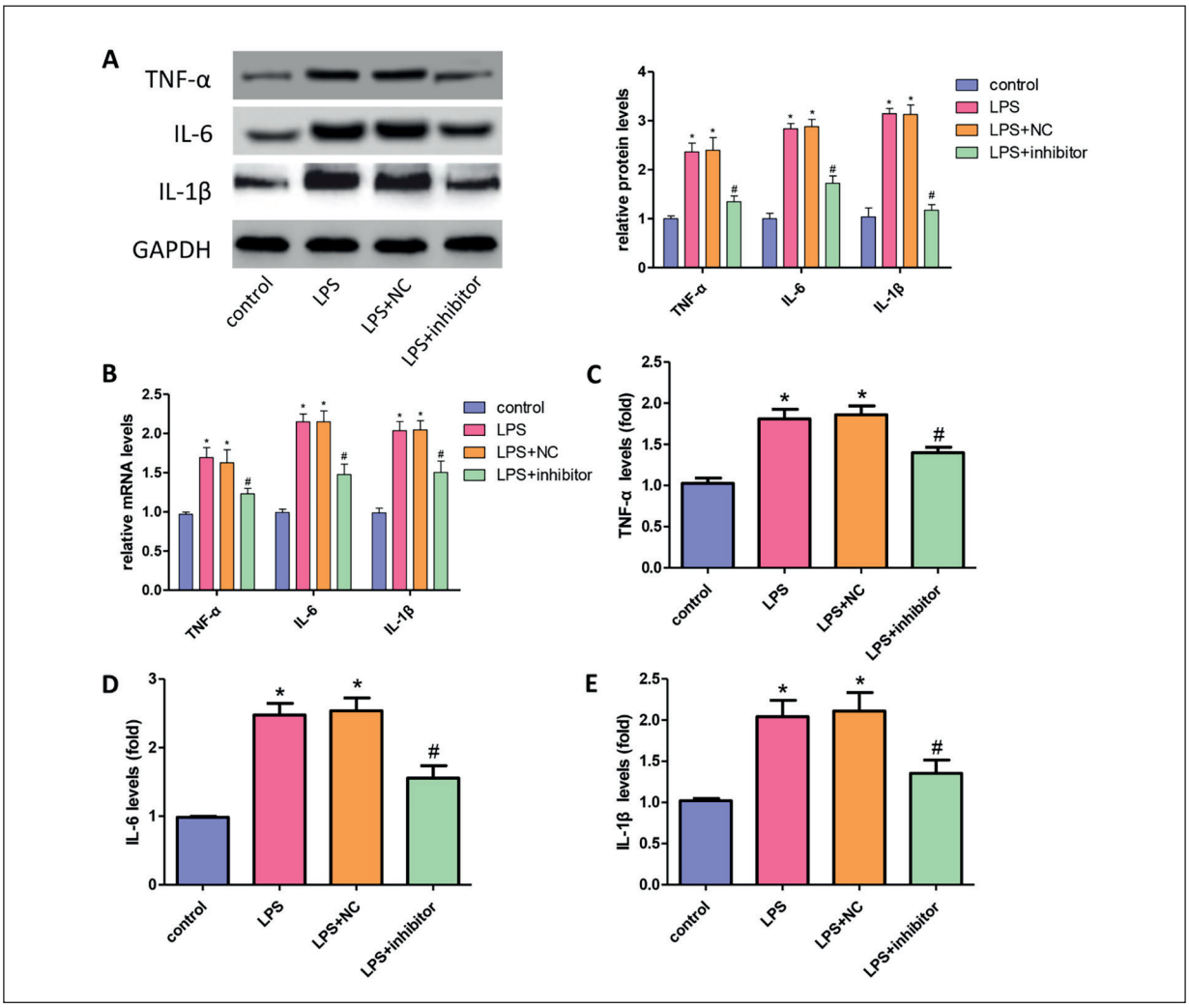

Figure 2. Downregulation of miR-543 inhibited LPS-induced inflammation of HK-2 cells. **A,** The expression of TNF-α, IL-6, IL-1β was detected using Western blot ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **B,** The expression of TNF-α mRNA, IL-6 mRNA, IL-1β mRNA was detected using RT-PCR ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **C,** The contents of TNF-α in the cell supernatant were detected using ELISA detection kit ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **D,** The contents of IL-6 in the cell supernatant were detected using ELISA detection kit ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **E,** The contents of IL-1β in the cell supernatant were detected using ELISA detection kit ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3).

ill disease in the clinic, and the mortality rate in clinical intensive care patients is as high as 70%, while 46% to 48% AKI is caused by sepsis[12]. Sepsis caused by bacterial LPS is a common cause of AKI.

The clinical complications of patients with sepsis complicated with AKI are far more than those with sepsis without AKI and non-septic AKI patients[13]. In patients with sepsis, inflammation induces cell hypoxia and oxidative stress, which further leads to changes in renal hemodynamics, renal blood perfusion insufficiency, and cytokines and chemokines mediate injury and apoptosis of renal tubular cells, eventually leading to kidney injury and the induction of AKI. Sepsis triggers a systemic cytokine-chemokine response. Inflammatory cytokines in the circulatory system can stimulate the endothelial capillary endothelium of the renal tubules, resulting in increased expression of endothelial adhesion molecules. These adhesion molecules can activate white blood cells and produce a vicious cycle in the inflammatory response[14]. In addition, activated leukocytes may stimulate the release of pro-inflammatory cytokines, thereby damaging microvessels and renal tubules[15].

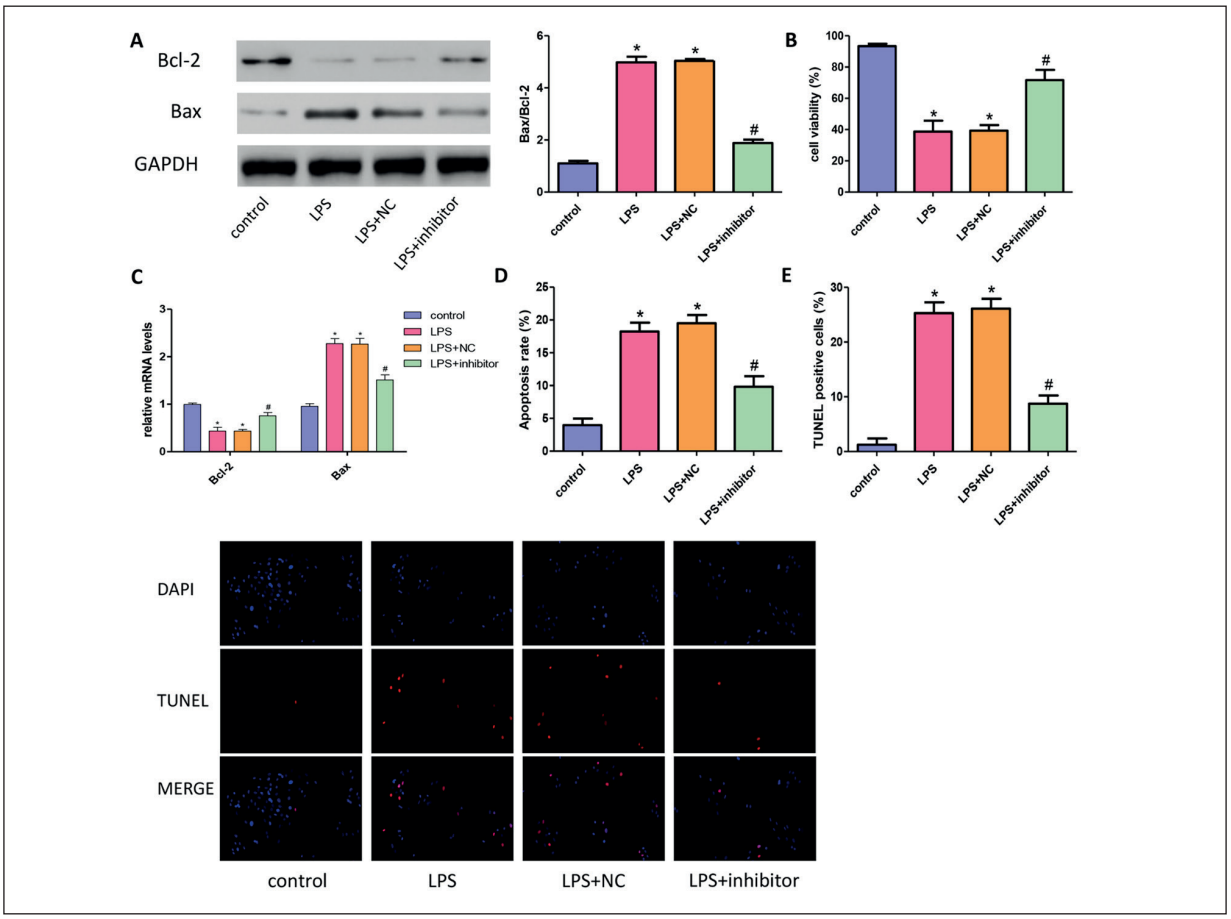

Figure 3. Downregulation of miR-543 inhibited LPS-induced apoptosis of HK-2 cells. **A,** The expression of Bcl-2 and Bax was detected using Western blot ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **B,** The cell viability was detected by CCK-8 assay ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **C,** The expression of Bcl-2 mRNA and Bax mRNA was detected using RT-PCR ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **D,** Apoptosis rate was detected by flow cytometry ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **E,** Apoptosis was observed by TUNEL staining (magnification: 200×) ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3).

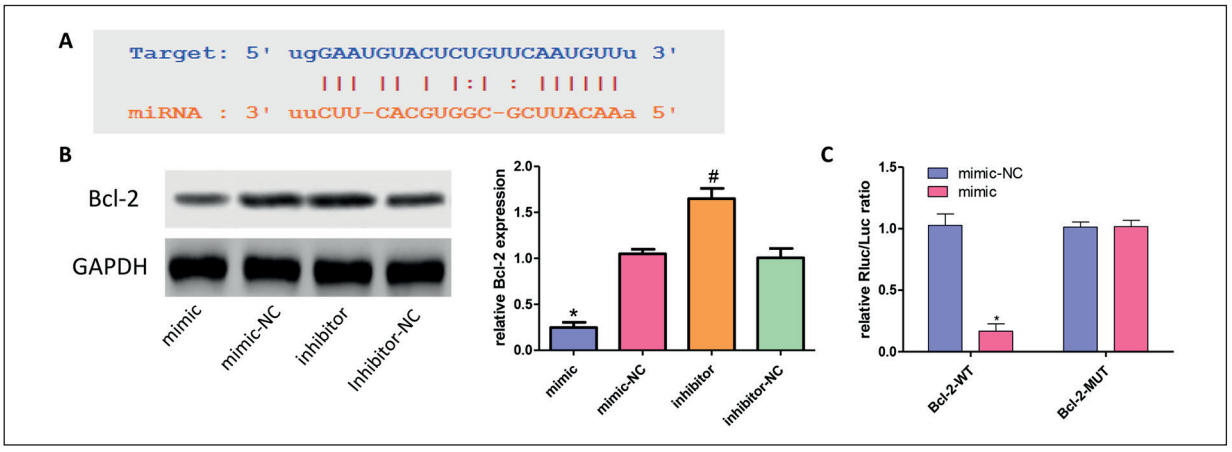

Figure 4. Downregulation of miR-543 inhibited LPS-induced inflammation and apoptosis by targeting Bcl-2. **A,** Binding site predicted by the Starbase database. **B,** The expression of Bcl-2 was detected using Western blot ("*" $p<0.05$ *vs.* mimic-NC, "#" $p<0.05$ *vs.* inhibitor-NC, n=3). **C,** MiR-543 overexpression significantly decreased the Luciferase activity in WT group, but did not decrease the Luciferase activity in MUT group ("*" $p<0.05$ *vs.* mimic-NC, n = 3).

Normal apoptosis is very important to maintain the integrity of cells and the homeostasis of the environment. In septic AKI, due to the pathological stimulation of the kidney, the apoptosis is disordered, resulting in excessive cell death and organ dysfunction.

MiRNAs, short-chain non-coding RNAs, play an important regulatory role in the occurrence and progression of septic AKI[10]. Fu et al[16] found that upregulation of miR-21 could protect kidney cell apoptosis in septic AKI. Zheng et al[17] showed that propofol could relieve septic AKI by regulating the expression of miR-290-5p. In this study, we found that treatment of HK-2 cells with LPS could induce inflammatory cytokines levels and promote apoptosis. In addition, miR-543 was upregulated in LPS-treated HK-2 cells. However, silencing miR-543 markedly reduced the levels of inflammatory cytokines and inhibited apoptosis of HK-2 cells *in vitro*.

Conclusions

The findings in this paper illustrated that restoring the expression of miR-543 could protect against LPS-induced inflammation and apoptosis in an *in vitro* model of AKI by targeting Bcl-2. However, the lack of *in vivo* functional verification of miR-543 was one of the limitations of our study. Although the results of this paper show a promising future treatment for septic AKI, more research is needed to further study the function and molecular mechanism of miR-543. Altogether the above results revealed that miR-543 expression was remarkably increased in septic AKI, and inhibition of miR-543 could inhibit LPS-induced inflammation and apoptosis of HK-2 cells via targeting Bcl-2.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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