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Effect of miR-132 on lung injury in sepsis rats *via* regulating Sirt1 expression

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Abstract. – OBJECTIVE: The aim of this study was to explore the effects of the expressions of micro ribonucleic acid (miR)-132 and sirtuin1 (Sirt1) on lung injury in sepsis rats, and to elucidate the regulatory relation between miR-132 and Sirt1.

MATERIALS AND METHODS: The model of sepsis-induced lung injury was successfully established in rats *via* injection of lipopolysaccharide (LPS) into the caudal vein (model group). Before modeling, the rats were infused with miR-132 antagomir *via* the trachea (miR-132 antagomir group) or intraperitoneally injected with the Sirt1 activator (SRT1720) (SRT1720 group). Meanwhile, the rats injected with an equal volume of normal saline *via* the caudal vein were enrolled in the control group. The expressions of the inflammatory factors interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-α (TNF-α) were determined using enzyme-linked immunosorbent assay (ELISA). Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) and Western blotting were applied to detect gene and protein expressions in lung tissues, respectively. Targeted relationship between miR-132 and Sirt1 was explored using Luciferase reporter assay. In addition, tissue sections of the right lung were stained with hematoxylin-eosin (HE) to observe the degree of lung injury.

RESULTS: The model of sepsis-induced lung injury was successfully established in rats by LPS. The results showed that the expressions of IL-6, IL-1β, TNF-α and miR-132 rose significantly in lung tissues ($p<0.01$), whereas the expression of Sirt1 significantly declined ($p<0.01$). Lung injury was alleviated by miR-132 antagomir and SRT1720. Both miR-132 antagomir and SRT1720 significantly reduced the expressions of miR-132, IL-6, IL-1β and TNF-α ($p<0.01$). However, the expression of Sirt1 was remarkably upregulated in rats with lung injury ($p<0.01$). Luciferase reporter gene assay indicated that miR-132 regulated Sirt1 in a targeted manner.

CONCLUSIONS: MiR-132 may cause lung injury in sepsis rats by regulating the expression of Sirt1.

Key Words:
MiR-132, Sirt1, Rats, Lung injury, Sepsis.

Introduction

Sepsis is a serious disease and a major cause of death worldwide. It triggers systemic inflammation, but the underlying mechanism remains unclear[1]. Sepsis is characterized by enhanced systemic inflammatory response to severe infection, which is usually accompanied with spiking fever and septic shock, as well as respiratory and organ failure[2]. In developed countries, the mortality rate of sepsis is 1 per 1000 people[3], accounting for approximately 2% of all hospitalized patients[4,5]. Sepsis causes acute lung injury, eventually leading to respiratory dysfunction featured by acute hypoxemic respiratory failure with bilateral pulmonary infiltration. Current statistics have shown that its mortality rate is over 38%[6]. Hence, exploring the molecular mechanism of sepsis-induced lung injury is of significant importance.

Micro ribonucleic acids (miRNAs) are a class of small, 18-nucleotide-long to 28-nucleotide-long, noncoding RNA molecules. They have been found to play critical roles in innate and adaptive immunity of pathological diseases, such as atherosclerosis, rheumatoid arthritis, diabetes mellitus, and bacterial infections[7]. Many scholars[8] have suggested that miRNAs directly target tumor necrosis factor (TNF) pathway, which are major regulators of the pro-inflammatory process in sepsis. Although reports on the expression of miR-132[9,10] and the role of Sirt1[11,12] in sepsis have been published, whether miR-132 has a regulatory effect on Sirt1 and their potential mechanisms in sepsis-induced lung injury have not been fully elucidated. In this study, the regulatory effect of miR-132 on Sirt1 was analyzed, and, their mechanisms in sepsis-induced lung injury were

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investigated. Our findings might help to provide effective molecular targets for potential treatment methods.

Materials and Methods

Main Materials

Sprague-Dawley rats and 293T cell lines were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China); Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA); SYBR Green Real-Time PCR Master Mix was purchased from TaKaRa (Komatsu, Japan); lipopolysaccharide (LPS) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA); TRIzol was purchased from Invitrogen (Carlsbad, CA, USA) and miRNA RT kit was purchased from Applied Biosystems (Foster City, CA, USA), Sirt1 antibody, Bax antibody, β-actin antibody and HRP-labeled anti-rabbit secondary antibodies were purchased from (Abcam, Cambridge, MA, USA) and Luciferase reporter assay system was purchased from Promega (Madison, WI, USA), and bioluminescent plate reader from Modulus™ (Scottsdale, AZ, USA).

Establishment of Rat Model of Lung Injury

This study was approved by the Animal Ethics Committee of Zibo Central Hospital Animal Center. Rats weighing 180-200 g were randomly divided into two groups, including: model group and control group. In the model group, the rats were first deprived of food for 8 h before modeling. Subsequently, they were anesthetized *via* intraperitoneal injection of 0.75% pentobarbital, fixed in a supine position, and injected with LPS (10 mg/kg) *via* the caudal vein. After that, the model of sepsis-induced lung injury was successfully established in rats. In the control group, the rats were injected with an equal volume of normal saline, and blood samples and lung tissues were harvested after 24 h. At 24 h before modeling, the rats were infused with 50 μL of NC antagomir or miR-132 antagomir (0.4 μg/μL) *via* the trachea (miR-132 antagomir group), or intraperitoneally injected with the Sirt1 activator (SRT1720, 20 mg/kg) (SRT1720 group).

293T Cell Culture and Luciferase Reporter Gene Assay

293T cells were resuscitated with DMEM containing 10% FBS, and cultured in an incubator with 5% CO_2 at 37°C. When the cells covered 90% of the plate bottom, they were washed twice with phosphate buffered saline (PBS) and digested with 0.25% trypsin, followed by cell passage (1:6). Subsequently, the cells in each group were transfected with miR-132 mimics or miR-NC and Sirt1-WT or Sirt1-MUT for 48 h. After discarding the medium, the cells were washed with PBS for 3 times, and lysed with 50 μL of newly-prepared lysis buffer for 30 min. Next, 10 μL of lysate was taken and added with 100 μL of luciferase assay reagent prepared already. Luciferase activity was finally measured using the Luciferase reporter gene assay system and bioluminescent plate reader. Each assay was repeated independently for 3 times, with 3 parallel controls.

Collection of Serum and Tissues

At 24 h after modeling, blood samples were drawn and centrifuged. The supernatant was collected to determine the expressions of inflammatory factors interleukin-6 (IL-6), IL-1β and tumor necrosis factor-α (TNF-α) using enzyme-linked immunosorbent assay (ELISA). After the rats were sacrificed, lung tissues were quickly taken. Some of collected tissues were fixed in formalin, while some were frozen in liquid nitrogen for later use.

RNA Extraction and qRT-PCR

The sequences of IL-6, IL-1β, TNF-α, Sirt1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), miR-132 and U6 were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and microRNA database (http://www.targetscan.org/). The primers used in this study were designed by Primer Premier 6.0 and synthesized by Sangon (Shanghai, China) (Table I). Total RNA in tissues and cells was extracted using TRIzol reagent. The concentration of extracted RNA was detected using a NanoDrop spectrophotometer. 5 ng of total RNA was synthesized into complementary deoxyribose nucleic acid (cDNA) using the miRNA RT kit for miR-132 and U6. Meanwhile, total RNA was synthesized into cDNA using the random primers of RT Master Mix for IL-6, IL-1β, TNF-α, Sirt1 and GAPDH. QRT-PCR was performed according to the manufacturer's instructions of SYBR Green Real-Time PCR Master Mix and ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The transcription level was evaluated using the cycle

Table I. Primer sequences of IL-6, IL-1β, TNF-α, Sirt1, GAPDH, miR-132, and U6.

Gene	Sequence
IL-6 F	5'-AGCGATGATGCACTGTCAGA-3'
IL-6 R	5'-GGAACTCCAGAAGACCAGAGC-3'
IL-1β F	5'-CACCTCTCAAGCAGAGCACA-3'
IL-1β R	5'-ACGGGTTCCATGGTGAAGTC-3'
TNF-α F	5'-CATCCGTTCTCTACCCAGCC-3'
TNF-α R	5'-AATTCTGAGCCCGGAGTTGG-3'
miR-132 F	5'-ATTAACAAGGAAGCAAAGGTCT-3'
miR-132 R	5'-CACAGCTCGTAGAACAGGAGG-3'
Sirt1 F	5'-ATCTCCCAGATCCTCAAGCCA-3'
Sirt1 R	5'-TTCCACTGCACAGGCACATA-3'
U6 F	5'-CTCGCTTCGGCAGCACA-3'
U6 R	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH F	5'-AGTGCCAGCCTCGTCTCATA-3'
GAPDH R	5'-GACTGTGCCGTTGAACTTGC-3'

threshold (Ct), and gene expression normalized to endogenous references was determined using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

Total protein was extracted from tissues and cell lines, and its concentration was measured using the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). After separation *via* 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), total proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. After sealing with 5% skim milk powder and 0.1% tris-buffered saline with Tween-20, the membranes were incubated with Sirt1 and β-actin primary antibodies at 4°C overnight. On the next day, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies. Immuno-reactive bands were finally exposed by enhanced chemiluminescence (ECL) reagent, with β-actin as an internal control.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was used for data processing. Experimental data in different groups were expressed as mean ± standard deviation ($\bar{x} \pm s$). Independent-samples *t*-test was performed for intergroup comparison. $p<0.05$ was considered statistically significant.

Results

Serological Detection After Modeling

The expressions of serum IL-6, IL-1β and TNF-α were detected *via* ELISA. As shown in Figure 1, LPS significantly upregulated the expressions of IL-6, IL-1β and TNF-α compared with control group ($p<0.01$).

RNA Expression in Lung Tissues After Modeling

The expression of genes in lung tissues after modeling was determined using qRT-PCR. As shown in Figure 2, LPS significantly increased miR-132 expression ($p<0.01$) but reduced Sirt1 expression ($p<0.01$) compared with control group.

Effect of MiR-132 Antagomir on Lung Injury in Sepsis Rats

QRT-PCR results indicated that miR-132 antagomir could evidently lower the expression of miR-132 in normal rats ($p<0.05$) (Figure 3A). LPS

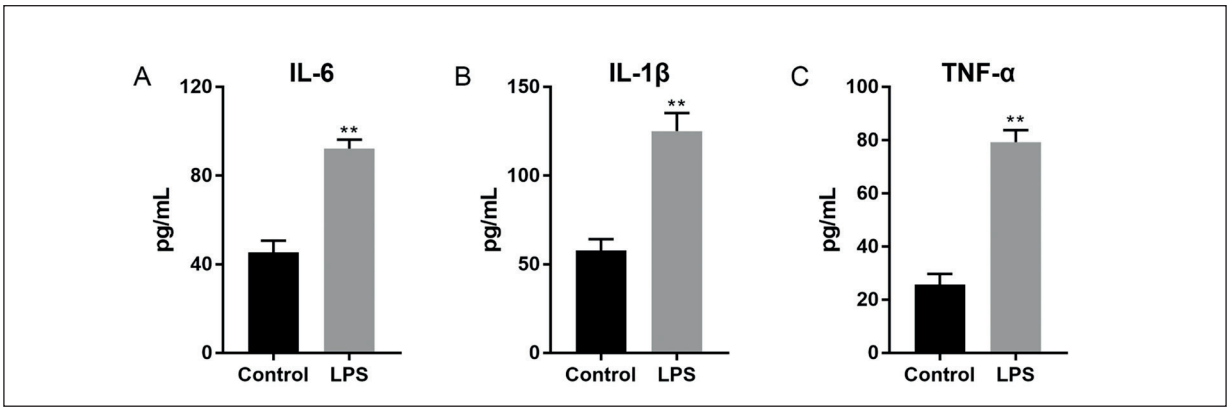

Figure 1. Expressions of serum IL-6, IL-1β and TNF-α detected *via* ELISA. **A,** IL-6 expression. **B,** IL-1β expression. **C,** TNF-α expression. $^{**}p<0.01$: a significant difference between the two groups.

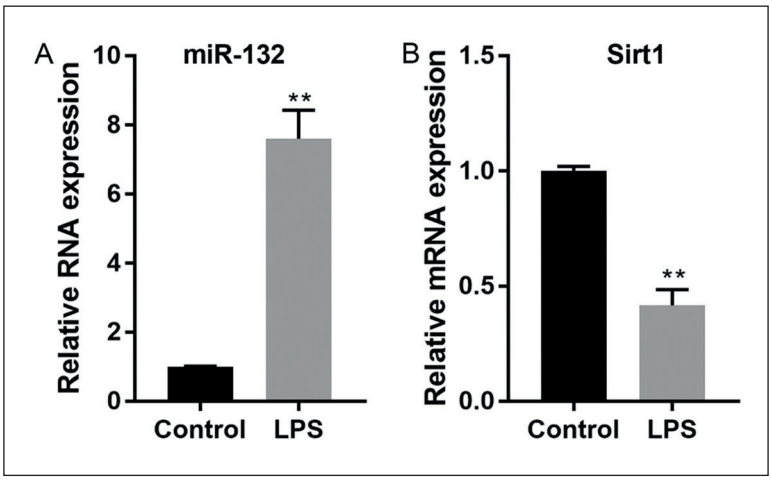

Figure 2. MiR-132 and Sirt1 expressions detected *via* qRT-PCR. **A,** MiR-132 expression. **B,** Sirt1 expression. **p<0.01: a significant difference between the two groups.

could significantly reduce the mRNA expression of Sirt1 (p<0.01) (Figure 3B), but increase the mRNA expressions of miR-132, IL-6, IL-1β and TNF-α (p<0.01) (Figure 3C-3E). However, miR-132 antagomir showed the opposite effects.

Targeted relation Between MiR-132 and Sirt1

The targeted regulatory relationship between miR-132 and Sirt1 was predicted, and the targeting sequences were shown in Figure 4A. Wild-

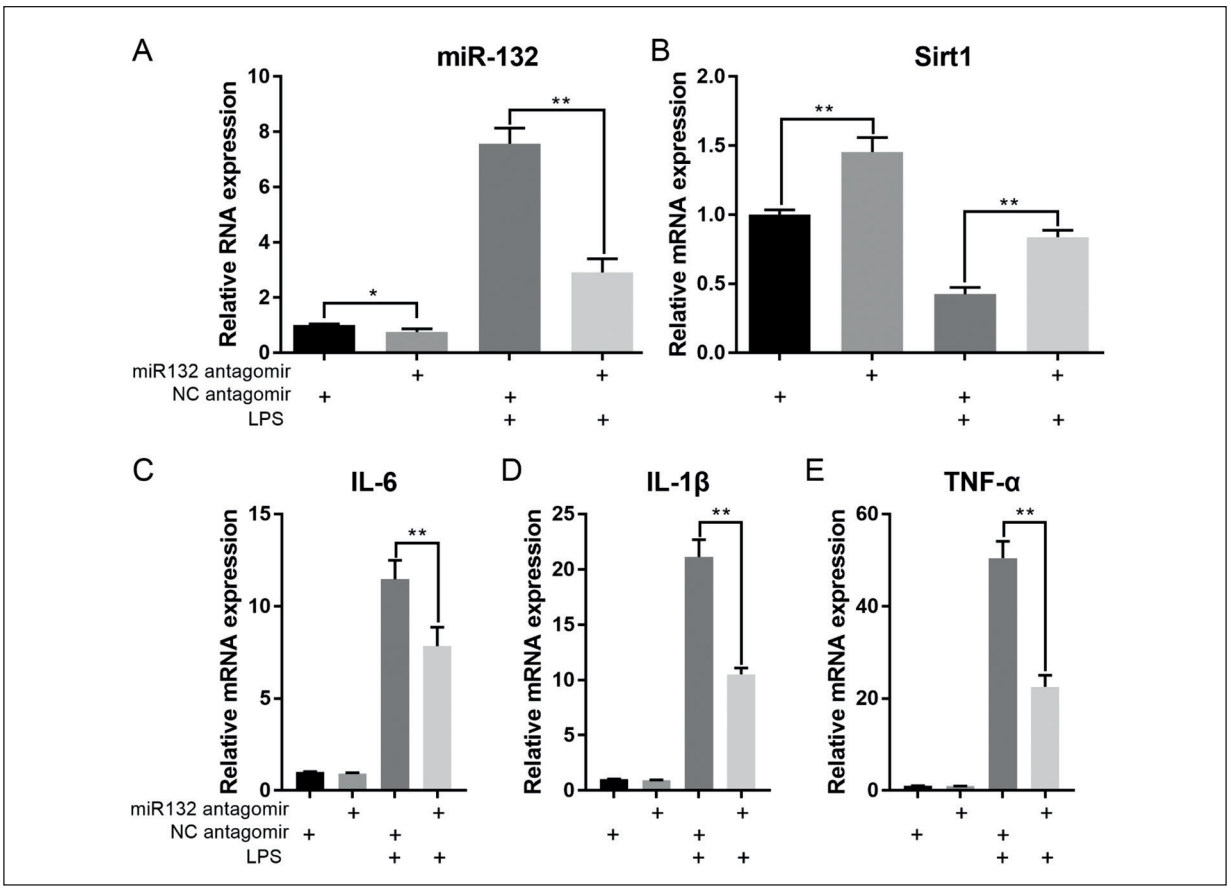

Figure 3. Gene expressions of miR-132, Sirt1, IL-6, IL-1β and TNF-α detected using qRT-PCR. **A,** MiR-132 gene expression. **B,** Sirt1 gene expression. **C,** IL-6 gene expression. **D,** IL-1β gene expression. **E,** TNF-α gene expression. *p<0.05 and **p<0.01: a significant difference among groups.

type Sirt1 plasmids (Sirt1-WT) and mutant-type Sirt1 plasmids (Sirt1-MUT) were constructed and transfected into cells with miR-132 mimics, respectively. The results of Luciferase reporter gene assay showed that Sirt1-WT Luciferase activity was remarkably inhibited by miR-132 mimics ($p<0.01$). However, no significant changes were observed in Sirt1-MUT Luciferase activity ($p>0.05$) (Figure 4B).

Effect of Sirt1 on Lung Injury in Sepsis Rats

The results of qRT-PCR manifested that SRT1720 markedly reduced the expressions of IL-6, IL-1β and TNF-α ($p<0.01$), but markedly raised the expression of Sirt1 in sepsis rats ($p<0.01$) (Figure 5).

Effects of MiR-132 and Sirt1 on Lung Injury in Sepsis Rats

HE staining demonstrated that LPS caused pulmonary alveolar atrophy and alveolar wall swelling in rats. Lung injury was remarkably alleviated by miR-132 antagomir and SRT1720. The protein expression of Sirt1 in lung tissues was remarkably reduced by LPS ($p<0.01$), whereas was significantly upregulated by miR-132 antagomir and SRT1720 ($p<0.01$). Similarly, the protein expression of Bax was remarkably raised by LPS ($p<0.01$), but reduced by miR-132 antagomir and SRT1720 ($p<0.01$) (Figure 6).

Discussion

Severe sepsis, with a high mortality rate, can lead to lung injury and even respiratory failure. LPS is a well-known inducing agent of lung injury, which has been used to establish the lung inflammation and injury model in various studies[12-14]. In this study, the elevation of IL-16, IL-1β and TNF-α expressions[15] was detected after injection of LPS into the caudal vein. Meanwhile, there were significant changes in lung tissues and lung injury observed by HE staining, indicating successful modeling.

As non-coding RNAs, miRNAs play key roles in post-transcriptional regulation of genes. This demonstrates different expression profiles and different expression levels in various diseases and inflammation, respectively[16,17]. MiR-132, an apoptosis inducer, induces cell apoptosis *via* targeting Bcl-20. It also participates in meditating inflammation and injury[18]. Taganov et al[19] have found that exposure of human monocytes to LPS will upregulate miR-132 expression. In a mouse model of multiple sclerosis, miR-132-deficient mice exhibit better immunity to experimental autoimmune encephalomyelitis, indicating the pro-inflammatory effect of miR-132[20]. On the contrary, Hanieh and Alzahrani[21] have indicated the anti-inflammatory effect of miR-132, which promotes brain anti-inflammation *via* targeting acetylcholinesterase. In septic patients, aberrantly expressed miRNAs including miR-146a, miR155, miR-150, miR-182, miR-342-5p, miR-486 and miR-132 are detected[22,23]. Rao et al[18] have observed severe inflammatory response in SEB-exposed mice and detected the upregulation of miR-132 by analyzing gene expression profiles. In this study, in the mouse model of LPS-induced lung injury, the remarkable upregulation of miR-132 was observed. Subsequent results showed that this was positively correlated with inflammatory factor levels. After miR-132 expression was inhibited, the downregulation of inflammatory factor levels and the expression of pro-apoptotic

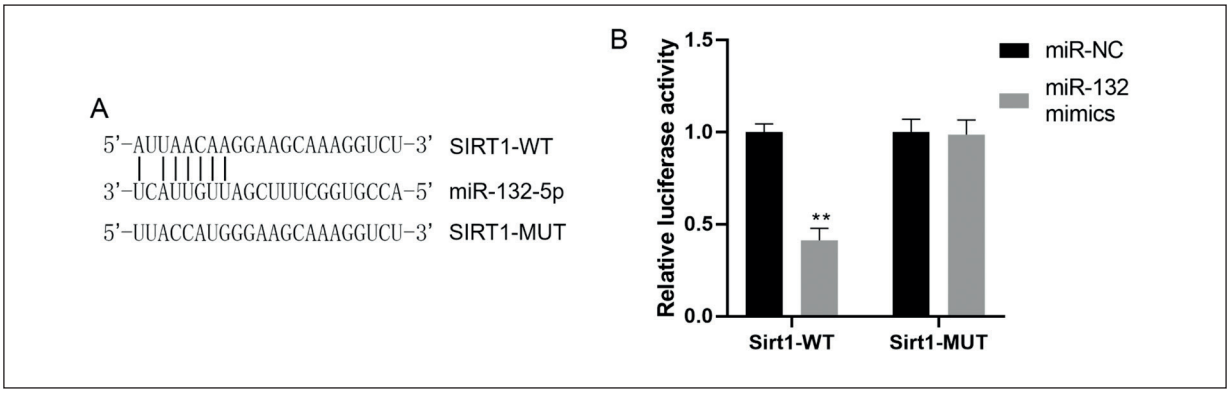

Figure 4. Targeted relation between miR-132 and Sirt1 verified *via* Luciferase reporter assay. **A,** Targeting sequences of miR-132 and Sirt1. **B,** Luciferase activity. **$p<0.01$: a significant difference between two groups.

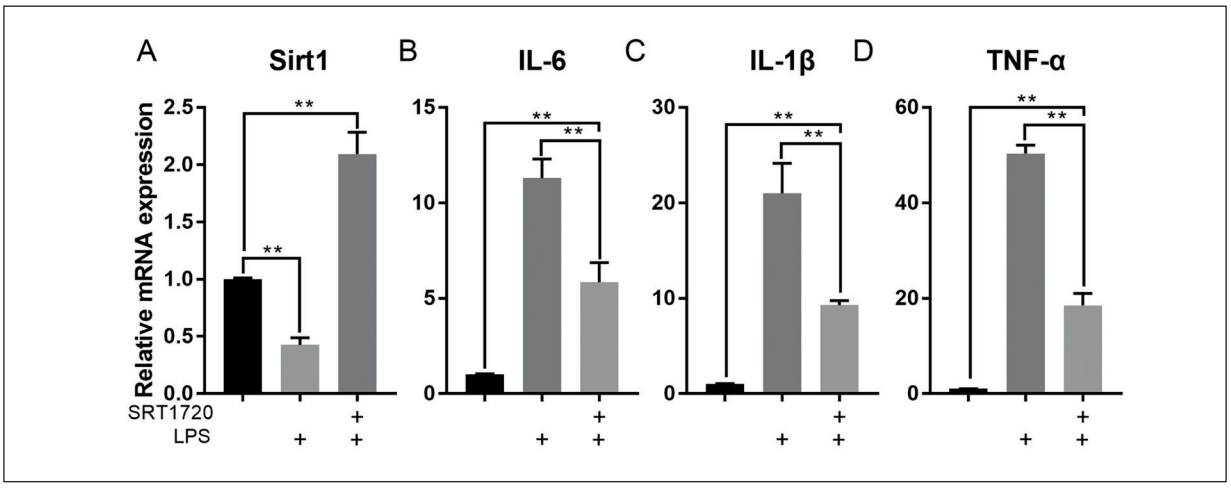

Figure 5. Gene expressions of Sirt1, IL-6, IL-1β and TNF-α detected using qRT-PCR. **A,** Sirt1 gene expression. **B,** IL-6 gene expression. **C,** IL-1β gene expression. **D,** TNF-α gene expression. ***p*<0.01: a significant difference between the two groups.

factor Bax was observed, thus alleviating lung injury.

MiRNAs, by targeting the 3'UTR of their target mRNAs, may inhibit the translation of target mRNAs or degrade mRNAs[7]. In this study, the targeting sequence of miR-132 and Sirt1 were predicted. Luciferase reporter gene assay was performed to verify the targeted regulation of miR-132 on Sirt1. Sirt1 is a NAD^+-dependent Class III protein deacetylase that regulates many cellular activities, including cell growth, differentiation, stress resistance, reduction of oxidative damage, and metabolism[24,25]. Previous reports[26,27] have indicated that Sirt1 possesses the effects of anti-inflammation, anti-oxidation, suppressing DNA damage, and reducing apoptosis in various types of cells. Gao et al[28] have found that sepsis enhances inflammatory lung injury in Sirt1-deficient mice. However, Sirt1, by inhibiting the activation pathway of inflammasomes, prevents over-production of pro-inflammatory mediators. This may eventually restrain acute lung inflammation induced by sepsis and reduce lung injury. Li et al[12] have found that resveratrol can effectively activate the expression of Sirt1, as well as reduce acute lung injury and inflammation in a mouse model of LPSinduced sepsis. Moreover, Sirt1 induces the deacetylation of DNA repair factor Ku70, which isolates the pro-apoptotic factor Bax in mitochondria, thereby suppressing stress-induced apoptosis[26]. In this study, the expression of Sirt1 was significantly upregulated by SRT1720 in sepsis mice. In addition, it significantly downregulated the expression levels of

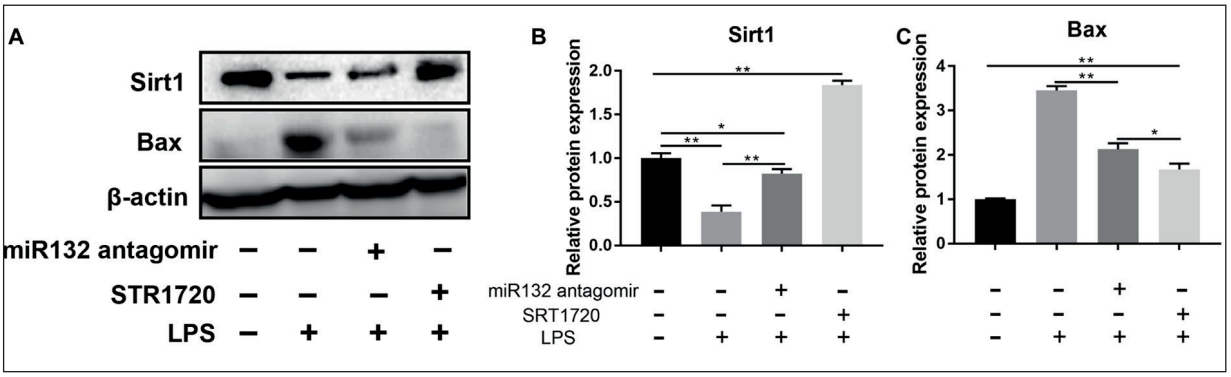

Figure 6. Effects of miR-132 and Sirt1 on lung injury in sepsis rats. **A,** Protein expression in lung tissues detected using Western blotting. **B,** Relative expression of Sirt1. **C,** Relative expression of Bax. **p*<0.05 and ***p*<0.01: a significant difference among groups.

inflammatory factors and Bax, thereby relieving LPS-induced lung injury.

Conclusions

In summary, miR-132, by inhibiting expression of Sirt1, promotes LPS-induced lung injury. The novelty of this study was that such a mechanism can provide a potential treatment target and theoretical basis for clinical prevention and treatment of lung injury induced by sepsis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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