

Original Research Article

Regulation of RhoA/ROCK1 signaling pathway by miR-26b in sepsis-induced acute lung injury

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Abstract

Purpose: To investigate the role of miR-26b in the regulation of RhoA/ROCK1 signaling pathway in acute lung injury (ALI) caused by sepsis.

Methods: Thirty male rats were randomized into sham group (SG), cecal ligation and puncture (CLP) group (CG) and miR-26b mimic group (MG). Hematoxylin and eosin (H & E) staining assay was performed to determine the pathological characteristics of rat lung tissues in each group, while enzyme-linked immunosorbent assay (ELISA) was conducted to determine TNF- α and IL-1 β levels. The miR-26b expression was evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR), while RhoA and Rock1 protein levels were assessed using western blotting.

Results: The CG had significant lung injury in comparison with the SG. There were significant elevation in TNF- α and IL-1 β levels ($p < 0.05$). RhoA and ROCK1 levels in lung tissue were noticeably elevated in CG ($p < 0.05$). After treatment, lung injury in MG was reduced in contrast to CG. The MG showed statistically significant decrease ($p < 0.05$) in the levels of TNF- α and IL-1 β , while the lung tissue mRNA expression and the RhoA and ROCK1 expression levels were significantly reduced in MG ($p < 0.05$).

Conclusion: The MiR-26b mimics plays an important role in the treatment of ALI induced by sepsis in rats by regulating RhoA/ROCK1 signaling pathway. Thus, the findings of this study provide a theoretical basis for clinical studies on the use of miR-26b in the therapy of sepsis.

Keywords: miR-26b, RhoA/ROCK1, Sepsis, Acute lung injury

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INTRODUCTION

Sepsis is a disorder in the host's response to an infection, and is a life-threatening medical emergency [1]. It can be a serious complication arising from trauma, burn, shock, infection, and major surgery for clinically critical patients. It also contributes to septic shock and multiple organ dysfunction syndrome (MODS). The lung is the most affected organ in sepsis induced multiple

organ injury. The appearance of acute lung injury (ALI) suggests the development of multiple organ dysfunction [2]. In sepsis, many inflammatory factors and mediators, including TNF- α and IL-1, IL-6, and IL-8, are released and activated in large quantities, resulting in pro-inflammatory and anti-inflammatory cytokine imbalance as well as impaired immune functions in the body [3].

The Ras homologous gene (Rho)/Rho kinase (ROCK) signaling pathway regulates endothelial cell permeability by regulating the remodeling of the endothelial cytoskeleton [4]. The ROCK is involved in various intracellular life activities and can regulate cytoskeleton proteins. RhoA/ROCK signaling pathway is closely associated with tumor invasion and metastasis, fibrosis of various tissues and organs, regeneration and reconstruction of neurites [5]. Rho/ROCK signaling pathway plays a crucial part in the increase of lung epithelial permeability induced by sepsis, and the resulting lung injury course [2]. Lipopolysaccharide (LPS) comprises part of the bacterial cell wall that can enter the organism to promote a chain reaction between monocytes and the specific CD14 receptor on the surface of macrophages, producing fatal septic shock, immune dysfunction, multi-organ failure, and other symptoms consistent with the physiological and pathological changes induced by sepsis [6]. Therefore, LPS is often used to establish septic animal models. LPS activates the RhoA/ROCK signaling pathway.

miRNA is a short non-coding, single-stranded RNA, comprising 19 - 22 nucleotides. A recent study observed that miR-26b contributes to tumor development and metastasis. Gao *et al* [7] found that miR-26b targeted hENT1 through RhoA/ROCK-1 signaling pathway and regulates the invasion and migration of lung cancer cells. At present, the exact mechanism underlying the occurrence and development of ALI in sepsis is not clear. Thus, studies are required to identify relevant molecular targets and determine the molecular mechanism underlying the development of ALI in sepsis. It was observed that no previous study has reported that miR-26b affects sepsis-induced ALI via the regulation of RhoA/ROCK1 signaling pathway. Therefore, this study aimed to regulate RhoA/ROCK1 signaling pathway in order to lay the foundation for the prevention and treatment of sepsis-induced ALI and MODS.

EXPERIMENTAL

Animals and grouping

A total of 30 male rats were randomized into the sham group (SG), cecal ligation and puncture (CLP) group (CG), and the miR-26b mimic group (MG). Twelve (12) rats were assigned to each group and two time points (6 and 12 h) were set, with 6 animals per time point. Permission for this study was granted by the Animal Ethics Committees (AEC) of The First People's Hospital of Wenling Animal Center (no. NCT02136524). All procedures of animal experiments were

implemented following the Guide for the Care and Use of Laboratory Animals [8].

Preparation of sepsis model and its treatment

The CLP was performed to prepare the sepsis model of rats [9], and 50 mL/kg of saline was injected subcutaneously to perform fluid resuscitation after operation. In the SG, the abdomen was opened without CLP, and the remaining procedures were the same. In the MG, commercial *in vivo* nucleic acid transfection reagents were used, and miR-26b was injected into the lungs of rats according to the instructions guiding how to establish an animal model overexpressing miR-26b in the lung tissue (LT).

Examination of histopathological changes in the lungs

The lung tissues were fixed with 4 % formaldehyde, dehydrated, cleared, paraffin-embedded, and sliced into 5- μ m sections. The slices were dewaxed, hydrated, and stained in hematoxylin for 10 min. After washing the anti-blue, the sections were counterstained with eosin, washed, and then mounted. The LT morphology was observed under a microscope.

Assessment of of TNF- α and IL-1 β

Blood samples were collected into tubes from the retrobulbar sinus or the tail vessel using a 23 gauge needle at 6 and 12 h after modeling, and centrifuged to acquire the supernatant as a sample. Then 0.1 mL of samples was added into the reaction well for incubation at 37 °C for 1 h. Next, 0.1 mL of enzyme-labeled antibody was diluted and added after washing, followed by incubation at 37 °C for 1 h. The substrate solution was added to develop color. After 30 min of incubation, the stop solution was added, followed by the measurement of absorbance.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA in the tissue was extracted according to TRIzol instructions (Invitrogen, Carlsbad, USA), and the ultra-micro spectrophotometer was used to determine RNA concentration. Primer 5 was used for miR-26b primer design, and with U6 as an internal control, successful reverse transcription was validated and cDNA templates were calibrated. Table 1 shows the used primers. Total RNA (100 ng) served as a template, and the reverse transcription of cDNA was carried out with the following reaction conditions: 37 °C for 15 min; 98 °C for 5 min. Afterwards, PCR was conducted. mRNA

expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method [10]. Experiments were repeated three times.

Table 1: Primer sequence

Primer	Sequence (5' - 3')
miR-26b	F CGCCGCTTCAAGTAATTCAGGAT
	R GTGCAGGGTCCGAGGTGTCCTA
U6	F CTCGCTTCGGCAGCACACA
	R AACGCTTCACGAATTTGCGT
β -actin	F AGGGAAATCGTGCGTGACATCAAA
	R ACTCAT CGTACTCCTGCTTGCTGA

Western blotting (WB)

RhoA and ROCK1 expressions were determined in LT samples using WB, with β -actin as internal control. After homogenization, the protein was extracted and its concentration was determined using bicinchoninic acid (BCA) method, with the kit supplied by Abcam (Cambridge, USA). Lysates were resolved on SDS-PAGE and then transferred to PVDF and nitrocellulose membranes. The blots were blocked in 0.1 % Tween® 20 Detergent (TBST) for 1 h at 25 °C and then probed with the primary and secondary antibodies in 5 % milk PBST for 12 h and 2 h respectively. The system analyzed the gel imaging and calculated the relative expression.

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) 22.0 (IBM, Armonk, USA), and are presented as mean \pm standard deviation (SD). Student's *t*-test was adopted to compare data between two groups. $P < 0.05$ was defined as statistically significant.

RESULTS

Histopathological changes in lung tissues

The lungs of the rats in the SG were pink with no notable abnormalities. Significant edema and bleeding were observed in rats in the CG after dissection, and the MG rats had less edema and bleeding in the LT and a smaller range than rats in the other groups. It was shown in H & E staining that the LT structure was complete in the SG, the alveolar cavity was clear, and there was no edema and inflammation in the alveolar space (Figure 1). The pathological changes in the LTs of rats in the CG were noticeable: Edema and hemorrhage were visible in the alveoli, the alveolar interval increased, and increased inflammatory cell infiltration was observed. The LTs of rats in the MG also showed some pathological changes, but the changes were fewer than those in the CG. Thus, the CG had

obvious lung injury. Lung injury in the MG was alleviated in contrast to the CG.

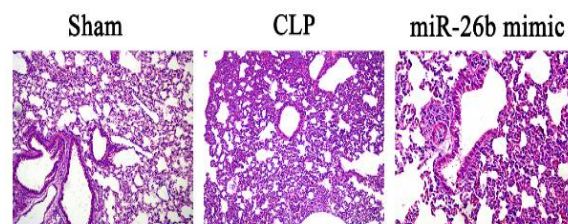


Figure 1: Histopathological changes in rat lung tissues rats; H & E staining ($\times 100$)

TNF- α and IL-1 β levels

Both the CG and the MG exhibited an elevation of TNF- α and IL-1 β at 6 and 12 h in contrast to the SG ($p < 0.05$). The MG exhibited remarkable reduction in TNF- α and IL-1 β at 6 and 12 h in contrast to the CG ($p < 0.05$). Thus, the CG showed significant increase in NF- α and IL-1 β in contrast to the SG. The MG had significant decrease in TNF- α and IL-1 β in comparison with CG (Figure 2 A and B).

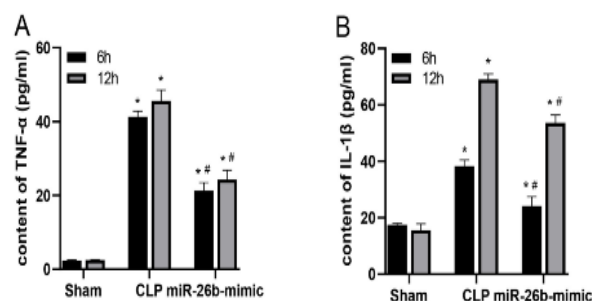


Figure 2: Comparison of TNF- α (A) and IL-1 β (B) levels. Both CLP and miR-26b mimic groups showed noticeably higher TNF- α and IL-1 β levels at 6 and 12 h than the sham group ($*p < 0.05$ versus sham group, $\#p < 0.05$ versus CLP group)

MiR-26b expression in rat lung tissues

The qRT-PCR results revealed significantly lower miR-26b expression in ALI tissues than in normal LTs (Figure 3). The CG showed significantly reduced miR-26b expression in the LTs at 6 and 12 h in contrast to the SG ($p < 0.05$). The MG showed elevated miR-26b expression in the LTs when compared to CG ($p < 0.05$) (Figure 3).

Expression of RhoA and ROCK1

The CG showed increase in Rho and ROCK1 expression in the LTs when compared to the SG ($p < 0.05$). The MG showed remarkable reduction in Rho and ROCK1 expression in the LTs when compared to the CG (Figure 4).

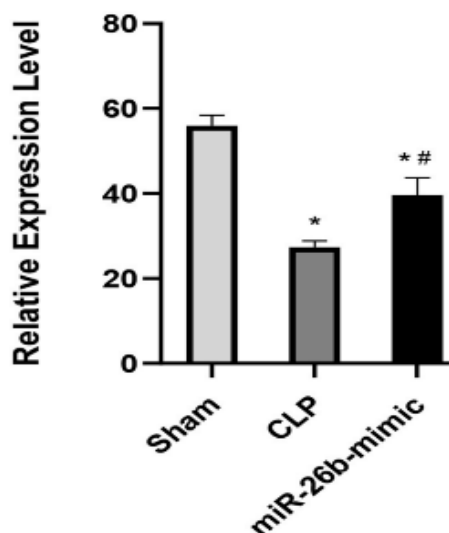


Figure 3: Comparison of miR-26b expression in the LTs. ALI tissue showed noticeably lower miR-26b expression than normal LT (* $P < 0.05$ versus sham group, # $p < 0.05$ versus CLP group)

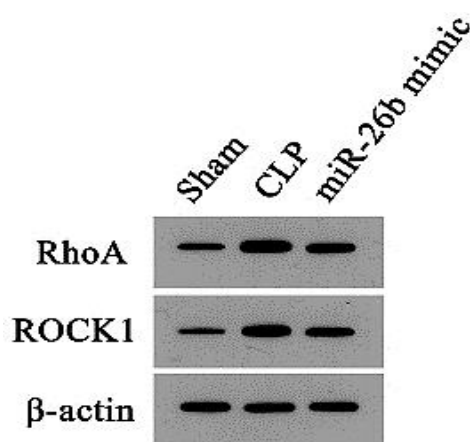


Figure 4: Expression of RhoA and ROCK1 detected by WB

DISCUSSION

Sepsis, as an inflammatory condition caused by an infection, destroys the autoimmune system, destabilizes immune function, and disrupts the body's internal environment. Zhang *et al* [11] found that LPS-induced pulmonary fibrosis in rats was characterized by alveolar space destruction and enlargement, inflammatory cell infiltration, and interstitial edema. In this study, the CG showed noticeable pathological changes, edema and hemorrhage occurred in the alveoli, the alveolar interval increased, and increased inflammatory cell infiltration was observed. The LTs of rats in the MG also showed some pathological changes, but the changes were fewer than those observed in the LTs of rats in

the CG, indicating a regulatory role of miR-26b in sepsis-induced ALI.

The occurrence of sepsis is closely related to immune system and organ dysfunction, and mortality caused by sepsis is the result of the interaction between inflammatory and anti-inflammatory responses [12]. Isopropyl propionate effectively inhibits TNF- α expression in LPS-induced sepsis rats and enhances IL-10 levels to prevent sepsis [13]. Therefore, effective suppression of the inflammatory response is expected to be crucial for preventing and treating sepsis. In this study, the miR-26b mimic remarkably decreased the levels of TNF- α and IL-1 β in LPS-induced sepsis rats.

miRNA is an endogenous, highly conserved single-stranded non-coding RNA. The production steps of miRNA are as follows: First, pri-miRNA was formed by the action of RNA polymerase; second, pri-miRNA was cleaved into pre-miRNA, which has a length of 60 - 70 nucleotides; and finally, pre-miRNA was cleaved by the enzyme dicer [14]. Studies have shown that more than 50 % of miRNA coding sequences are located in the genomic regions of tumor-related genes, suggesting that miRNAs play crucial roles in the occurrence and development of tumors [15]. Different miRNAs serve as molecular targets for early diagnosis, treatment, and prognosis of tumors [16]. In this study, by determining miR-26b expression in ALI and normal LTs, the importance of miR-26b in the occurrence of sepsis-induced ALI was observed.

The Rho/ROCK signaling pathway exerts a regulatory role in cell morphology, cytoskeletal remodeling, and cell migration via regulating actin movement [17]. Studies have shown that in septic ALI animal models, the ROCK activity of the LTs is significantly increased, and Rho/ROCK signaling pathway critically participates in the pathological process [2]. In this study, remarkable decrease occurred in the RhoA and ROCK1 expressions after the septic ALI cells were treated with miR-26b mimic.

CONCLUSION

The findings of this study show that miR-26b treatment provides protection against lung injury in rats with sepsis. The mechanism of action may be the suppressive action of miR-26b on the activity of RhoA/ROCK signaling pathway in lung tissues (LT), thereby improving endothelial permeability and reducing inflammation and oxidative stress, resulting in reduction of lung injury. The results provide new ideas and methods for developing drugs to treat sepsis-induced ALI in the future.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Yi Mao and Yi Gao contributed equally to this work.

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