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Influence of LincRNA-p21 on acute lung injury in sepsis

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Abstract. – OBJECTIVE: Acute lung injury (ALI) is one of the most serious complications of sepsis and remains refractory. It is of great significance to discuss the pathogenesis of acute lung injury in sepsis and look for more effective drugs for treatment. The purpose of this study was to investigate the role of LincRNA-p21 on acute lung injury in sepsis.

MATERIALS AND METHODS: Lung histology was detected by HE staining to evaluate sepsis-induced ALI model in rats. The miRNA expression of LincRNA-p21 in septic model in vivo and in vitro was detected by RT-qPCR. Cell apoptosis, inflammatory responses and oxidative stress were detected to uncover the influence of LincRNA-p21 on LPS-induced septic model *in vitro*.

RESULTS: The expression of LincRNA-p21 was significantly increased in septic model *in vivo* and *in vitro*. Cell apoptosis, inflammatory responses and oxidative stress were alleviated by LincRNA-p21 interference in LPS-treated BEAS-2B cells.

CONCLUSIONS: All the results in the current study proved that LincRNA-p21 interference could alleviate the acute lung injury in septic model. It raised the conclusion that LincRNA-p21 may act as a novel regulator in the pathological process and a potential therapeutic target in sepsis-induced ALI.

Key Words:
LincRNA-p21, Lung injury, Sepsis.

Introduction

Sepsis refers to the systemic inflammatory response syndrome caused by severe infection, trauma, blood loss, and neoplasm[1]. It is often life-threatening multiple organ dysfunction in patient due to body's unbalanced response to infection, being featured with acute onset, rapid progress and high rates of morbidity and mortality in intensive care unit (ICU)[2]. Acute lung injury (ALI), characterized by severe dysfunction of the diffusion of lung, is one of the most serious complications of sepsis. About half of the patients with severe sepsis will develop ALI and even acute respiratory distress syndrome (ARDS)[3]. As a difficult problem in the field of critical care medicine, the research of anti-sepsis drugs is one of the hot spots in the international medical field all over the world[4]. At present, liquid therapy, resuscitation, anti-infection therapy are commonly used in clinical treatment of sepsis, but most complications of sepsis remain refractory[5]. Therefore, it is of great significance to explore the pathogenesis of ALI in sepsis and look for effective drugs for treatment.

In recent years, more and more studies have shown that lung oxidative stress, inflammatory response, apoptosis or necrosis of cell and other mechanisms play an extremely important role in the development of sepsis-induced ALI[6]. A large number of clinical and basic researches confirmed systemic inflammatory response caused by the interactions and subsequent cascade activation of pro and anti-inflammatory cytokines as the key of ALI pathogenesis[7]. Oxidative stress is basically defined as imbalance between pro-oxidant and antioxidant[8]. This imbalance in ALI will result in excessive accumulation of ROS and injury of lung and important organelles[9]. Studies have proved that inflammation is closely related with oxidative stress and could promote

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the pathological process of sepsis[10,11]. Besides, oxidative stress could cause cellular apoptosis via mitochondria-dependent and mitochondria-independent pathways[8]. Pro-apoptotic signals could cleave and activate caspases to ultimately dismantle cells by the effector caspases[12]. Pathologic changes of ALI include the infiltration of inflammatory cells, the release of peroxide, the injury of cells, the production of pro-inflammatory factors and so on[13]. Therefore, it is reasonable to believe that inhibition of oxidative stress, inflammatory response and apoptosis may contribute to the prevention and treatment of ALI.

Long noncoding RNAs (lncRNAs) are generally considered to be transcripts longer than 200 nucleotides, lacking protein-coding function. They can play crucial roles in cell biology by regulating a variety of processes including gene expression, chromatin remodeling, post-transcriptional processing and transcription[14]. With the development of research on the treatment of sepsis, it is discovered that lncRNAs play an increasingly important role in the process of sepsis[15]. Zhuang et al[16] proved that lncRNA MALAT1 could enhance the level of TNF-α and apoptosis in sepsis model. LincRNA p21 was a direct transcriptional target of p53 and functions as a component of p53 pathway[17]. Tu et al[18] reported that knockdown of lincRNA p21 could greatly reduce liver fibrosis and inflammation and inhibit apoptosis *in vitro* and *in vivo*. Besides, knockdown of lincRNA p21 could mitigate SH-SY5Y cell apoptosis, inflammation, oxidative stress and enhance cell viability[19]. However, the definite effect and mechanism of lincRNA p21 on sepsis-induced ALI have not yet been reported. Therefore, the current study aims to determine whether lincRNA p21 could affect inflammation, oxidative stress, apoptosis and lung pathological changes in the process of sepsis-induced ALI.

Materials and Methods

Establishment of Sepsis Model

Sepsis model was established by cecal ligation and puncture (CLP). Rats were fasted and water deprived for 12 h before operation. Then, rats were anesthetized by chloral hydrate (300 mg/kg of body weight). After ventral midline incision, the cecum was isolated and ligated its root, and punctured twice with an 18-gauge needle. Then, we reset the cecum and closed the abdominal cavity. Sham procedure only consisted of laparotomy without cecal manipulation or perforation. All animal experiments were conducted according to the Ethical Guidelines of the First Affiliated Hospital of Guangxi University of Traditional Chinese Medicine and the "3R" principle.

Cell Transfection

According to the manufacturer's instructions, the shRNA-NC and shRNA-LincRNA-p21 were transfected into BEAS-2B cells by using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, 11668019, Carlsbad, CA, USA) when the cells were cultured until 50% confluence. Un-transfected BEAS-2B cells were employed as a blank control group.

HE Staining

All lung samples were fixed in 10% neutral-buffered formalin, subsequently embedded in paraffin, and sectioned into 4-μm thick sections. The sections were deparaffinized with xylene and hydrated gradually with ethanol. After washing in distilled water, the sections were stained with hematoxylin for 3 minutes, differentiated in 1% hydrochloric-alcohol for 3-5 minutes. Next, the sections were blued with ammonium hydroxide, followed by eosin staining for 1 min. Then, the sections were dehydrated and permeabilized by gradient ethanol, and xylene transparentization twice for 10 min. The lung structures were observed under optical microscopy.

Cell Counting Kit-8 (CCK8) Assay

The CCK8 assay was applied to measure BEAS-2B cell proliferation that underwent different types of processing. The transfected BEAS-2B cells were inoculated into 96-well plates at an appropriate density of 5×10^4 cells per well and each step was set with 3 replicates. After BEAS-2B cells attached, 10 μl CCK-8 solution was added to each well and incubated at 37°C for 1 h. Finally, the absorbance of each well at 450 nm (OD450) was recorded using a microplate reader (Bio-Rad, Hercules, CA, USA).

Flow Cytometry Analysis of Cell Apoptosis

Cell apoptosis was analyzed using an Annexin V-FITC/PI apoptosis detection kit (KeyGen Biotech, CN). The transfected BEAS-2B cells were digested with trypsin and centrifuged at 1500 rpm for 10 min. After fixed in 75% ethanol, cells were re-suspended and washed twice with phosphate-buffered saline (PBS). Apoptotic cells

were stained in propidium iodide (PI) (10 μg/ml) / FITC-Annexin V (50 μg/ml) for about 15 min at room temperature in the dark according to the manufacturer's instructions. Data were accessed using a flow cytometer (Bio-Rad, Hercules, CA, USA).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from differently treated BEAS-2B cells using TRIzol reagent (Invitrogen, 15596018, Carlsbad, CA, USA). Real-Time RT-PCR for LincRNA-p21 was measured using an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Green Master Mix (Roche, 04913914001, Basel, Switzerland). The PCR reaction mixture contained 25 μl Dream Taq Green PCR master Mix, 1 μl forward/reverse primer, 4 μl cDNA and 19 μl nuclease-free H_2O. The thermocycling conditions were as follows: pre-denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s and 60°C for 30 s. Expression was calculated β-actin. The relative RNA expression of LincRNA-p21 was normalized with the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and analyzed by the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers used were as follows: LincRNA-p21 (forward 5'-GGGTGGCTCACTCTTCTGGC -3', reverse 5'-TGGCCTTGCCCGGGCTTGTC-3'), GAPDH (forward 5'-GCTCTCTGCTCCTCCTGTTC -3', reverse 5'-ACGACCAAATCCGTTGACTC-3').

Western Blot Analysis

Total protein was extracted from cultured BEAS-2B cells using RIPA Lysis Buffer (Beyotime, P0013B, Shanghai, China) in the presence of protease inhibitors (Beyotime, P1006, Shanghai, China). The protein concentration of each sample was determined using bicinchoninic acid (BCA) assay. Equal amounts of protein samples (30 μg per lane) were loaded and separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then subsequently transferred to the polyvinylidene difluoride (PVDF) membrane (GE, 10600023, Boston, MA, USA). The membrane was incubated with Tris-Buffered Saline and Tween-20 (TBST) blocking solution containing 5% skimmed milk at room temperature for 1 h, followed by separate incubation with primary antibodies against bcl2 (Abcam, Cambridge, MA, USA, ab32124, 1:1000), bax (Abcam, Cambridge, MA, USA, ab32503, 1:5000), cleaved caspase3 (Abcam, Cambridge, MA, USA, ab2302, 1:1000) and caspase-3 (Abcam, Cambridge, MA, USA, ab13847, 1:1000) overnight at 4°C. Then, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam, Cambridge, MA, USA, ab6721, 1:10000) for 1 h at room temperature. Protein bands were detected with an enhanced chemiluminescence (ECL) kit (Beyotime, P0018FM, Shanghai, China) in line with the manufacturer's protocol and scanned by Quantity one software.

Analysis of Inflammatory Cytokines

Inflammation is involved in the pathophysiology of lung disorders, including acute lung injury. To detect the effects of LincRNA-p21 on inflammation in LPS-associated ALI, the concentration of inflammatory cytokines including TNF-a (Mlbio, ml002859, Shanghai, CN), IL-1β (Mlbio, ml063132, Shanghai, CN), IL-6 (Mlbio, ml063159, Shanghai, CN), MCP-1 (Mlbio, ml002960, Shanghai, CN) and ICAM-1 (Mlbio, ml202830, Shanghai, CN) in cell culture were determined using an enzyme-linked immunosorbent assay (ELISA) Kit in line with the manufacturer's protocol. The absorbance of each well was measured at 450 nm on a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Detection of ROS Levels

The production of intracellular reactive oxygen species (ROS) was measured using 2, 7-dichlorofluorescin diacetate (DCFH-DA). After exposure to different experimental conditions, BEAS-2B cells were collected and washed 3 times with PBS. Next, the cells were labeled with 10 μM DCFH-DA (Beyotime, S0033, Shanghai, CN) at 37°C for 30 min. Then, ROS was investigated by flow cytometer (Bio-Rad, Hercules, CA, USA).

Determination of LDH, SOD, MDA Activities

The cells were lysed with radioimmunoprecipitation assay (RIPA) buffer and maintained on ice for 30 min. Then, enzyme solution of cell was collected by centrifugation (10,000×g for 10 min at 4°C). According to the manufacturer's instructions, MDA (A003-1-2), SOD (A001-3-2) and LDH (A020-1-2) levels were examined using the respective kits (Nanjing Jiancheng Bioengineering Institute, CN). The LDH activity was evaluated by colorimetric methods. The SOD

level was evaluated by hydroxylamine method. The MDA content was determined by barbituric acid method. The readings were recorded by a microplate reader (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

All data were presented as mean values ± standard deviation and analyzed by Statistical Product and Service Solutions (SPSS) software (Version 17.0, SPSS Inc., Chicago, IL, USA). The statistical differences among groups were assessed by *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. When a value of $p< 0.05$, differences were considered to be of significance.

Results

Successful Establishment of Sepsis-Induced ALI Model In Rats

Pathological observations under light microscopy showed no evident pathological changes in control group. In contrast, rats in the model group showed serious hyperemia in the lung tissues and marked red blood cell and inflammatory cell infiltration in the alveolar spaces (Figure 1A). All the results above indicated that these pulmonary histopathological changes were significantly enhanced in sepsis-induced ALI model in rats, compared with the control group. The W/D ratios were significantly increased in the model group, compared with that in the control group (Figure 1B).

The Expression of LincRNA-p21 Was Significantly Increased In Pulmonary Lavage Fluid and Blood Serum of Septic Rats

To further evaluate the specific role of LincRNA-p21 in sepsis-induced ALI model, we detected the miRNA expression of LincRNA-p21 by RT-qPCR. Compared with the control group, the miRNA expression of LincRNA-p21 was markedly upregulated in the pulmonary lavage fluid (Figure 2A) and blood serum (Figure 2B)

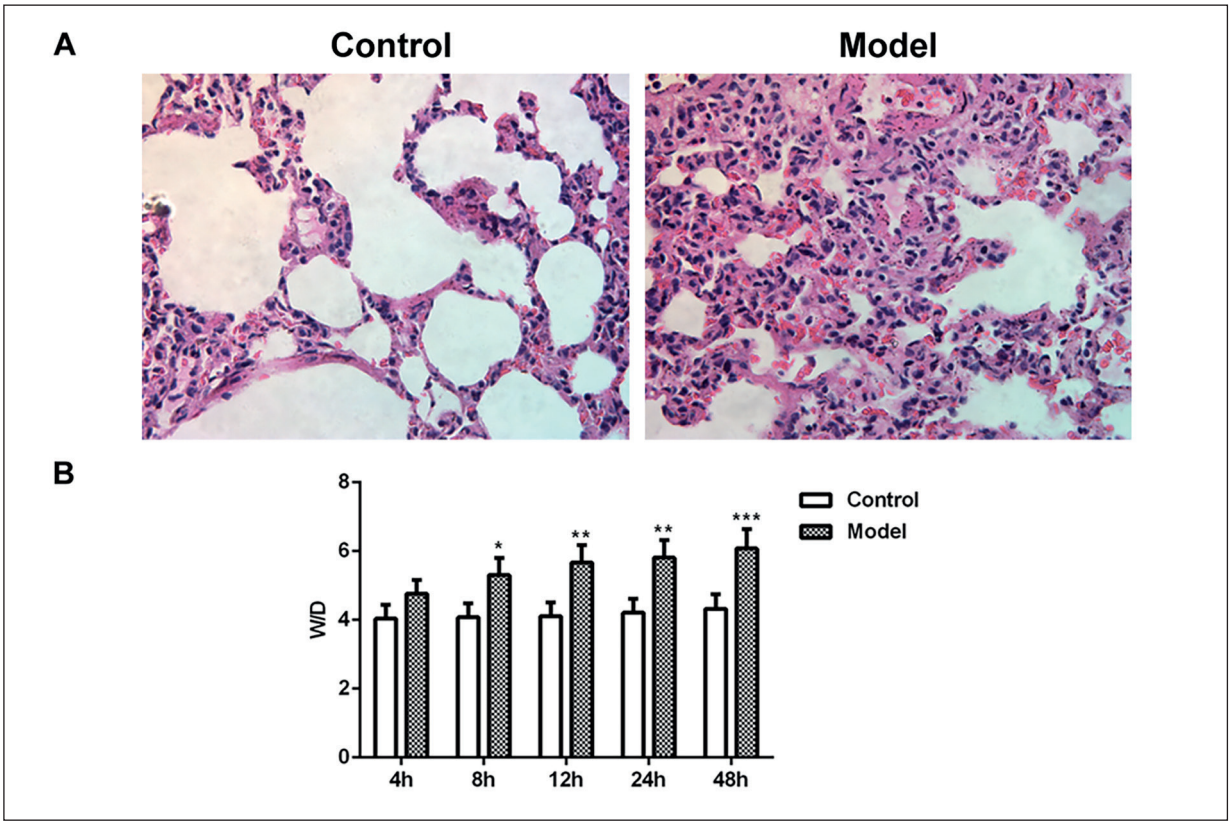

Figure 1. Successful establishment of sepsis-induced ALI model in rats. **A**, Lung histology detected by hematoxylin and eosin staining (200×). **B**, Lung wet to dry weight (W/D) ratios. *p<0.05, ** p<0.01, *** p <0.001 *vs.* control group.

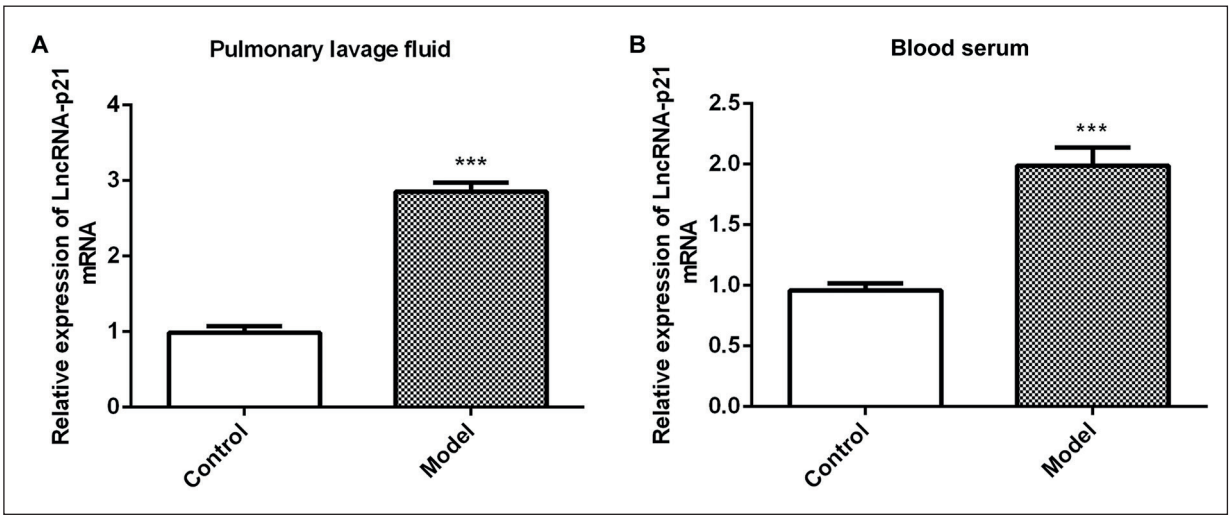

Figure 2. The expression of LincRNA-p21 was significantly increased in pulmonary lavage fluid and blood serum of septic rats. **A**, The miRNA expression of LincRNA-p21 in pulmonary lavage fluid of septic rats was detected by RT-qPCR. **B**, The miRNA expression of LincRNA-p21 in blood serum of septic rats was detected by RT-qPCR. ***p<0.001 *vs*. control group.

of septic rats. These results suggested that LincRNA-p21 inhibitor may protect against sepsis-induced acute lung injury by inhibiting LincRNA-p21 expression level.

LincRNA-p21 Expression Was Markedly Upregulated In LPS-Treated BEAS-2B Cells

BEAS-2B cells were prepared and subjected to LPS treatment, simulating LPS-induced septic model *in vitro*, and the change of LincRNA-p21 expression in the supernatant was analyzed accordingly. A similar result was observed in LPS-treated BEAS-2B cells, including increased LincRNA-p21 expression in cells (Figure 3A). These results indicated that LincRNA-p21 might play somewhat important role in LPS-induced sepsis. To further elucidate the biological role of LincRNA-p21 in sepsis-induced ALI, we investigate the effect of LincRNA-p21 on cell proliferation in LPS-treated BEAS-2B cells. CCK-8 assay data showed that cell proliferation was significantly inhibited in LPS-treated BEAS-2B cells in a time-dependent manner (Figure 3B).

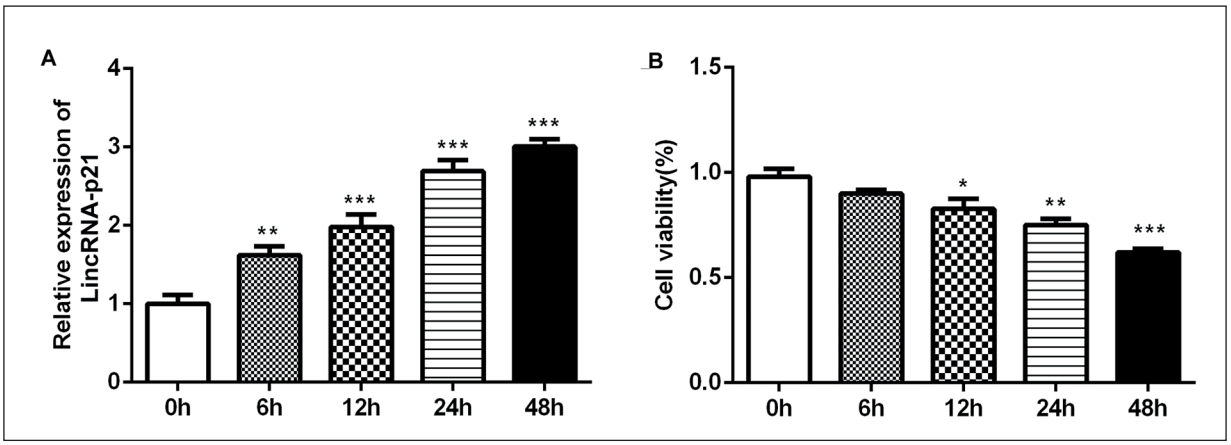

Figure 3. LincRNA-p21 expression was markedly upregulated in LPS-treated BEAS-2B cells. **A**, The miRNA expression of LincRNA-p21 in LPS-treated BEAS-2B cells was detected by RT-qPCR. **B**, The time-dependent cell viability of LPS-treated BEAS-2B cells was determined by CCK8 assay. *p<0.05, **p<0.01, ***p<0.001 *vs*. 0 h.

LincRNA-p21 Interference Inhibited Apoptosis In LPS-Treated BEAS-2B Cells

To determine whether LincRNA-p21 contributed to the apoptosis of LPS-treated BEAS-2B cells, we analyzed cell apoptosis by flow cytometry and Western blot assay. As shown in Figure 4A, we inhibited the LincRNA-p21 expression by transfecting with LincRNA-p21 inhibitor and measured LincRNA-p21 level by RT-qPCR assay. The result of flow cytometry showed that the apoptosis levels of LPS-treated BEAS-2B cells were significantly increased in LPS group, which was reversed by LincRNA-p21 interference (Figure 4B and Figure 4C). Subsequently, the related proteins including Bcl-2, Bax and cleaved capased3 were detected using Western blot assay. The WB results discovered that LPS significantly decreased the protein levels of Bcl-2 and increased the levels of Bax and cleaved caspase3, which was reversed by LincRNA-p21 interference (Figure 4D).

LincRNA-p21 Interference Alleviated Inflammatory Responses In LPS-Treated BEAS-2B Cells

Serum levels of TNF-a, IL-1β, IL-6, MCP-1 and ICAM-1 were determined using ELISA detection in order to explore the effect of LincRNA-p21 interference on these aforementioned inflammation-related gene levels. Our results suggested that LincRNA-p21 interference could effectively reduce TNF-α, IL-1β, IL- 6, MCP-1 and ICAM-1, which were induced by LPS treatment (Figure 5).

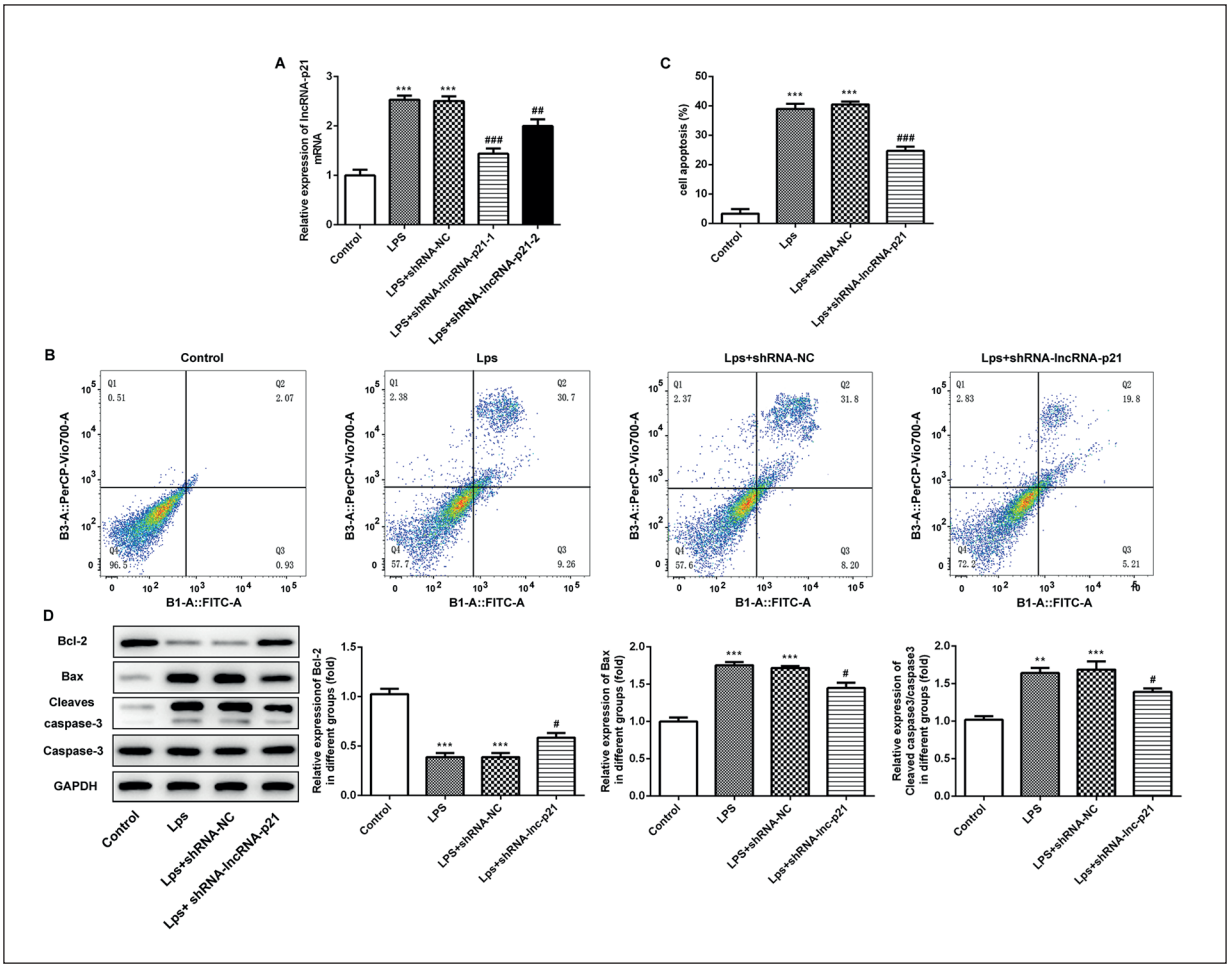

Figure 4. LincRNA-p21 interference inhibits the apoptosis in LPS-treated BEAS-2B cells. **A**, The mRNA level of LincRNA-p21was detected by RT-qPCR. **B**, The apoptosis counts of LPS-treated BEAS-2B cells were determined by flow cytometry. **C**, Quantitative statistics of cell apoptosis. **D**, The protein related to cell apoptosis was detected by Western blot. $^{**}p<0.01$, $^{***}p<0.001$ *vs.* control group, $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* LPS group.

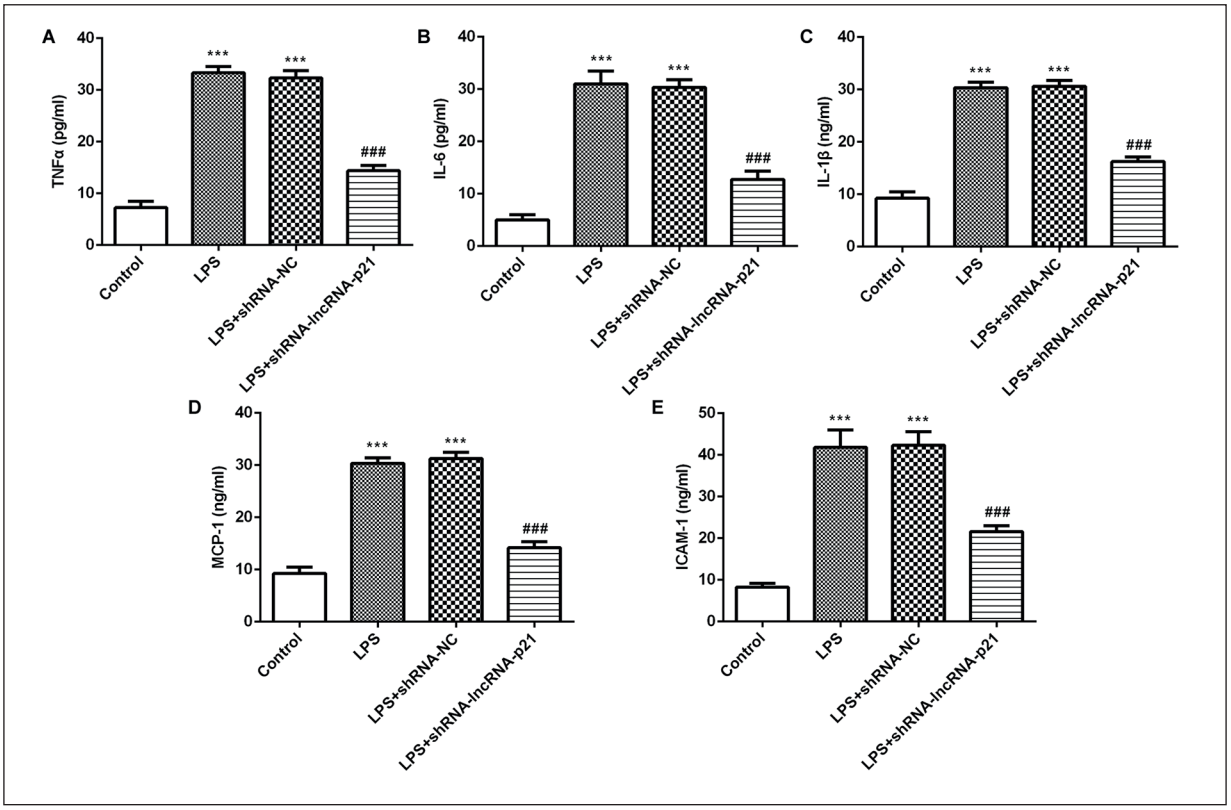

Figure 5. LincRNA-p21 interference alleviated inflammatory responses in LPS-treated BEAS-2B cells. Levels of cytokines (TNF-α, IL-1β, IL- 6, MCP-1 and ICAM-1) secretion were detected by ELISA. *** $p<0.001$ *vs.* control group, ### $p<0.001$ *vs.* LPS group ***$p<0.001$ *vs.* control group, ###$p<0.001$ *vs.* LPS group.

LincRNA-p21 Interference Alleviated Oxidative Stress In LPS-Treated BEAS-2B Cells

Considering that the oxidative stress is an important pathological change in sepsis-induced acute lung injury, ELISA assay was adopted to detect the expression of ROS, MDA, SOD and LDH levels in LPS-treated BEAS-2B cells. According to the results, the expression of ROS, MDA and LDH in the LPS group was markedly higher than that in the control group, which was significantly inhibited by LincRNA-p21 interference (Figure 6). The expression of SOD in the LPS group was markedly lower than that in the control group, which was significantly reversed by LincRNA-p21 interference (Figure 6). The above results indicate that LincRNA-p21 interference can alleviate the oxidative stress level in LPS-induced septic model *in vitro.*

Discussion

Sepsis is a common critical disease in clinic, which has the characteristics of high incidence, complicated pathogenesis, serious condition and high fatality rate[20]. Its essence is the imbalance of host immune response caused by infection, and manifests itself as a clinical syndrome of life-threatening organ dysfunction[21]. Lungs are usually susceptible to injury and about half of patients with sepsis will develop ALI[22]. The present medication or operation treatment can only improve the ALI symptoms without achieving the purpose of cure[5]. From the present study, pathologic examination revealed serious injury to the lung tissues in rat model with severe sepsis.

Nowadays, lncRNAs have become a research hotspot in the field of noncoding RNAs. It has been observed that many lncRNAs express differentially in a number of diseases. Meanwhile, lncRNAs play an important role in the gene-regulatory network of diseases through a variety of mechanisms and are involved in development and progress of diseases[23]. However, the research on relationship between lncRNA and sepsis-induced ALI is still in its early stages. Our study proved that the expression of lincRNA p21 was significantly upregulated in the rat sepsis model and

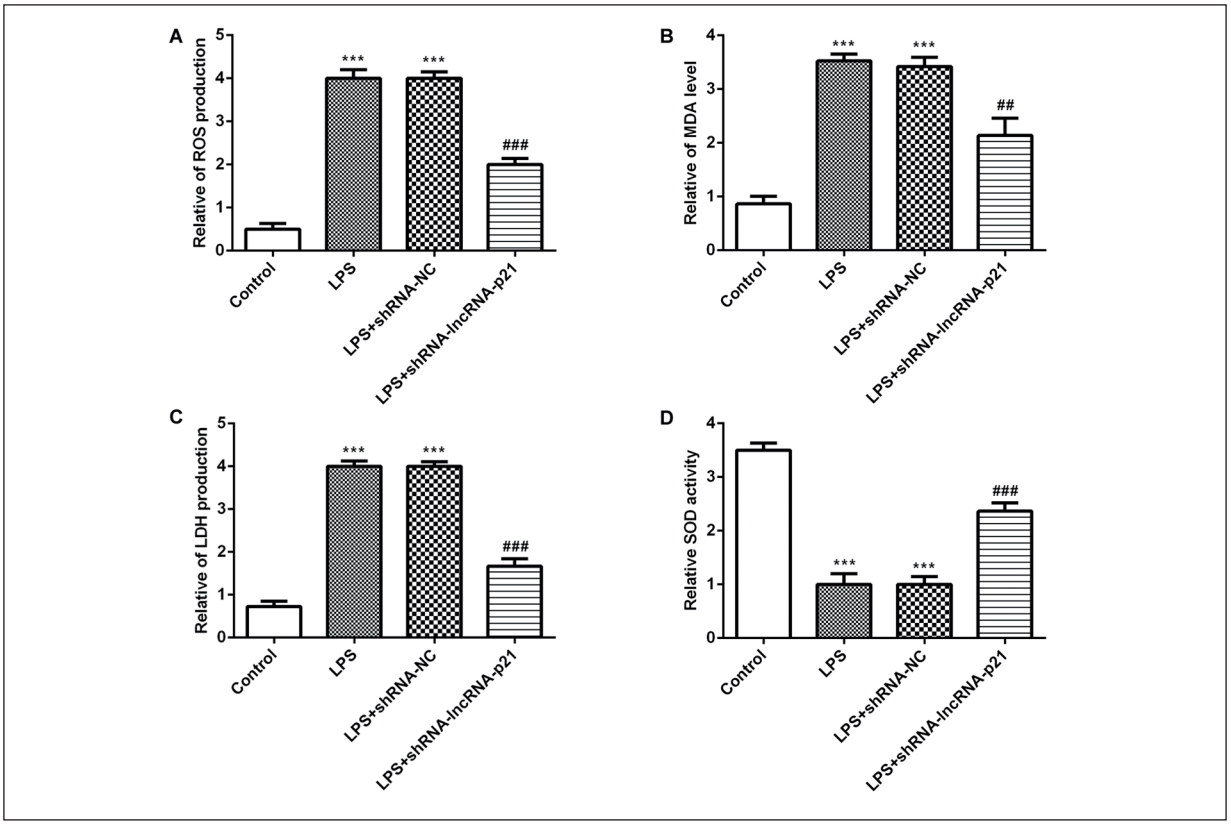

Figure 6. LincRNA-p21 interference alleviated oxidative stress in LPS-treated BEAS-2B cells. Measurement of ROS, MDA, SOD and LDH levels. ***$p<0.001$ *vs.* control group, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* LPS group.

pulmonary epithelial cell damage model induced by LPS. This result provided one possibility that lincRNA p21 might be a potent therapeutic target for ALI.

In vivo research showed that knockdown of hepatocyte lincRNA p21 could greatly reduce CCl4-induced liver inflammation and fibrosis[18]. In the present study, we found that there is a significantly positive correlation between lincRNA p21 expression and inflammation. It is generally known that inflammation and oxidative stress are closely related. Besides, lincRNA p21 was proved to affect SH-SY5Y cell oxidative stress[19]. We showed that knockdown of lincRNA p21 could inhibit oxidative stress in BEAS-2B cells. Recent reports have emphasized that silencing of lincRNA p21 could significantly increase cell viability and inhibit cell apoptosis rate, while the overexpression of lincRNA p21 reversed this effect[24]. In this study, we investigated the cell apoptosis after knockdown of lincRNA p21 in BEAS-2B cells. The result showed that silencing of lincRNA p21 could significantly inhibit cell apoptosis and influence the protein expression of bcl-2, bax, cleaved caspase3.

Conclusions

Our current study validated that LincRNA-p21 could serve as a novel regulator in the process of sepsis-induced ALI. Therefore, the present result that provides an evidence that lincRNA-p21 could be a potential therapeutic target in sepsis-induced ALI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Availability of Data and Materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' Contribution

All the authors made substantial contributions to the design of this study, performed the experiments, analyzed the data and prepared the figures.

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