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Influences of miR-155/NF-κB signaling pathway on inflammatory factors in ARDS in neonatal pigs

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Abstract. – OBJECTIVE: Acute respiratory distress syndrome (ARDS) is greatly threatening human health with high morbidity and mortality. The pathogenesis of ARDS is closely related to the inflammatory response in patients. The micro-ribonucleic acid (miR)-155/nuclear factor-κB (NF-κB) signaling pathway is crucial in regulating the expression of inflammation-related genes. Therefore, the influences of miR-155/NF-κB signaling pathway on inflammatory factors in ARDS in neonatal pigs were explored in this study.

MATERIALS AND METHODS: The model of ARDS in neonatal pigs was established first. The expression levels of miR-155, NF-κB-related proteins, and inflammatory factors in model group and control group were detected, and their differences were compared. Moreover, after treatment with the miR-155/NF-κB signaling pathway inhibitor, the changes of the inflammatory factors expression in ARDS neonatal pigs were observed at different time points.

RESULTS: In the model group, the levels of miR-155 and NF-κB-related proteins were significantly increased, and the levels of inflammatory factors, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and IL-6, were also increased synchronously. However, the levels of IL-4 and IL-10 declined significantly. In addition, it was proved that after treatment with the inhibitor in model group the mRNA expressions of miR-155/NF-κB signaling pathway-related proteins were significantly inhibited, and the levels of IL-1β, TNF-α, and IL-6 were also significantly inhibited ($p<0.05$). The levels of IL-4 and IL-10 remarkably rose after treatment with the inhibitor for 24 h ($p<0.05$).

CONCLUSIONS: The miR-155/NF-κB signaling pathway influenced the changes of inflammatory factors in ARDS in neonatal pigs, which might be a potential target for eliminating the inflammatory response after ARDS in neonatal pigs.

Key Words:

MiR-155/NF-κB signaling pathway, ARDS in neonatal pigs, Inflammatory factors, influences.

Introduction

Acute respiratory distress syndrome (ARDS) has high morbidity and mortality in the world, imposing huge human and economic costs[1-3]. According to a large number of reports[4-6], the number of new ARDS cases is about 150,000 every year in America. The mortality of ARDS patients was reported between 10% and 90%. The morbidity and diagnosis of ARDS are determined by the heterogeneity of the underlying disease process. Due to the lack of a precise definition of ARDS, a considerable number of patients with ARDS cannot be identified[4]. Therefore, more and more researchers are trying to understand the actual morbidity and outcome of this clinical syndrome[7].

Nuclear factor-κB (NF-κB) is a basic transcription factor that is crucial for the expression of inflammation-related genes, such as inducible nitric oxide synthase, and inflammatory cytokines[8]. NF-κB is usually retained in the cytoplasm, and it is called inhibitory protein of NF-κB α (IκB-α) there[9]. The stimulation of the IκB kinase led to the phosphorylation, ubiquitination and degradation of IκB-α, so that NF-κB could be transformed into nucleus and induce transcription[9]. Previous studies[10] have demonstrated that most drugs that activate NF-κB mediated the effects through inhibiting IκB-α phosphorylation and subsequent degradation.

Pro-inflammatory cytokines, especially tumor necrosis factor-α (TNF-α), have been proved to be key mediators of ARDS[11-13]. Great progress has been made in understanding these pro-inflammatory mediators. However, the influence of the micro-ribonucleic acid (miR)-155/NF-κB signaling pathway on the inflammatory response in ARDS remains unclear. Therefore, this work aimed to study and explored for this purpose.

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Materials and Methods

Laboratory Animals

A total of 15 neonatal pigs born within 1 week were selected as the model animals and divided into 3 groups: control group (no modeling, blank control), model group (ARDS model was established) and inhibitor group (treated with the miR-155/NF-κB inhibitor based on the treatment in model group). This investigation was approved by the Animal Ethics Committee of Shengli Oilfield Central Hospital Animal Center.

Main Reagents and Consumables

The pressure sensor MS45xx series was purchased from Intersema (Berne, Switzerland). The pancuronium bromide was obtained from Shanghai Boyle Chemical Co., Ltd. (Shanghai, China). The Enzyme-Linked Immunosorbent Assay (ELISA) kits for cytokines were provided by Shanghai Lengton Biotechnology Co., Ltd. (Shanghai, China). The ELISA kits for NF-κB-related proteins were bought from Shanghai Guduo Biotechnology Co., Ltd. (Shanghai, China). Western blotting equipment and reagents were produced by Bio-Rad (Hercules, CA, USA). The enhanced chemiluminescence (ECL) solution was purchased from Engreen (Beijing, China).

Modeling

After sedated *via* intramuscular injection of ketamine (10 mg/kg) and acepromazine (0.1 mg/kg), the neonatal pigs were anesthetized *via* subcutaneous injection of 2% lidocaine in the anterior cervical region was. The pigs were then dissected and the 4Fr catheter was inserted into the carotid artery for the continuous measurement of blood pressure, heart rate, and arterial blood gas, and it was also inserted into the jugular vein for continuous infusion of 5% glucose solution (4 mL/kg/h). Then, the trachea was exposed, and the tracheal catheter (inner diameter: 3.5 mm or 4.0 mm) was threaded and fastened tightly. The body temperature was maintained at 38-39.5°C using a heating pad, and the inspiratory pressure was recorded using a pressure sensor. The tidal volume was evaluated through integrating the time and flow signals obtained from the pneumotachograph connected to the pressure sensor and the same data acquisition software as above. After ventilation started, pancuronium bromide (0.1 mg/kg) was intravenously injected for muscular paralysis and applied repeatedly every time when the spontaneous respiratory effort was observed. The lung injury was induced according to the method originally described by Lachmann. The lung was lavaged using the saline (30 mL/kg) at 37°C. Before lavage, the ventilator was disconnected to the animal, and the warm normal saline was imported through the tracheal catheter and gravity under the maximum pressure of 40 cm H_2O. The lavage fluid was then discharged as much as possible by chest massage. The lung lavage lasted for 60 s every time, including infusion for 20 s and drainage for 40 s. The process was repeated for many times at an interval of 5 min till the establishment of ARDS model (PaO_2/FiO_2 ≤100 detected in arterial blood gas measurement). To confirm the establishment of ARDS model, the arterial blood gas was measured 10 min after the lung lavage last time. The lung lavage was repeatedly performed each time the parameter did not meet the standard. The heart rate and arterial blood pressure were continuously monitored and recorded before lung lavage every time.

Detection of Inflammatory Factors

The concentration of cytokines in serum was detected *via* ELISA. The whole blood samples of pigs were collected and mixed with the tablet containing the protease inhibitor, followed by centrifugation at 2,000 rpm for 15 min. Then, the supernatant was taken for detection.

Expression of NF-κB p50, NF-κB p65, and IκB-α

The peripheral blood serum was isolated in the same way as above. ELISA was performed according to the instructions. The optical density (OD) was read at 450 nm, and the corresponding sample concentration was determined according to the standard curve regression equation.

Detection of mRNA Expression Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and then reversely transcribed into complementary DNA (cDNA) according to instructions of the M-MLV reverse transcriptase kit. The expression of miR-155 was detected using the SYBR PrimeScriptTM RT-PCR kit (TaKaRa, Otsu, Shiga, Japan) on the Roche LightCycler480 fluorescence quantitative PCR system. At least 3 repeated wells were set for each specimen. The relative expression of miRNAs was calculated using $2^{-\Delta\Delta CT}$ method. U6 was selected as the internal reference. The

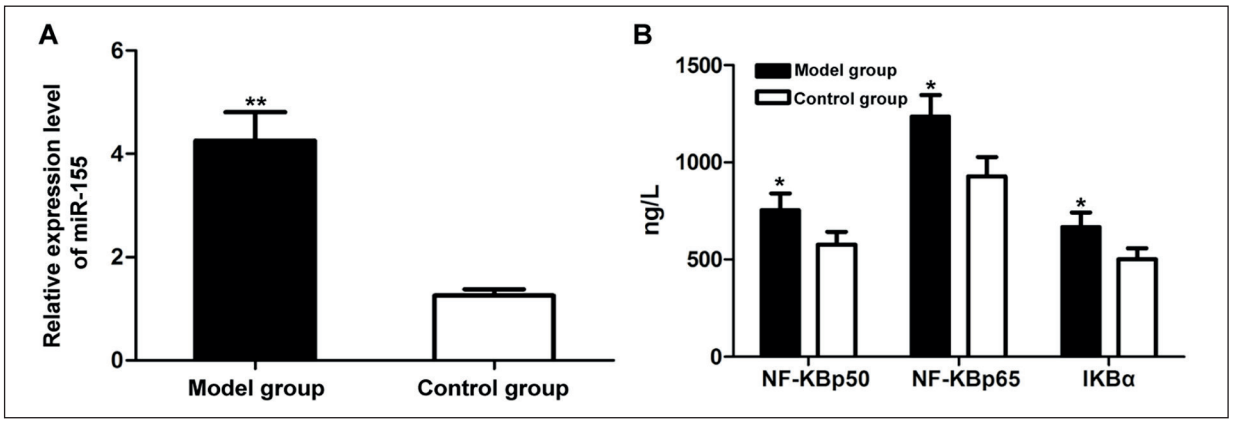

Figure 1. Expression of miR-155 and NF-κB signaling pathway-related proteins in peripheral blood in ARDS in neonatal pigs. ***A,*** Relative expression level of miR-155, ***B,*** Expression of NF-κB-related proteins (NF-κB p50, NF-κB p65, and IκB-α), *p<0.05, **p<0.01.

primers used in this study were as follows: GTCGTATCCAGTGCGTGTCGTGGAG and TCGGCAATTGCACTGGATAGGACCCCCTC, primer of U6: TGTTGGCGTGGAGTFG.

Western Blotting

The whole cell protein extraction, subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blotting were performed as already described[10]. In brief, the total cell protein was extracted with lysis buffer [0.1 M KH_2PO_4 (pH 7.5) and 1% NP-40, added with the complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) and 0.1 mM β-glycerophosphate], and detected *via* Bio-Rad protein assay (Hercules, CA, USA). The protein immobilized on the nitrocellulose membrane was incubated with the primary antibody at 4°C overnight, followed by further reaction with the horseradish peroxidase (HRP)-labeled secondary antibody. Finally, the image was developed using the ECL solution.

Statistical Analysis

GraphPad Prism 5.0 (La Jolla, CA, USA) was used for data analysis, and data were expressed as mean ± standard deviation. The t-test was adopted for the analysis. p<0.05 suggested that the difference was statistically significant.

Results

Expression of MiR-155 and NF-κB Signaling Pathway-Related Proteins in Peripheral Blood in ARDS in Neonatal Pigs

First, the model of ARDS in neonatal pigs was established in this study, and the miR-155 and NF-κB-related proteins in the miR-155/NF-κB signaling pathway were analyzed in model group and control group. As shown in Figure 1A and 1B, the levels of miR-155/NF-κB pathway-related proteins in model group were significantly higher than those in control group (p<0.01), and the miR-155 level was increased to 3 times compared to that in control group.

Expression of Inflammatory Factors in Peripheral Blood in ARDS in Neonatal Pigs

The expression features of inflammatory factors in the blood of model group were detected using the ELISA kit. As shown in Figure 2, the levels of pro-inflammatory factors (IL-1β, TNF-α, and IL-6) in model group were all markedly increased (p<0.05), while the levels of IL-4 and IL-10 declined significantly (p<0.05) compared with those in control group.

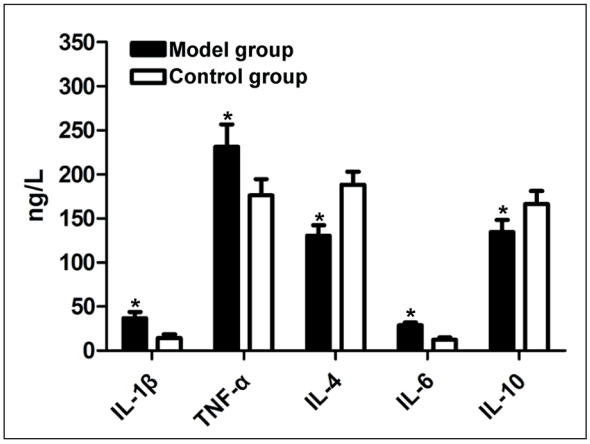

Figure 2. Expression of inflammatory factors in peripheral blood in ARDS neonatal pigs. *p<0.05.

Influence of MiR-155/NF-κB Inhibitor on MiR-155 mRNA Expression

To further study the influence of miR-155/NF-κB signaling pathway on the inflammatory response, the expression of miR-155 mRNA in ARDS of neonatal pigs was detected *via* RT-PCR after treatment with the miR-155/NF-κB inhibitor (inhibitor group). The results revealed that the miR-155 mRNA expression showed a significant increase in the model of ARDS in neonatal pigs ($p<0.05$) (Figure 3). However, the miR-155 expression remarkably declined in inhibitor group ($p<0.05$), suggesting that the miR-155/NF-κB inhibitor inhibited the increase of miR-155 in ARDS in neonatal pigs.

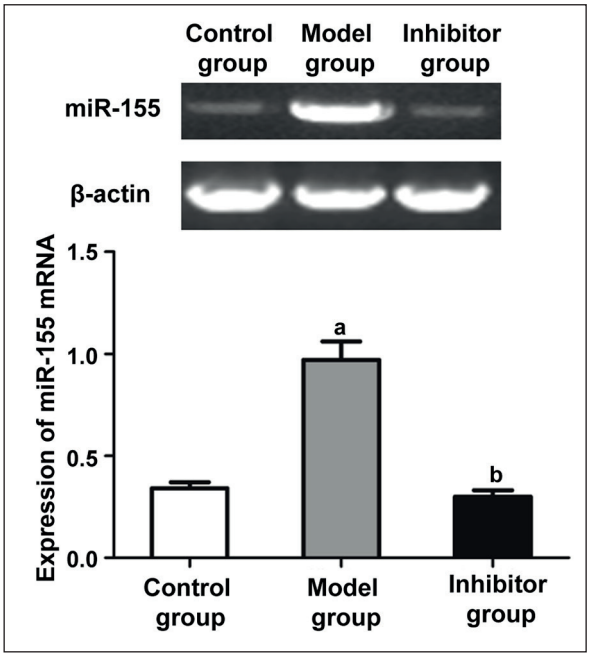

Figure 3. Influence of miR-155/NF-κB inhibitor on miR-155 expression detected *via* RT-PCR. ***A,*** Comparison between model group and control group, *$p<0.05$ ***B,*** Comparison between inhibitor group and model group, *$p<0.05$.

Influence of MiR-155/NF-κB Inhibitor on NF-κB-Related Proteins in ARDS in Neonatal Pigs

The influence of miR-155/NF-κB inhibitor on the mRNA levels of NF-κB-related factors in pigs of the model group was further detected *via* RT-PCR. The mRNA of NF-κB-related factors in inhibitor group was markedly reduced ($p<0.05$). However, the difference was not significant between inhibitor group and control group (Figure 4A and 4B), suggesting that the miR-155/NF-κB inhibitor inhibits the increase in mRNA and protein expression of NF-κB p50, NF-κB p65, and IκB-α in ARDS in neonatal pigs.

Influence of MiR-155/NF-κB Inhibitor on Expression of Inflammatory Factors

The neonatal pigs in model group were treated with the miR-155/NF-κB inhibitor to explore the

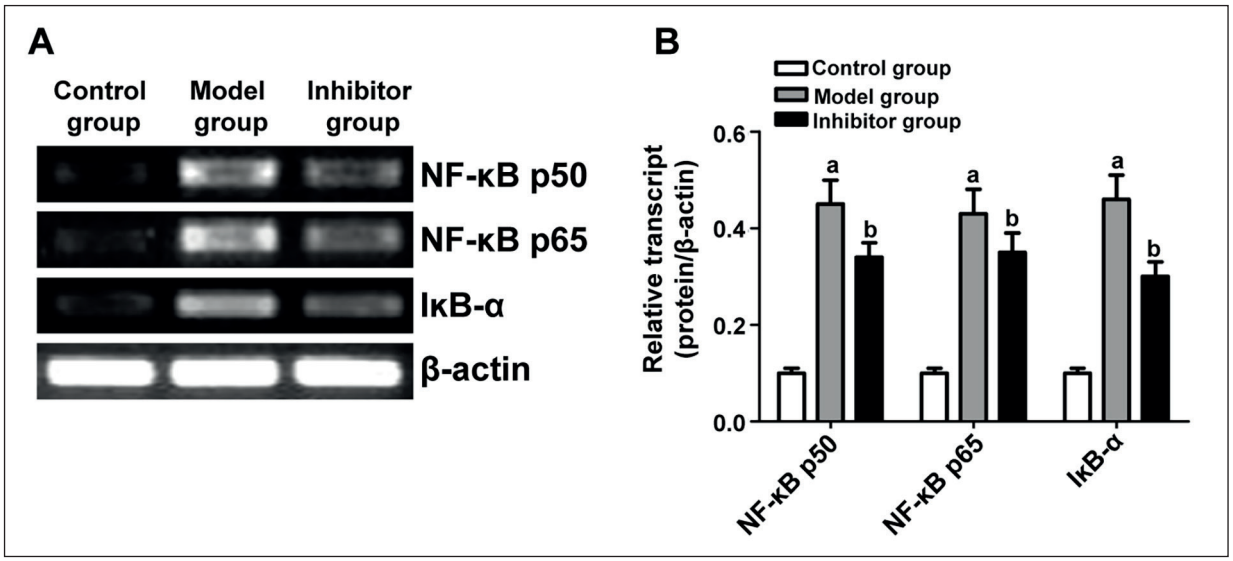

Figure 4. Influence of miR-155/NF-κB inhibitor on mRNA and protein expression of NF-κB p50, NF-κB p65, and IκB-α in the model group. ***A,*** Expression of NF-κB p50, NF-κB p65, and IκB-α mRNA in the control group, model group and inhibitor group detected *via* RT-PCR, ***B,*** Expression of NF-κB p50, NF-κB p65, and IκB-α protein detected *via* Western blotting. a, The levels are significantly increased in model group compared with those in control group, *$p<0.05$; b, the levels significantly decline in inhibitor group compared with those in model group, *$p<0.05$.

persistent effect of the inhibitor on cytokines in the blood of ARDS neonatal pigs. As shown in Figure 5A-5C, the inhibitor treatment remarkably reduced the expression of pro-inflammatory factors (IL-1β, TNF-α, and IL-6) ($p<0.05$), and the differences were extremely significant at 36 h ($p<0.01$). The levels of IL-4 and IL-10 also markedly rose after inhibitor treatment for 24 h ($p<0.05$) (Figure 5D-5E), while the IL-10 level was increased twice higher than before after inhibitor treatment for 48 h ($p<0.01$).

Discussion

The results of this study showed that the miR-155/NF-κB signaling pathway inhibitor could significantly weaken the expression of pro-inflammatory cytokines, and its mechanism was realized through the interference with the NF-κB pathway signal transduction. The inhibition on NF-κB is related to the anti-inflammatory effect, and the miR-155/NF-κB inhibitor reduced the release of pro-inflammatory cytokines through inhibiting NF-κB[15].

Adwanikar et al[16] have demonstrated that the miR-155/NF-κB signaling cascade played an important role in the initiation of the inflammatory response. Koranteng et al[17] revealed that inhibiting miR-155/NF-κB might be effective in alleviating the inflammatory response. Guo et al[18] indicated that the NF-κB inhibitor could suppress the IL-6 and TNF-α expression in monocytes and mastocytes. It is hypothesized that the miR-155/NF-κB pathway might be an effective target for the anti-inflammatory treatment of ARDS in neonatal pigs. We showed that the effect of the miR-155/NF-κB inhibitor on inflammatory response might be realized through regulating the NF-κB pathway, lowering the expression of NF-κB p50, NF-κB p65, and IκB-α and inhibiting the expression of IL-1β, TNF-α, and IL-6.

MiR-155 has been considered to exert extensive effects in the regulation of inflammation and immune system. For example, miR-155 is often up-regulated in leukemia cells and solid tumors through the targeted inhibition on κB-Ras1, increasing the NF-κB activation, inducing the persistent inflammatory state and leading to proliferative diseases. MiR-155 could be induced by a variety of inflammatory factors, including LPS and TLR, and its increase in the miR-155 expression is positively correlated with the increased production of cytokines, especially IL-1β and TNF-α[19-21].

Meduri et al[22] reported that inflammatory cytokines were related to the development of adult ARDS, shock and multiple organ dysfunction syndromes. A total of 27 patients with severe ARDS were studied, and the expression levels of

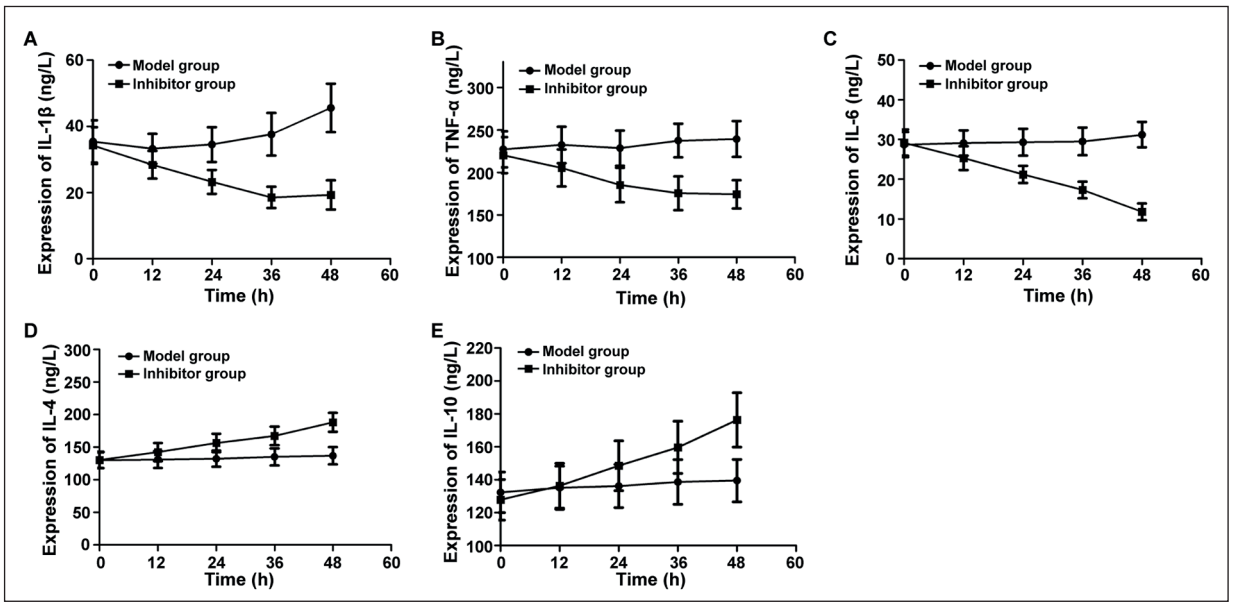

Figure 5. Changes of cytokines in the blood at different time points after treatment with the miR-155/NF-κB inhibitor. ***A-E,*** Expression changes in inflammatory factors (IL-1β, TNF-α, IL-4, IL-6, and IL-10) at different time points (12 h, 24 h, 36 h, 48 h, and 60 h), *$p<0.05$.

TNF-α and ILs (IL-1β, 2, 4, 6, 7, 8, 10, and 12) in the plasma were measured at 1, 2, 3, and 5 d. The results revealed that the overall mortality of patients was higher, the mean initial level of TNF-α, IL-1β, IL-6, and IL-8 in the plasma of non-survival and sepsis patients were significantly increased, and the correlation between ARDS and levels of plasma IL-1β (p<0.01) and IL-6 (p=0.03) was very high. The levels of plasma TNF-α, IL-1β, IL-6, and IL-8 continuously rose with time (p<0.0001). The research results of plasma IL-1β and IL-6 levels were consistent, so both of them are effective predictive factors. IL-1β could also function through inducing interferon-γ[23]. According to the clinical data, IL-6 might be an important early predictive factor for the mortality of patients with fatal and non-fatal sepsis[24]. This study also pointed out that the miR-155/NF-κB inhibitor continuously reduced the levels of TNF-α, IL-1β, and IL-6 in ARDS. It was also found that the levels of IL-4 and IL-10 were significantly increased and reached the peak at 24 h after treatment with the miR-155/NF-κB inhibitor. The inhibitor maintained the TNF-α, IL-1β, and IL-6 at low levels. Therefore, the inhibition of the expression of cytokines might be the potential mechanism of reducing multiple organ dysfunctions after microbial sepsis.

Conclusions

We showed that the miR-155/NF-κB signaling pathway influenced the changes in inflammatory factors in ARDS in neonatal pigs and could be considered as the potential target for eliminating the inflammatory response after ARDS in neonatal pigs. The miR-155/NF-κB inhibitor significantly reduced the pro-inflammatory factors.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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