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Effects of MiR-26a on respiratory distress syndrome in neonatal rats via the wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: Micro ribonucleic acids (miRNAs) are crucial to post-transcriptional regulation of the gene expression. Whether miR-26a affects respiratory distress syndrome (RDS) in neonatal rats through the Wnt/β-catenin signaling pathway was investigated in this study.

PATIENTS AND METHODS: The neonatal rat model of RDS was established, and the expressions of miR-26a and glycogen synthase kinase-3β (GSK-3β) in RDS in neonatal rats and their correlation were analyzed. The cascade relationship between miR-26a and the Wnt/β-catenin signaling pathway and the influence of miR-26a on the expression of inflammatory cytokines were subsequently verified. Finally, the influences of miR-26a on the expressions of important markers, receptor for advanced glycation endproducts (RAGE), high mobility group box 1 (HMGB1), and plasminogen activator inhibitor-1 (PAI-1), through the Wnt/β-catenin signaling pathway were analyzed.

RESULTS: Compared with those in normal tissues, the expression of miR-26a in lung tissues of neonatal rats with RDS was significantly decreased ($p<0.05$), while the expression of GSK-3β messenger RNAs (mRNAs) was notably increased ($p<0.01$), and the GSK-3β expression was negatively correlated with the miR-26a expression (r=-0.6693, $p=0.0064$). In addition, miR-26a mimics significantly inhibited the GSK-3β protein expression and activated the Wnt/β-catenin signaling pathway. Moreover, miR-26a could reduce the expressions of tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and IL-6, as well as RAGE, HMGB1, and PAI-1.

CONCLUSIONS: MiR-26a can affect inflammatory responses and markers through the Wnt/β-catenin signaling pathway in neonatal rats with RDS.

Key Words:
MiR-26a, Wnt/β-catenin signaling pathway, Respiratory distress syndrome.

Introduction

Acute respiratory distress syndrome (ARDS) is a serious lung disease that can lead to a decrease in blood oxygen content[1]. The occurrence of ARDS often leads to septicemia or multi-functional failure of other organs[2,3]. The physiological sign of ARDS is the destruction of the alveolar-capillary membrane barrier, leading to the development of non-cardiogenic pulmonary edema, in which protein exudate fills the alveolar space, impairs gas exchange and causes respiratory failure[4]. Therefore, the repair and regeneration of the alveolar epithelium is related to the treatment of ARDS[5]. The main sites of cell injury in ARDS are the vascular endothelium and alveolar epithelium[6-8].

A micro ribonucleic acid (miRNA) is an important regulator of gene expression after transcription. MiR-17, miR-92a, miR-26a, and miR-127 have been shown to be able to regulate lung development[9,10]. Receptor for advanced glycation endproducts (RAGE), high mobility group box 1 (HMGB1), and plasminogen activator inhibitor-1 (PAI-1) are important biomarkers of RDS[11]. MiRNA analysis is used to identify miRNAs involved in the pathogenesis of various lung diseases, such as ventilator-induced lung injury, bronchopulmonary dysplasia, chronic obstructive pulmonary disease, and idiopathic pulmonary fibrosis[12]. However, whether miRNA participates in the pathogenesis of ARDS remains to be studied.

The Wnt/β-catenin signaling pathway involves cell proliferation, differentiation, embryo development, and other physiological processes[13]. The classical Wnt pathway is the basic regulatory pathway for the development, differentiation and other physiological functions of cells and organisms[13]. The activation of the Wnt pathway mainly depends on the accumulation of β-catenin. Bin-

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ding of Wnt pathway ligands to low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6 results in the inhibition of glycogen synthase kinase-3β (GSK-3β) and the accumulation of β-catenin[13]. β-catenin is then transferred into the nucleus to regulate target gene expression[13]. Therefore, GSK-3β and β-catenin are considered as key signal regulators in the classical Wnt pathway. Several recent papers have shown that Wnt and its downstream typical signal transduction have an important impact on ARDS[14].

In this work, therefore, the effects of miR-26a on GSK-3β and β-catenin expressions and the cascade association between the two were studied by the establishment of a neonatal rat model of RDS, and the relationship between cytokines and biomarkers (RAGE, HMGB1, and PAI-1) was also studied.

Materials and Methods

Main Chemical Reagents

RNeasy Mini and miRNeasy Mini kits were purchased from Qiagen (Duesseldorf, Germany), Applied Biosystems TaqMan miRNA and Applied Biosystems hsa-miR-26a assay kits from Thermo Fisher Scientific (Waltham, MA, USA), and miR-26a mimics and control mimics from Ribobio Co, Ltd. (Guangzhou, China)

Sources of Experimental Animals

Adult Sprague Dawley (SD) rats were purchased from Chongqing Medical University Laboratory Animal Center. A total of 50 neonatal rats produced under the adequate supply of food and water after mating were used in the experiment of this study. This investigation was approved by the Animal Ethics Committee of Chongqing Medical University Animal Center.

Establishment of the Neonatal Rat Model of RDS

The rat model of ARDS was induced by saline irrigation and mechanical ventilation[15]. In short, the rats were anesthetized by intraperitoneal injection of ketamine and xylazine. Then, they were placed on a heated water pad at 37°C, a tracheotomy was performed, and a blunt cannula was inserted and fixed. Ventilation with 100% oxygen at a breathing rate of 30 breaths/minute was carried out, with a volume (Vt) of 8 mL/kg, an inspiration/expiration ratio of 1:2 and PEEP of 3 cm H_2O. Pancuronium bromide was injected into muscles to relax muscles and prevent spontaneous breathing. After 15 min of ventilation, the Vt was increased to 16 mL/kg, and PEEP was increased to 8 cm H_2O, followed by ventilation for 15 min. The lungs were then washed 10 times with preheated salt water to deplete the pulmonary surfactant, and ventilation was performed for another 3.5 h. Anesthesia and muscle relaxation were maintained by intraperitoneal administration of ketamine/ xylazine and pancuronium at half of the initial dose every 45 min, respectively. Lung parameters were detected, the oxygenation index was less than 150 mmHg, and dynamic lung compliance was decreased to 50%.

Experimental Grouping

The successfully prepared neonatal rat model of RDS was randomly divided into four groups, namely, control mimic group, miR-26a mimic group, control inhibitor group, and miR-26a inhibitor group, with 10 rats in each group. MiR-26a mimic group and control mimic group were injected with 100 nM medicine in equal amount, while miR-26a inhibitor group and control inhibitor group were injected with 50 nM medicine in equal amount.

Polymerase Chain Reaction (PCR) Detection

The RNeasy Mini and miRNeasy Mini kits were used to extract the total RNA and miRNA, respectively. The expressions of miR-26a and GSK-3β were determined by RT and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) using kits. Primers are as follows: miR-26a: sense: TTGGATCCGTCAGAAATTCTCTCCCGAGG and antisense GGTCTAGATGTGAACTCTGGTGTTGGTGC, and GSK-3β: sense: GGAGACTGGTCTACAAG-3' and antisense: ACATTGGGTTCTCCTCGGACC, with U6 (sense: GCGCGTCGTGAAGCGTTC and antisense: GTGCAGGGTCCGAGGT) as control. QRT-PCR data were normalized using the $2^{-\Delta\Delta Cq}$ method.

Western Blotting

The lung tissues were lysed using ice-cold lysis buffer, and the protein in the supernatant was quantified after centrifugation, separated using 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred onto the nitrocellulose membrane. After blocking with 0.5% phosphate-buffered saline-bovine serum albumin (PBS-BSA), the membrane was bound with GSK-3β, β-catenin, Wnt, and β-actin

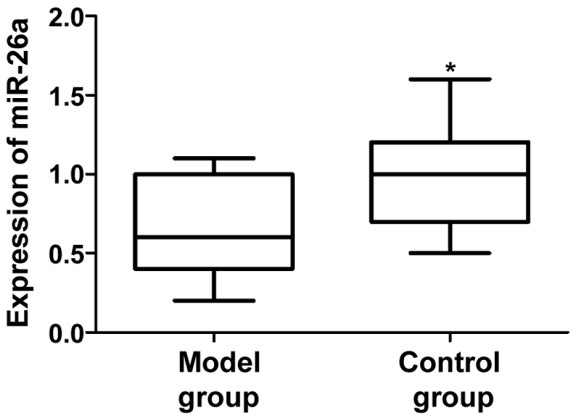

Figure 1. Expression of miR-26a in neonatal rats with RDS. **$p<0.05$: a significant difference *vs.* control group.

antibodies. Then, the goat anti-rabbit IgG secondary antibody linked with horseradish peroxidase was used for Western blotting. A chemiluminescent substrate kit was applied to detect signals. The protein level of β-actin was used as the loading control, and the relative expression level of protein was analyzed.

Cytokine Detection

With reference to the instructions, the cytokine detection kits were used to detect the cytokine content in serum of the rats in model group treated under different conditions.

Determination of Biomarkers Such as RAGE, HMGB1, and PAI-1

Rapidly frozen tissues of neonatal rats with RDS were homogenized in precooled lysis solution. After centrifugation, the supernatant was taken out, and the protein was diluted to 1 mg/mL and stored at -80°C. The expressions of RAGE, HMGB1, and PAI-1 in lung tissues of neonatal rats with RDS were detected.

Statistical Analysis

Data were expressed as mean ± standard deviation and analyzed using the Student's-*t* test to check the statistical significance. $p<0.05$ represented a statistically significant difference. The Spearman's rank correlation coefficient was used to evaluate the correlation between the GSK-3β expression and the miR-26a expression.

Results

Expression of miR-26a in neonatal rats with RDS

The expression of miR-26a in lung tissues of 15 neonatal rats with RDS and control rats was determined by qRT-PCR. Compared with that in normal control tissues, the miR-26a expression in lung tissues of neonatal rats with RDS was significantly reduced ($p<0.05$) (Figure 1).

Correlation Between the MiR-26a Expression and the GSK-3β Expression

The GSK-3β messenger RNA (mRNA) expression in lung tissues of 15 neonatal rats with RDS and control rats was measured. The results revealed that the GSK-3β mRNA expression in miR-26a model group was evidently increased ($p<0.01$) (Figure 2A). The GSK-3β expression was negatively correlated with the

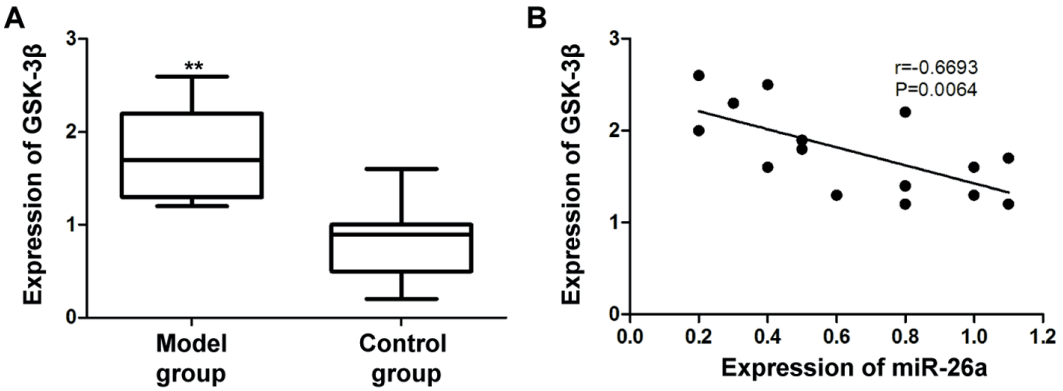

Figure 2. MiR-26a expression is negatively correlated with the GSK-3β expression. ***A,*** GSK-3β mRNA level detected *via* qRT-PCR. ***B,*** Correlation between the GSK-3β mRNA level and the miR-26a expression. *$p<0.05$ *vs.* control group.

miR-26a expression (r=-0.6693, p=0.0064) (Figure 2B).

Cascade Relationship Between MiR-26a and the Wnt/β-Catenin Signaling Pathway

To detect the cascade relationship between miR-26a and the Wnt/β-catenin signaling pathway, miR-26a mimics or inhibitors were used to treat rats in model group. First, qRT-PCR was conducted to detect their regulatory effects on the miR-26a expression. The results (Figure 3A-C) manifested that the miR-26a expression was remarkably decreased in miR-26a inhibitor group compared with that in control inhibitor group (p<0.05). On the contrary, miR-26a mimics markedly increased the expression of miR-26a compared with control mimics (p<0.01).

Moreover, miR-26a inhibitors significantly promoted the GSK-3β protein expression but decreased the expressions of β-catenin and Wnt compared with control inhibitors (p<0.01). Compared with control mimics, miR-26a mimics markedly suppressed the GSK-3β protein expression, but promoted the expressions of β-catenin and Wnt (p<0.01), indicating that miR-26a mimics promote the activation of signal pathways (p<0.01) (Figure 2B).

Effect of MiR-26a on the Expression of Inflammatory Cytokines

The change in inflammatory cytokines is an important index of RDS in neonatal rats. Therefore, the effect of miR-26a on the expression of inflammatory cytokines in the neonatal rat model of RDS was explored in this part. The levels of TNF-α, IL-1β, and IL-6 were quantitatively detected using enzyme-linked immunosorbent assay (ELISA). According to the results (Figure 4), miR-26a inhibitors significantly promoted the expression levels of TNF-α, IL-1β, and IL-6 compared with control inhibitors (p<0.05). In contrast, miR-26a mimics notably decreased the expression levels of TNF-α, IL-1β, and IL-6 compared with control mimics (p<0.05).

Effects of MiR-26a on RDS in Neonatal Pigs Via the Wnt/β-Catenin Signaling Pathway

RAGE, HMGB1, and PAI-1 are important markers of RDS. The effect of miR-26a on biomarkers of RDS in neonatal pigs was further studied. As shown in Figure 5, the results manifested that miR-26a inhibitors significantly promoted the expression levels of RAGE, HMGB1, and PAI-1 compared with control inhibitors (p<0.05). On the contrary, miR-26a mimics significantly reduced the expressions of RAGE, HMGB1, and PAI-1 compared with control mimics (p<0.05), indicating that miR-26a markedly regulates the expression of biomarkers of RDS.

Discussion

ARDS is a common respiratory disease in newborns, which is related to many factors including oxidants, growth factors, and cytokines[16]. The vascular endothelium and alveolar epithelium are the two main sites of ARDS cell injury, especially alveolar epithelial cells.

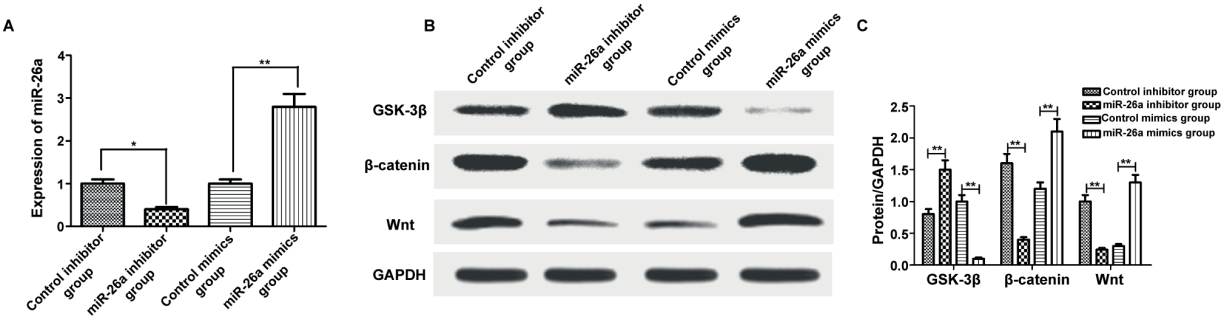

Figure 3. Cascade relationship between miR-26a and the Wnt/β-catenin signaling pathway. ***A,*** After model group is treated with control inhibitors, miR-26a inhibitors, control mimics, and miR-26a mimics, RT-PCR is adopted to detect the miR-26a expression. *p<0.05: a significant difference. ***B,*** After model group is treated with control inhibitors, miR-26a inhibitors, control mimics, and miR-26a mimics, Western blotting is used to detect and analyze the protein levels of GSK-3β, β-catenin, Wnt, and GAPDH. ***C,*** Each protein level is quantitatively analyzed using Quality One software (target protein/GAPDH). **p<0.01: an extremely significant difference.

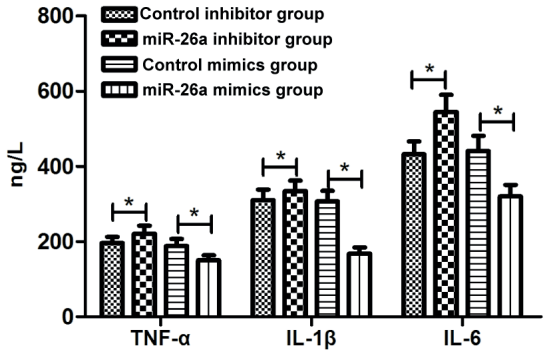

Figure 4. Expression levels of TNF-α, IL-1β, and IL-6 after model group is treated with control inhibitors, miR-26a inhibitors, control mimics, and miR-26a mimics. $^{**}p<0.05$, displaying an extremely significant difference.

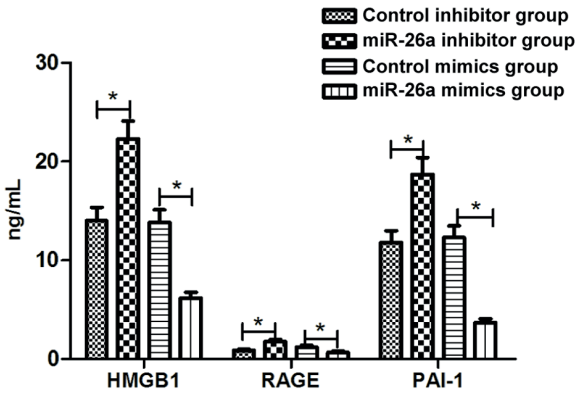

Figure 5. Expressions of RAGE, HMGB1 and PAI-1 after model group is treated with control inhibitors, miR-26a inhibitors, control mimics, and miR-26a mimics. $^{*}p<0.05$: a significant difference.

This study confirmed the role of miR-26a in RDS in neonatal rats. It was found that the miR-26a expression in lung tissues of neonatal rats with RDS was significantly reduced ($p<0.05$), and miR-26a mimics and inhibitors played roles in the changes in the Wnt/β-catenin signaling pathway, inflammatory cytokines, and biomarkers in neonatal rats with RDS.

Previous studies[17-19] have shown the role of miR-26a in different cell functions, which has biphasic effects on various functions of tumor cells. It is reported that miR-26a has anti-proliferation properties that weaken the growth of cancer cells, including hepatocellular carcinoma, nasopharyngeal carcinoma, and breast cancer. In addition to its important role in cancer, miR-26a also plays a role in regulating the hypertrophy of airway smooth muscle cells and promoting apoptosis induced by reactive oxygen species in cardiomyocytes[20]. MiR-26a has been previously reported to exert a crucial effect on regulating the synthesis of pulmonary surfactant, suggesting that miR-26a may potentially be used to treat ARDS. MiR-26a was abundantly expressed in rat and human heart tissues, and the level of ischemic preconditioning is decreased[20]. Studies on the role of miR-26a have also been carried out in many cancer cell types. In breast cancer cells, miR-26a has been shown to initiate apoptosis through external and internal pathways *via* caspase-8 and caspase-9 activation, respectively[17]. In nasopharyngeal carcinoma C666-1 cell line, apoptosis induced by ionizing radiation depends on reactive oxygen species, and the exogenous miR-26a expression results in significant cytotoxicity[18].

The Wnt pathway is closely related to the inhibition of GSK-3β and the accumulation of β-catenin[13]. β-catenin is then transferred into the nucleus to regulate target gene expression. GSK-3β and β-catenin are considered as key signal regulators in the classical Wnt pathway. In the presence of Wnt, β-catenin is stable and can induce gene transcription. Wnt binds its common receptors Fz and LRP5/LRP6. When Wnt is present, β-catenin is stable and can induce gene expression by compounding with various transcription factors including TCF/LEF, TBX5, and HIF-1α[21]. This study further confirmed the regulatory relationship between GSK-3β and Wnt/β-catenin in RDS.

HMGB1 is released to the outside of the cell during cell injury and induced an inflammatory reaction. The HMGB1 expression is increased when ARDS occurs[22]. RAGE and PAI-1 significantly increase the expression level of cardiogenic pulmonary edema in the development of pulmonary edema in ARDS[23]. MiR-26a mimics significantly reduced the expressions of RAGE, HMGB1, and PAI-1, further verifying the potential role of miR-26a in the treatment of RDS.

Inflammation plays a vital role in the pathogenesis of ARDS, which is a serious form of acute lung injury characterized by leukocyte activation, endothelial and epithelial barrier dysfunction, leakage of protein-rich exudate from circulation to the alveolar cavity and interstitial tissues, lung injury, and gas exchange disability[24]. Overactivated white blood cells, endothelial cells or epithe-

lial cells can produce and release a large number of inflammatory mediators responsible for initiating and accelerating secondary inflammatory responses[25]. In this study, miR-26a mimics reduced the expressions of TNF-α, IL-1β, and IL-6 in neonatal rats with RDS.

Conclusions

We demonstrated that miR-26a can affect inflammatory responses and markers through the Wnt/β-catenin signaling pathway in neonatal rats with RDS.

Conflict of interest

The authors declare no conflicts of interest.

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