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MiR-200a improves respiratory distress syndrome in newborn rabbits *via* the Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of micro-ribonucleic acid (miR)-200a on respiratory distress syndrome (RDS) in newborn rabbits by regulating the Wnt/β-catenin signaling pathway.

MATERIALS AND METHODS: In this work, newborn rabbits aged three days were selected from our laboratory as research objects. The messenger RNA (mRNA) and protein expression levels of miR-200a, β-catenin and interleukin-10 (IL-10) in blood samples of healthy newborn rabbits and newborn rabbits with RDS were determined by fluorescence quantitative Polymerase Chain Reaction (PCR) and Western blotting, respectively. Lentivirus-packaged plasmids containing miR-200a were then injected into newborn rabbits suffering from RDS. After 2 d, the mRNA and protein expression levels of miR-200a, β-catenin and IL-10 in blood samples of newborn rabbits in different treatment groups were measured. Meanwhile, lung sections were collected from newborn rabbits in different treatment groups. After that, the sections were observed *via* hematoxylin and eosin (H&E) staining. At the same time, lung coefficient of newborn rabbits in different treatment groups was also measured.

RESULTS: Compared with healthy newborn rabbits, the mRNA and protein expression levels of miR-200a and IL-10 in the blood of newborn rabbits with RDS decreased significantly ($p<0.05$), while β-catenin increased markedly ($p<0.05$). The mRNA and protein expression levels of β-catenin and IL-10 in newborn RDS rabbits with miR-200a over-expression and knockout were detected as well. The results revealed that lowly expressed miR-200a could remarkably promote the expression level of β-catenin, whereas inhibiting the expression of IL-10. However, highly expressed miR-200a could significantly inhibit the expression level of β-catenin and promote the expression level of IL-10. H&E staining results manifested that miR-200a knockout markedly promoted the increase of pulmonary alveoli with increased lung coefficients. However, the up-regulation of miR-200a could reduce lung coefficients and remarkably improve RDS.

CONCLUSIONS: MiR-200a regulates RDS in newborn rabbits by regulating the Wnt/β-catenin signaling pathway.

Key Words:
MiR-200a, Wnt/β-catenin, Signaling pathway, Newborn rabbits, Respiratory distress syndrome (RDS).

Introduction

Acute respiratory distress syndrome (ARDS) is a high-risk symptom of acute lung and tissue hypoxia caused by many factors, such as severe traumatic infection[1] and shock. At present, clinical studies have found that the main clinical manifestations of ARDS have increased pulmonary capillary and microvascular permeability, accompanied by symptoms including local alveolar atrophy[2]. According to the analysis of clinical follow-up statistics of ARDS, the mortality rate of patients with ARDS after hospitalization is as high as 43.5%. Since the molecular mechanism of ARDS remains currently unclear, there is no effective treatment for ARDS[3-5]. Previous studies have indicated that the main clinical treatment for ARDS is surgery.

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In recent years, with the rapid development of molecular biology, the relationship between gene expression regulation and the occurrence of has attracted more and more attention. Messenger ribonucleic acids (mRNAs) are a kind of non-coding amino acid genes in the human body. By studying the molecular mechanism of different diseases, it has been found that mRNAs can participate in the regulation of multiple genes. For example, they can combine with target gene mRNAs to inhibit their translation processes[4-7], thus ultimately achieving the regulation of target genes. Through the determination of miRNAs in various malignant tumors, it has been found that miR-200a is closely related to the occurrence of breast cancer and colon cancer. However, few authors[8,9] have investigated the correlation between miR-200a and RDS at present.

In this work, the role of miR-200a in the pathogenesis of RDS was first investigated. Meanwhile, the regulatory relationship between miR-200a and the Wnt/β-catenin signaling pathway in the pathogenesis of RDS was preliminarily explored. Our study aimed to provide a theoretical and experimental basis for further research on RDS.

Materials and Methods

Animals

16 male, three-day-old newborn rabbits raised in our laboratory were selected as research objects. To simulate RDS, 12 randomly selected rabbits were injected with 0.2 mL/kg oleic acids through the auricle vein. After treatment, 4 rabbits were randomly selected and intravenously injected with miR-200a coated with lentivirus. Then, cell lines with miR-200a over-expression were constructed. At the same time, 4 rabbits were randomly selected and intravenously injected with miR-200a coated with lentivirus. Cell lines with miR-200a knockout were constructed for subsequent experiments as well. All the above experiments were approved by the Animal Ethics Committee of Medical College of Xi'An Jiaotong University Animal Center.

Main Reagents

In this experiment, Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Roche (Basel, Switzerland), 0.25% trypsin and EDTA (Ethylene Diamine Tetraacetic Acid) reagent from Invitrogen (Carlsbad, CA, USA), the lentiviral vector system and transfection kits from TaKaRa (Otsu, Shiga, Japan), and Crisp-Case9 kit from Applied Biological Materials (abm) Inc. (Richmond, BC, Canada). Relevant recognition sites were predicted and designed through the online website (https://chopchop.rc.fas.harvard.edu/index.php). β-catenin and interleukin-10 (IL-10) antibodies and 3-(4,5)-dimethylthiahiazol (-z-yl)-3,5-diphenytetrazoliumromide (MTT) detection kits were bought from Roche (Basel, Switzerland), the animal cell total protein extraction kit and hematoxylin and eosin (H&E) staining kit from Thermo Fisher Scientific (Waltham, MA, USA), and the fluorescence quantitative Polymerase Chain Reaction (PCR) kit and the intracellular RNA extraction kit from Axygen (Tewksbury, MA, USA).

Construction of Cell Lines With MiR-200a Overexpression

In this study, primers used to construct miR-200a were synthesized by Shanghai Sangon Co., Ltd. (Shanghai, China). The primer sequences were shown in Table I. Other related molecular operations were based on the *Molecular Cloning: A Laboratory Manual, 3rd Edition*.

Construction of Cell Lines With MiR-200a Knockout

Cell lines with miR-200a interference were constructed using Crisp-Case9 technique. First, different targets in miR-200a were selected through the Crisp-Case9 online design website (https://chopchop.rc.fas.harvard.edu/index.php). Subsequently, the targets were cut and knocked out. The sgRNA sequences were shown in Table II. Two groups of recognition sites were selected for each gene with reference to the instructions[9].

RNA Extraction

The RNA extraction scheme was operated according to the instructions of the Axygen kit (Tewksbury, MA, USA). The specific procedure was as follows:

(1) About 0.1 g frozen tissue samples were taken out from liquid nitrogen and dissolved on ice. 0.45 mL of RNA Plus was added. Then, the mixture was grounded in a pre-cooled mortar and moved into a 1.5 mL Eppendorf (EP; Eppendorf,

Table I. MiR-210a gene cloning primers.

Primer name	Primer sequence
MiR-200a-F	TGCTGATGCTGATCGTAGCTAG
MiR-200a-R	GCTGATCGTAGCTAGCAGCTG

Table II. MiR-200a gene recognition target sequences.

Name	Sequence
MiR-200a-F1	ATCGGATCGTCAGCTACGTACGATCG
MiR-200a-R1	CGTAGCCGGCATCGATCAGCTAGCTA
MiR-200a-F2	CGAGCTACGAGCGCACTACGATCGC
MiR-200a-R2	CGGGAGCTACGAGCATCGACTAGCTG

Hamburg, Germany) tube. After that, 0.45 mL of RNA Plus was added to the mortar, followed by transfer into a centrifuge tube after washing. (2) 200 μL of chloroform was added to the centrifuge tube, shaken vigorously for 15 s, and let stand on ice for 15 min. (3) The mixture was centrifuged at 12000 rpm and 4°C for 15 min. (4) The supernatant was transferred into an EP tube with RNase removed. Meanwhile, the same amount of isopropyl alcohol was added, mixed upside down, and let stand on ice for 10 min. (5) The mixture was centrifuged at 12000 rpm and 4°C for 10 min. (6) The supernatant was discarded, and 750 μL of 75% ethanol was added and gently mixed, followed by centrifugation at 12000 rpm and 4°C for 10 min. (7) The supernatant was discarded, and the residual ethanol was removed as much as possible. (8) A proper amount of RNase-free water was added. The quality of extracted RNA was measured, and the remaining was used for reverse transcription[10].

Fluorescence Quantitative Polymerase Chain Reaction

Fluorescence quantitative Polymerase Chain Reaction (PCR) kit was purchased from TaKaRa (Otsu, Shiga, Japan). Total RNA was extracted using the TRIzol Reagent and then reversely transcribed into complementary DNA (cDNA) according to the instructions. The expressions of mRNAs were detected using the SYBR PrimeScript TM RT-PCR kit (TaKaRa, Otsu, Shiga, Japan) on the Roche LightCycler 480 fluorescence quantitative PCR system (Basel, Switzerland). 3 repeated wells were set for each sample. The relative expression of miRNAs was calculated by the $2^{-\Delta\Delta CT}$ method. The primers used in this study were shown in Table III.

Western Blotting

The animal cell protein extraction kit (Roche, Basel, Switzerland) was used to extract the total protein in tissue and cells[11]. The concentration of extracted protein was determined by the bicinchoninic acid (BCA) method. Protein samples were electrophoresed on polyacrylamide gels and then transferred onto polyvinylidene difluoride (PVDF)

Table III. MiR-200a gene recognition target sequences.

Name	Sequence
β-catenin	F: 5'-CGCGCTAGCATCGATCAGCTAGC-3' R: 5'-CGGGCTAGCTACGATCGCTACG-3'
IL-10	F: 5'-CGGGCATCGATCGATAAGCTAC-3' R: 5'-CGGCGCATGCTACGATCGACTCG-3'
GAPDH	F: 5'-TCATGGGTGTGAACCATGAGAA-3' R: 5'-GGCAGGACTGTGGTCATGAG-3'

membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. On the next day, the membranes were incubated with corresponding secondary antibody after rinsing with Tris-Buffered Saline and Tween solution (TBST; Sigma-Aldrich, St. Louis, MO, USA). Enhanced chemiluminescence (ECL) was used to expose protein bands on the membrane.

Lung Coefficient Determination

The middle of the neck was cut at 2-3 cm below the edge of thyroid cartilage in different subjects to separate the trachea. Then, the trachea and esophagus were clamped and freed. Subsequently, the chest was opened and the heart and lung were carefully removed. The esophagus, heart and tissues around the lung hilum were carefully cut out. After that, lung appearance was observed and weighed, and the lung coefficient was calculated. Lung coefficient = lung weight (g)/body weight (kg)[11].

H&E Staining

Immunohistochemical staining showed yellow particles in the cell membrane or cytoplasm, indicating that the cells were positive. The criteria for immunohistochemical evaluation were as follows[12]: (1) tumor cells with membrane staining < 10% or showing negative after staining were judged negative, and (2) only the cell membrane being stained, or the stained membrane being found in more than 10% of tumor cells was judged positive. The results were quantitatively determined by the KI index (the number of positive cells in each field of vision).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Experimental data were expressed as ($\bar{x}\pm s$). One-way analysis of variance was used to compare the difference among different groups. The *t*-test was performed

to compare the difference between the two groups. The pairwise comparison was carried out by q test. $p<0.05$ was considered statistically significant.

Results

The mRNA Expression Levels of MiR-200a, β-Catenin and IL-10 in Healthy Newborn Rabbits and Newborn Rabbits With RDS

To explore the correlation between miR-200a and RDS in newborn rabbits, the mRNA expression levels of miR-200a, β-catenin and IL-10 in healthy newborn rabbits and simulated newborn RDS rabbits treated with oleic acid were first measured. The results (Figure 1) revealed that, compared with healthy newborn rabbits, the mRNA expression level of miR-200a in the blood of newborn rabbits with RDS decreased, showing a significant difference ($p<0.05$). At the same time, the changes in the transcription levels of β-catenin (a key gene in the Wnt/β-catenin signaling pathway) and IL-10 were detected as well. It was found that compared with healthy newborn rabbits, the mRNA expression of IL-10 in the blood of newborn rabbits with RDS remarkably declined, showing a significant difference ($p<0.05$). However, the mRNA expression level of β-catenin in the blood of newborn rabbits with RDS was markedly up-regulated ($p<0.05$). The above results indicated that miR-200a, β-catenin and IL-10 were all associated with RDS.

The Protein Expression Levels of β-Catenin and IL-10 in Healthy Newborn Rabbits and Newborn Rabbits With RDS

Western blotting was adopted to detect the protein expression levels of β-catenin and IL-10 in the rabbit of the experimental group and control group. The results (Figure 2) demonstrated that the protein level of β-catenin in newborn rabbits with RDS was remarkably higher than that of healthy newborn rabbits ($p<0.05$). Meanwhile, the protein level of IL-10 in newborn rabbits with RDS was reduced when compared with that in healthy newborn rabbits, and there was a significant difference ($p<0.05$).

Changes in β-Catenin and IL-10 mRNAs in Cells With MiR-200a Inhibition and Overexpression

To investigate the mechanism of miR-200a on RDS, the changes in β-catenin and IL-10 mRNAs in cells with miR-200a inhibition and overexpression were determined, respectively. As shown in Figure 3, miR-200a inhibition could markedly promote the improvement of β-catenin transcription level when compared with controls.

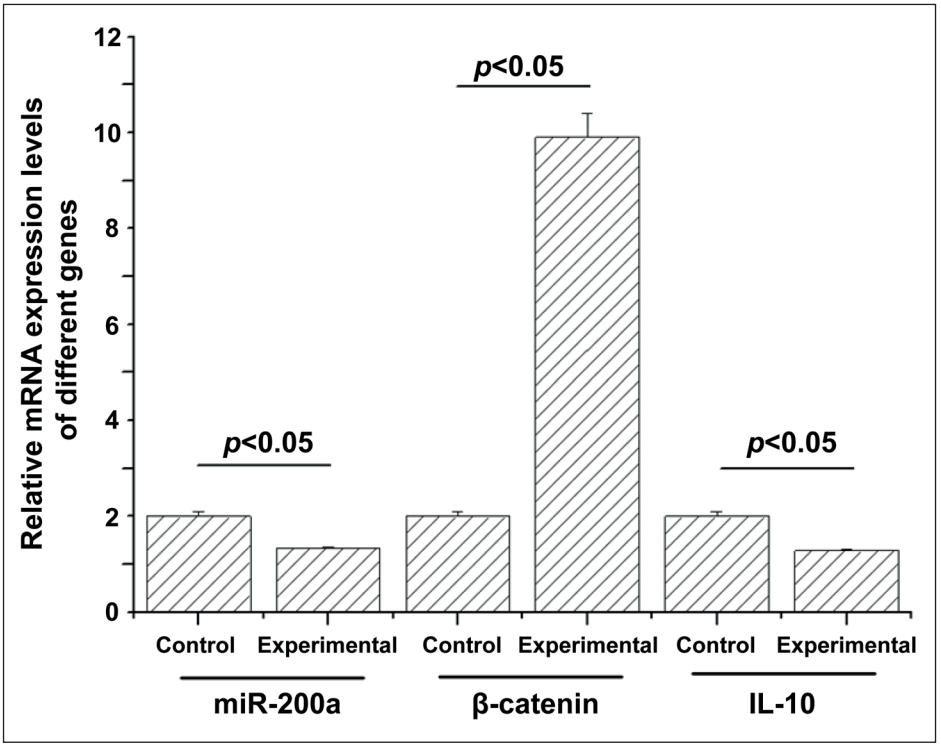

Figure 1. Transcription levels of miR-200a, β-catenin and IL-10 in different research objects detected *via* qRT-PCR.

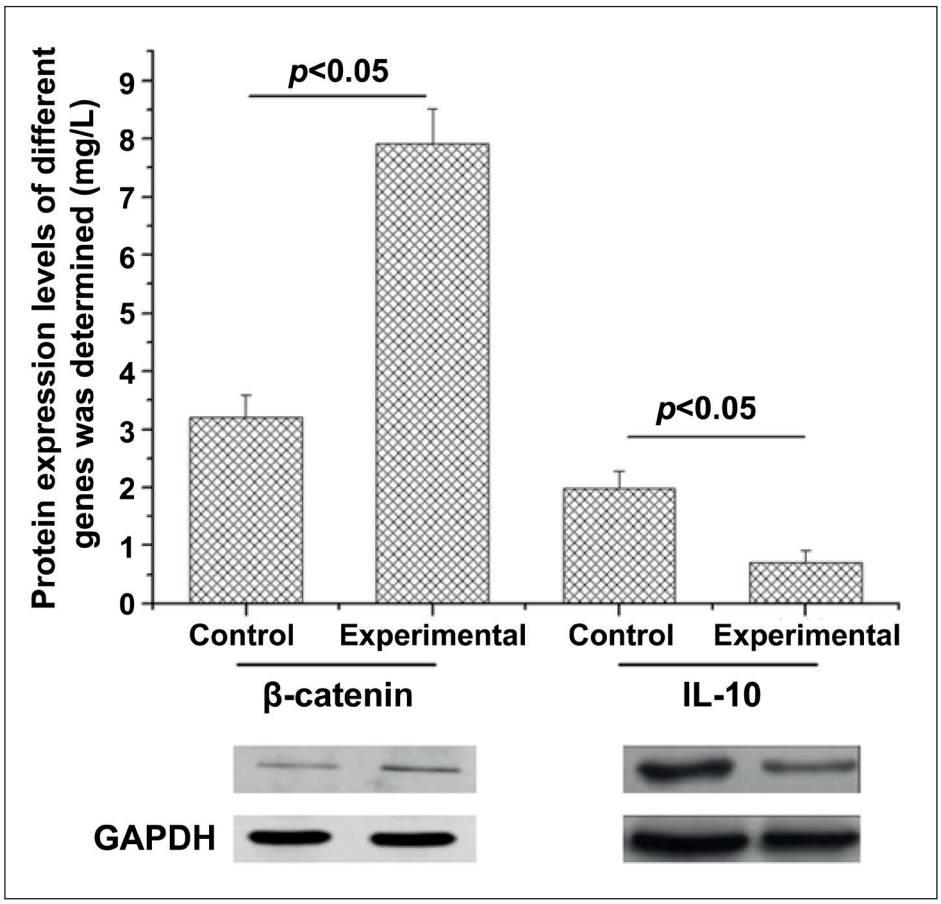

Figure 2. Protein expression levels of β-catenin and IL-10 in different research objects detected *via* Western blotting.

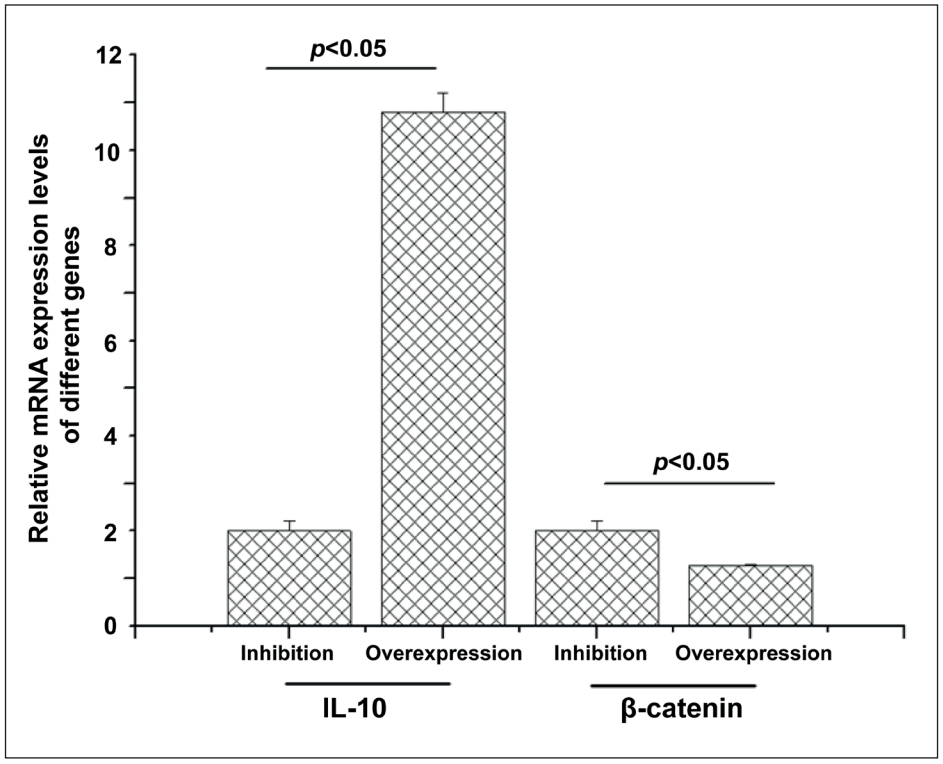

Figure 3. Changes in β-catenin and IL-10 mRNAs in cells with miR-200a inhibition and overexpression.

However, miR-200a overexpression could inhibit the increase in β-catenin transcription level. The results indicated that miR-200a, as a key gene in the Wnt/β-catenin signaling pathway, could inhibit the transcription level of β-catenin. The inhibition of miR-200a, a key indicator of resisting inflammation *in vivo* and monitoring RDS, was capable of significantly inhibiting the elevation of IL-10 transcription level. However, miR-200a overexpression could markedly promote the elevation of IL-10 transcription level. The above results suggested that miR-200a might participate in the pathogenesis of RDS by regulating β-catenin at the mRNA level.

Changes in β-Catenin and IL-10 Proteins in Cells With MiR-200a Inhibition and Overexpression

To explore the mechanism of miR-200a on RDS at the protein level, the changes in β-catenin and IL-10 in cells with miR-200a inhibition and overexpression were determined, respectively. As shown in Figure 4, the protein expression level of β-catenin in cells with miR-200a inhibition was notably elevated when compared with that in controls. However, it was significantly reduced in cells with miR-200a overexpression. This indicated that miR-200a could inhibit the protein expression of β-catenin. In addition, the inhibition of miR-200a was found to markedly inhibit the up-regulation of IL-10 protein level. However, miR-200a overexpression could promote the up-regulation of IL-10 protein level. The above results suggested that miR-200a might participate in the pathogenesis of RDS by regulating β-catenin at the translational level.

Effects of MiR-200a Overexpression and Inhibition on the Lung Coefficient of Newborn Rabbits

As an index of the lung condition, lung coefficients of different subjects were measured in this study. As shown in Table IV, compared with healthy newborn rabbits (0.43±0.12), the lung coefficient of newborn rabbits with miR-200a overexpression (0.65±0.11) decreased remarkably, showing a significant difference (p<0.05). At the same time, the lung coefficient of newborn rabbits

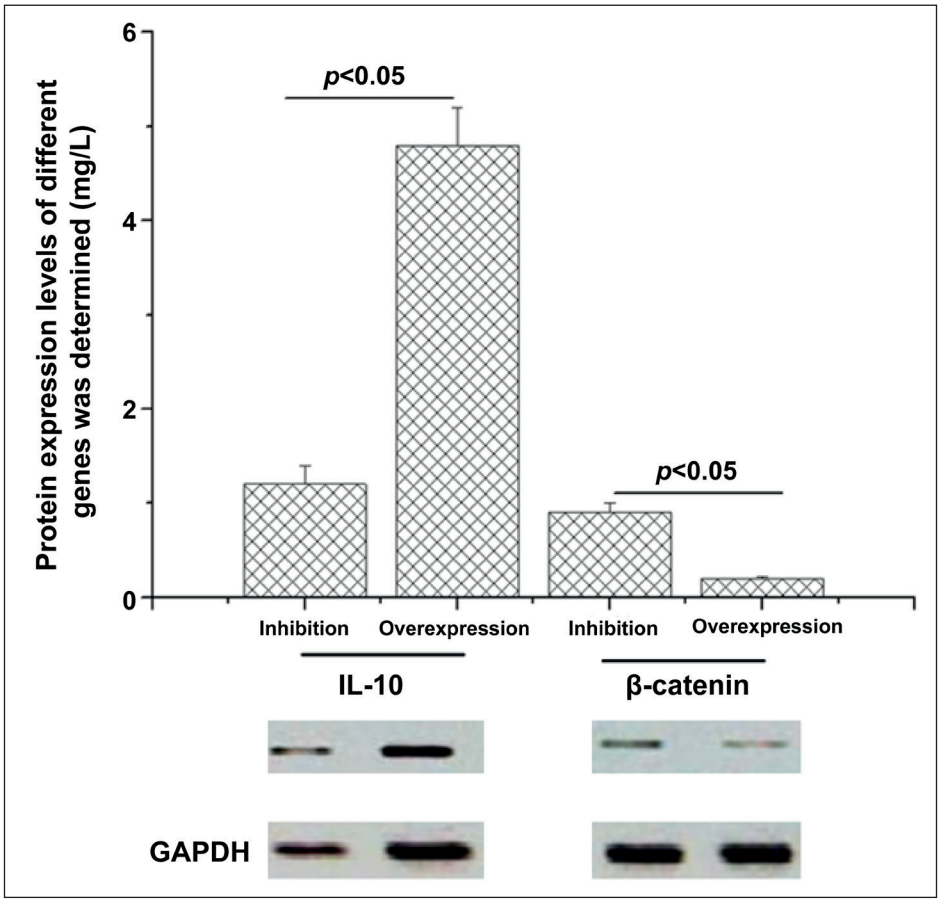

Figure 4. Changes in β-catenin and IL-10 proteins in cells with miR-200a inhibition and overexpression.

with miR-200a (3.2±0.16) knockout was higher than that of healthy newborn rabbits (0.43±0.12; $p<0.05$). The above results suggested that miR-200a could markedly affect the lung coefficient. Meanwhile, the decrease in lung coefficient could indicate the remission of RDS from the side.

Effects of MiR-200a Overexpression and Inhibition on RDS in Newborn Rabbits Detected via H&E Staining

Lung coefficient measurement illustrated that miR-200a overexpression could significantly improve RDS. To further confirm this, the lung condition of different subjects was measured by H&E staining. According to the results in Figure 5, the alveolar size of newborn rabbits with miR-200a overexpression was remarkably smaller than that of newborn rabbits with miR-200a inhibition. However, the alveolar size of newborn rabbits with miR-200a overexpression was similar to that of healthy newborn rabbits. Meanwhile, the alveolar size of newborn rabbits with miR-200a inhibition was notably larger than that of healthy newborn rabbits. The results were consistent with those of lung coefficient measurement, demonstrating that miR-200a overexpression could remarkably improve RDS.

Discussion

ARDS is an important and harmful complication of emergency treatment and high-risk diseases, such as severe mechanical injury and car accident[13]. Clinical statistics have manifested that the mortality rate of ARDS is about 35%-65%. However, the pathogenesis of ARDS is not clear and there is no significant treatment[14]. Therefore, it is of great practical significance to strengthen the research on ARDS.

MiRNA is a kind of single-stranded non-coding RNA with 15-30 base sequences in length, which exists in organism cells. In recent years, with continuous progress of molecular biotechnology, miRNA has been found widely present in eukaryotes, especially in the human body[15-17]. Currently, researches on miRNAs have demonstrated that although miRNAs cannot encode proteins in the human body, they can bind to mRNAs transcribed from certain target genes to form double-stranded RNAs. Eventually, this may prevent ribosomes and other translation-related organelles from binding to mRNAs, leading to a decrease in the expressions of target proteins. For example, a study has shown that the expressions of various miRNAs in the above-mentioned tumors are significantly higher or lower than those in healthy cells[18] through transcriptome sequencing. These results indicate that miRNAs may be involved in the pathogenesis of tumors and cancer cells. Among them, miR-183, miR-200 and miR-145 have all been proved to be related to non-small cell lung cancer. MiR-200a, as an important member of the miR-200 family, is located in the 1p36.33 region of chromosome 1. Mateescu et al[19] have indicated that it plays an important role in the metastasis of tumor cells, such as liver cancer cells. Chen et al[20] focusing on the function of miR-200a have demonstrated that miR-200a mainly exerts its functions in the lung. Based on this, the correlation between miR-200a and RDS was explored in this study.

Our results showed that the transcriptional level of miR-200a in newborn rabbits with RDS was significantly lower than that of healthy newborn rab-

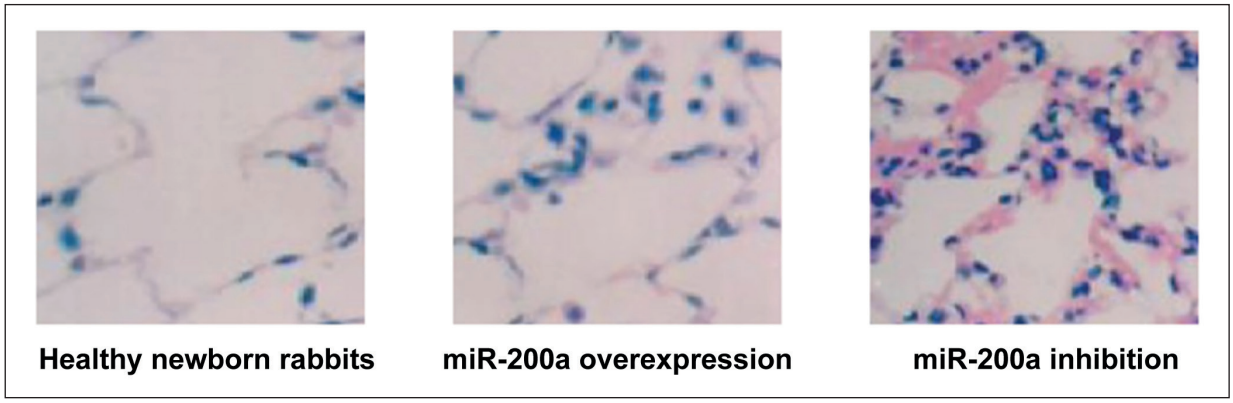

Figure 5. Effects of miR-200a overexpression and inhibition on RDS in newborn rabbits detected *via* H&E staining (magnification: 100×).

bits, suggesting that miR-200a was related to RDS. After that, lung coefficient and other indicators characterizing RDS in newborn rabbits with miR-200a overexpression and inhibition were detected as well. It was found that miR-200a overexpression could markedly improve RDS, while miR-200a inhibition could aggravate RDS. The above data further proved that miR-200a had a negative correlation with RDS. To explore the interaction mechanism of miR-200a and RDS, the expression of β-catenin was determined. The results revealed that the transcriptional and translational levels of β-catenin in newborn rabbits with RDS were remarkably improved when compared with those in healthy newborn rabbits, showing that RDS could activate the Wnt/β-catenin signaling pathway. However, it was found that the transcriptional and translational levels of β-catenin declined significantly in cells with miR-200a overexpression, while rose markedly in cells with miR-200a inhibition. Based on the above findings, it was believed that miR-200a could affect the development of RDS in newborn rabbits by regulating the transcriptional and translational levels of β-catenin. Although miR-200a was verified to participate in the development of RDS in rabbits by regulating the Wnt/β-catenin signaling pathway, how the Wnt/β-catenin signaling pathway participated in rabbit RDS remained unclear, which requires further research.

Conclusions

Our results revealed that miR-200a regulates RDS in newborn rabbits by regulating the Wnt/β-catenin signaling pathway.

Conflict of Interests

The authors declare that they have no conflict of interest.

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