Abstract. – OBJECTIVE: This study aims to investigate the miR-26a effects on H\textsubscript{2}O\textsubscript{2}-induced apoptosis of Type II alveolar epithelial cells (AEC-II) and the potential mechanism.

MATERIALS AND METHODS: AEC-II cells were treated with 0.5 mmol/L H\textsubscript{2}O\textsubscript{2} to mimic cellular model of acute lung injury. Transmitting electron microscopy (TEM) was employed to observe the change of morphological structures. After infecting with miR-26a mimics, flow cytometry was performed to detect cell apoptosis. Western blot was also done to explore mitochondrial apoptosis-related markers: Caspase-3, B-cell lymphoma-2 (Bcl-2) and Bax. AEC-II cells treated with 0.5 mmol/L H\textsubscript{2}O\textsubscript{2} exhibited significant cell apoptosis. Overexpression using miR-26a mimics partially reversed the effects of H\textsubscript{2}O\textsubscript{2}-induced apoptosis in AEC-II cells, evidenced by flow cytometry results.

RESULTS: Further Western blot results revealed increased levels of Caspase-3 and Bax, and the decreased Bcl-2 level after infecting with miR-26a mimics, indicating miR-26a has protective effects against mitochondrial apoptosis in AEC-II cells.

CONCLUSIONS: MiR-26a protected AEC-II cells against apoptosis via mitochondrial pathway. Thus, miR-26a promises to be a potential therapy in treatment of Acute Respiratory Distress Syndrome (ARDS).

Key Words: Acute Respiratory Distress Syndrome, Acute lung injury, microRNA, Alveolar epithelial cells, Apoptosis, Mitochondria, miR-26a

Introduction

Acute Respiratory Distress Syndrome (ARDS) is a common severe lung diseases among neonates, which often causes low level of oxygen in blood\textsuperscript{1,2}. ARDS mostly occurs accompanied by other major complications, thus resulting in the multiple organ dysfunction\textsuperscript{1}. Characteristic features of the ARDS are hypoxemia, lung inflammation, and non-cardiogenic pulmonary edema formation\textsuperscript{1,3}. As a special developmentally regulated lipoprotein, the pulmonary surfactant deficiency was very important in the development of ARDS. Synthesis of pulmonary surfactant is done by type II epithelial cells (AEC-II), which is a very complex process followed multifactorial control and then regulated by a variety of factors and hormones\textsuperscript{4}.

MicroRNAs, approximately 22-25 nt in length, belong to the non-coding small RNAs and are crucial mediators on the gene expression of post-transcriptional\textsuperscript{7,8}. The mature microRNAs regulate the gene expression through binding to the 3’-UTR of their target genes, leading to either decreased mRNA degradation or protein translation. Various microRNAs are highly expressed in the lung tissue\textsuperscript{9,10}. For example, miR-127 and miR-92a have been demonstrated to play a vital role in the lung development\textsuperscript{11,12}.

Among, miR-26a was previously reported to participate in regulating the lung alveolar surfactant synthesis via targeting Sekelsky Mothers Against Dpp 1 (SMAD1)\textsuperscript{13}. However, the role of miR-26a and its correlation with the type II alveolar epithelial cells (AEC-II) apoptosis has not yet been documented.

In the current study, we used H\textsubscript{2}O\textsubscript{2} to induce the cellular model of acute pulmonary injury. Then, we investigate the miR-26a changes after H\textsubscript{2}O\textsubscript{2} treatment. The purpose of this work was to explore the miR-26a effects on AEC-II cells apoptosis.

Materials and Methods

Cell Culture and H\textsubscript{2}O\textsubscript{2}, Treatment

Alveolar epithelial cells type II (AEC-II) were purchased from ShangHai Model Cell Bank.

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(Shanghai, China) and cultivated in the medium containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 5% CO₂ and 37°C. To mimic the process of the acute pulmonary injury, 0.5 mmol/L H₂O₂ was used to treat the cells for 12 h to induce apoptosis. None-treated cells were served as control group (Control group). After treatment with H₂O₂, cells were transfected with phosphate buffered saline (PBS) (H₂O₂ group), negative control miRNA (NC group) or miR-26a mimic (miR-26a mimic group) using Lipofectamine 3000 according to the manufacturer's protocol.

**MiR-26a Mimic Transfection**

MiR-26a mimic and a negative control (NC) were synthesized by Shanghai GenePharma (Shanghai, China). For transfection, CFs were planted in a 12-well plate at 5×10⁴/ml density and were transfected with 100 nM of miR-26a mimic or NC using Lipofectamine 3000 (Life Technologies Corporation, Gaithersburg, MD, USA) and then incubated for 6 h. Following transfection, medium were changed to 2% fetal bovine serum-Dulbecco's Modified Eagle Medium (FBS-DMEM) without antibiotics.

**Detection of Cell Apoptosis**

Transmission Electron Microscope (Olympus, Tokyo, Japan) was employed to observe the cellular microstructures and to detect apoptotic body. Flow cytometry was further performed to explore the apoptosis of the cells. Detailed protocols were according to a previous report14.

**RT-PCR**

After isolation of the total RNA from AEC-II cells using Qiazol reagent (Qiagen, Venlo, the Netherlands), 2.0 μg total RNA was reversely transcribed following the PrimeScript™ RT reagent Kit introduction (TaKaRa, Dalian, China) for cDNA synthesis. The following detailed protocols were previously described15. For miRNA detection, we used a TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA, USA). U6 was used as internal control.

**Western Blot**

A total of 40 μg denatured cell lysates were run on 10% gels and then transferred to the polyvinylidene difluoride (PVDF) membranes. Then, membranes were treated with 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline (TBS-T) containing 0.05% tween, and incubated with the primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 0.5% TBS-T overnight.

Primary rabbit anti-Bcl-2 antibody (dilution: 1/1000; Catlog#: ab32124), primary rabbit anti-Bax antibody (dilution: 1/1000; Catlog#: ab32503) and primary mouse anti-Caspase-3 antibody (dilution: 1/1000; Catlog#: ab2171) were all purchased from Abcam (Cambridge, MA, USA). After washing for 3 times with TBS-T, secondary horseradish peroxidase (HRP)-conjugated antibodies were incubated with membranes for 1 h with TBST. Secondary antibodies were detected using ECL Plus (Amersham, Arlington Heights, IL, USA) and imaged with the GelDoc XRS (Bio-Rad, Hercules, CA, USA). The relative band densities were normalized to β-actin.

**Statistical Analysis**

SPSS 20.0 software (IBM, Armonk, NY, USA) was used for statistical analyses. All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by Post-Hoc test (Least Significant Difference). Percentage (%) was used to express the enumeration data and X² test was used for data analysis. p-values < 0.05 were considered statistically significant.

**Results**

**H₂O₂ Induced Significant Apoptosis in AEC-II Cells**

To mimic the process of ARDS, we first used 0.5 mmol/L H₂O₂ to treat AEC-II cells. TEM showed that the number of microvillus on the surface of the cells was remarkably reduced. Cytoplasmic retraction and chromatin cohesion were also observed (Figure 1). These results suggested that cells treated with H₂O₂ presented as early apoptosis.

**MiR-26a Was Down-Regulated in AEC-II Cells After H₂O₂ Treatment**

Previous evidence reported that miR-26a expression in alveolar tissues was reduced in rats with ARDS compared with normal ones. To verify whether H₂O₂ can also induce decrease of miR-26a, RT-PCR was performed after H₂O₂ treatment for 12 h. Results revealed a significant decrease of miR-26a in H₂O₂ group compared with Control group (Figure 2), which was consistent with previous studies86.
MiR-26a Inhibited Apoptosis of AEC-II Cells

To explore the miR-26a effects on AEC-II cells apoptosis, cells were further transfected with the miR-26a mimics or the negative control (NC) after treatment with 0.5 mmol/L H$_2$O$_2$. Transfection efficiency was observed under the fluorescent microscope (Figure 3A). After infecting with miR-26a, we found the expression of miR-26a was significantly enhanced, evidenced by RT-PCR results (Figure 3B). Next, we employed flow cytometry to detect cells apoptosis. The results showed that H$_2$O$_2$ induced a significant apoptosis in AEC-II cells, while miR-26a mimics partially reverse its effects (Figure 4). These findings indicated that miR-26a may have a role in protecting AEC-II cells from apoptosis.

MiR-26a Protects AEC-II Cells Against Apoptosis Via Mitochondrial Pathway

To confirm the above hypothesis and clarify the underlying mechanism of miR-26a in this process, Western blot assay was conducted to explore expression of three mitochondrial apoptosis-related markers: Bcl-2, Caspase-3 and Bax. The results showed a marked increase of Bcl-2 and a significant decrease of Caspase-3 and Bax after infecting with miR-26a (Figure 5). All above results demonstrated that the protective effects of miR-26a against apoptosis in AEC-II cells were achieved via mitochondrial pathway.

Discussion

ARDS is a common respiratory disease in neonates, which is associated with various factors, including oxidants, growth factors, and cytokines\(^7\). Vascular endothelium and alveolar epithelium are two main sites of cell injury in ARDS, especially alveolar epithelium. Damage to AEC-II cells causes decreased fluid clearance from alveolar airspace and increased fluid flow into the alveolar lumens, and decreased the surfactant production\(^16\). H$_2$O$_2$ is one of the com-
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Figure 3. MiR-26a was overexpressed after infecting with miR-26a mimics. A, Transfection efficiency detected by fluorescent microscope. B, RT-PCR was performed to confirm the transfection efficiency of miR-26a mimics. (*p < 0.01, compared with NC group).

Figure 4. Overexpression of miR-26a inhibited H₂O₂-induced apoptosis of AEC-II cells.

Figure 5. Apoptosis-related proteins detected by Western blot.
mon injuries to AEC-II cells, which can induce excessive oxidative stress in cells, thus leading to cell apoptosis. In the current study, we established the cellular injury model using H2O2 in AEC-II cells. Current functional genomics techniques have provided novel insights concerning the environmental interactions among genes regulating this complicated pathological process. Previous evidence has demonstrated genes regulating this complicated pathological process. Previous evidence has demonstrated genes regulating this complicated pathological process. Previous evidence has demonstrated genes regulating this complicated pathological process. Previous evidence has demonstrated genes regulating this complicated pathological process. Previous evidence has demonstrated genes regulating this complicated pathological process. Previous evidence has demonstrated genes regulating this complicated pathological process. Previous evidence has demonstrated genes regulating this complicated pathological process. Previous evidence has demonstrated genes regulating this complicated pathological process. Previous evidence has demonstrated genes regulating this complicated pathological process. Previous evidence has demonstrated genes regulating this complicated pathological process.

Conclusions

Taken above, miR-26a is down-regulated in the process of H2O2-induced apoptosis of the AEC-II cells. MiR-26a has a significant protective effect against AEC-II cells apoptosis. MiR-26a might be a potential therapeutic in treating ARDS.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

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